Widening the prostacyclin paradigm: tissue fibroblasts are a critical site of production and anti-thrombotic protection

Authors:

Maria Vinokurova[1#], Maria Elisa Lopes-Pires[1#], Neringa Cypaite[1], Fisnik Shala[1], Paul C. Armstrong[2], Blerina Ahmetaj-Shala[1], Youssef Elghazouli[1], Rolf Nüsing[3], Bin Liu[4], Yingbi Zhou[4], Chuan-ming Hao[5], Harvey R. Herschman[6], Jane A. Mitchell[1], Nicholas S. Kirkby[1*]

Affiliations:

1: National Heart & Lung Institute, Imperial College London, London, UK

2: Blizard Institute, Queen Mary University of London, London, UK

3: Clinical Pharmacology and Pharmacotherapy Department, Goethe University, Frankfurt, Germany

4: Cardiovascular Research Centre, Shantou University Medical College, Shantou, China

5: Division of Nephrology, Huashan Hospital, Fudan University, Shanghai, China

6: Department of Molecular and Medical Pharmacology, University of California Los Angeles, USA

#: These authors contributed equally to the study

*: To whom correspondence should be addressed

Short title: Non-endothelial sources of prostacyclin

Correspondence to:

Dr Nicholas S Kirkby, National Heart & Lung Institute, Imperial College London, Sir Alexander Fleming Building, Exhibition Road, London SW7 2AZ, UK. n.kirkby@imperial.ac.uk

Abstract

Background: Prostacyclin is a fundamental signalling pathway traditionally associated with the cardiovascular system and protection against thrombosis but which also has regulatory functions in fibrosis, proliferation and immunity. Prevailing dogma states that prostacyclin is principally derived from vascular endothelium, although it is known that other cells can also synthesise it. However, the role of non-endothelial sources in prostacyclin production has not been systematically evaluated resulting in an underappreciation of their importance relative to better charecterised endothelial sources.

Methods: To address this we have used novel endothelial cell- and fibroblast-specific cyclo-oxygenase and prostacyclin synthase knockout mice and cells freshly isolated from mouse and human lung tissue. We have assessed prostacyclin release by immunoassay and thrombosis *in vivo* using an $FeCl_3$-induced carotid artery injury model.

Results: We found that in arteries, endothelial cells ***are*** the main source of prostacyclin but that in the lung, and other tissues, prostacyclin production occurs largely independently of endothelial and vascular smooth muscle cells. Instead, in mouse and human lung, prostacyclin production was strongly associated with fibroblasts. By comparison, microvascular endothelial cells from the lung showed weak prostacyclin synthetic capacity compared to those isolated from large arteries. Prostacyclin derived from fibroblasts and other non-endothelial sources was seen to contribute to anti-thrombotic protection.

Conclusions: These observations define a new paradigm in prostacyclin biology in which fibroblast/non-endothelial-derived prostacyclin works in parallel with endothelium-derived prostanoids to control thrombotic risk and potentially a broad range of other biology. Although generation of prostacyclin by fibroblasts has been shown previously, the scale and systemic activity was not tested and unappreciated. As such, this represents a basic change in our understanding and may provide new insight into how diseases of the lung result in cardiovascular risk.

6ketoPGF$_{1\alpha}$	6-keto prostaglandin F$_{1\alpha}$ (prostacyclin break-down product)
COX	Cyclo-oxygenase
EC	Endothelial cell
EGFP	Enhanced green fluorescent protein
EpCAM	Epithelial cell adhesion molecule (CD326)
HETE	Hydroxyeicosatetraenoic acid
PDGFR	Platelet-derived growth factor receptor
PG D$_2$/E2/F$_{2\alpha}$	Prostaglandin D$_2$/E2/F$_{2\alpha}$
PPP	Platelet poor plasma
PRP	Platelet rich plasma
SMC	Smooth muscle cell
TXB$_2$	Thromboxane B$_2$ (thromboxane A$_2$ breakdown product)
VEC	VE-cadherin/Cdh5

Introduction

Prostacyclin is a powerful endogenous inhibitor of platelet activation and represents one of the bodies fundamental anti-thrombotic and cardioprotective pathways[1,2]. Inhibition/deficiency of the prostacyclin receptor, IP, results in a pro-thrombotic phenotype in animal models[3,4] and an increased risk of heart attacks and strokes in man[5]. Prostacyclin can also, in some contexts, act as a vasodilator[1] and protect against atherogenesis[6]. Dysfunction of the prostacyclin pathway is a feature of vascular pathologies, including pulmonary hypertension where exogenous prostacyclin is an established therapy[2]. In addition to its cardiovascular actions, prostacyclin is important in lung, gastrointestinal and renal function, in pain/inflammation, and the regulation immunity. As such, understanding prostacyclin biology is essential not only for cardiovascular health, but also for the proper functioning of a broad range of organ systems.

Prostacyclin is produced as part of a family of prostanoid mediators by the activities of phospholipases, cyclo-oxygenases and prostacyclin synthase, through step-wise metabolism of membrane lipids[2]. Phospholipases, principally the cytosolic phospholipase A_2 isoform[7], liberate the substrate, arachidonic acid, from cell membranes which is then converted to an unstable intermediate, prostaglandin H_2, by two isoforms of cyclo-oxygenase enzyme. Cyclo-oxygenase-1 is, like cytosolic phospholipase A_2, widely expressed as a physiological house-keeping enzyme. In contrast, constitutive cyclo-oxyenase-2 expression is restricted to certain regions including the kidney, brain and gut[8] but can be induced elsewhere during inflammation and proliferation. Both cyclooxygenase isoforms contribute to prostacyclin generation and cardiovascular protection through distinct but parallel pathways. Cyclo-oxygenase-2 drives prostacyclin metabolites in the urine[9] (the origin of which is controversial[10]) but where studied directly, does not appear to mediate prostacyclin production by isolated systemic mouse[11,12] or human arteries[13] except under conditions of gross inflammation[14]. It has been suggested that vascular cyclo-oxygeanse-2 is rapidly lost when vessels are taken away from the influence of blood flow since laminar shear increases cyclo-oxygeanse-2 in endothelial cells[15]. However, whilst acute application of shear to static endothelial cultures has been shown to increase cyclo-oxygenase-2 expression[15]. this is not seen in endothelial cells subject to chronic laminar shear stress[16]. Nonetheless, cyclo-oxygenase-2 plays an unequivocal role in cardiovascular health; its inhibition in man increases cardiovascular risk[17] and vascular cyclo-oxygenase-2 deletion in mouse models increases thrombosis[4,18]. Cyclo-oxygenase-1 has a more clearly defined role in bulk systemic prostacyclin generation; cyclo-oxygenase-1 deletion/inhibition abolishes prostacyclin production by isolated mouse[11,12] and human vessels[13] and consequently, vascular cyclo-oxygenase-1 deficiency is associated with acceleration of the thrombosis[4].

The final step in prostacyclin synthesis is conversion of prostaglandin H_2 to prostacyclin by the enzyme prostacyclin synthase which has narrower expression pattern, resulting in spatial differences

in prostacyclin production. Prostacyclin was first discovered from arterial endothelial cells[4,19] and since then, arterial tissue *ex vivo* and arterial endothelial cells *in vitro* have been almost universally observed to possess a robust capacity for prostacyclin synthesis. By comparison, platelets[1] and leucocytes[20] are almost entirely deficient in their ability to physiologically produce prostacyclin. It was quickly realised that prostacyclin was also produced by isolated tissues; it is abundant in tissue perfusates and the major prostanoid generated by the lung and the heart[21,22]. *In vitro* evidence has indicated many cell types, including epithelial cells[23], fibroblasts[24-26] and smooth muscle cells[27], have at least some prostacyclin synthetic capacity, but it remains unknown within intact tissues whether these sources contribute meaningfully to prostacyclin production or whether this is instead associated with their vascular compartments. This question has remained unanswered because there have been no tools available that allow the contribution of individual cell types to be assessed for prostacyclin production in intact tissues or *in vivo*. We have recently described mice in which cyclo-oxygeanse-1, cyclo-oxygenase -2[4], or prostacyclin synthase[28] can be deleted specifically from vascular endothelial cells, which provide new tools to identify the cellular origins of prostacyclin within tissues. In the current study we have used them to determine whether prostacyclin synthesis is purely a product of the endothelium, or whether there are additional, underappreciated depots of prostacyclin production in the body that contribute to local organ function and/or systemic anti-thrombotic protection.

Materials & Methods

Animal studies

Studies were performed on 8-12 week old male and female mice housed in individually ventilated cages with free access to standard laboratory diet and water and 12h day/night cycle. All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act (1986) Amendment (2013) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and after local approval from the Imperial College Animal Welfare Ethical Review Board (UK Home Office License Project Licenses 70/7013 and PP1576048) or Fudan University. Unless otherwise indicated, animals were euthanised by CO_2 narcosis. Tissue samples were collected and used to measure prostaglandin content, prostaglandin release and gene expression[4,8,12,29,30] and thrombotic responses in vivo measured using the $FeCl_3$ carotid artery injury model[4,29] according to our previously published methodology and as detailed in the Supplemental Methods section.

Global cyclo-oxygenase-1 knockout mice (*Ptgs1*$^{-/-}$)[31] were generated as previously described and compared to age and sex matched wild-type C57Bl/6J controls (Charles River, UK). Endothelial-specific cyclo-oxygenase-1 knockout mice (*Ptgs1*$^{flox/flox}$*; VEC-iCre*) were generated as previously described[4] using VE-cadherin Cre^{ERT2} which is thought to be highly selective for endothelial cells with no off-target expression reported[32]. Because this Cre requires activation by tamoxifen, at 4-6 weeks

of age, all mice from this line were treated with tamoxifen (50mg/kg, ip, once daily for 5 days; Sigma, UK) and allowed to recover to 2 weeks before further use. Smooth muscle-specific cyclo-oxygenase knockout mice (*Ptgs1*$^{flox/flox}$*; Sm22a-Cre*)[33] were generated as previously described[4] using Sm22a Cre which is thought to have some potential off-target expression in cardiomyocytes[4], platelets, adipocytes and myeloid cells[34]. Endothelial/platelet-specific cyclo-oxygenase-1 knockout mice (*Ptgs1*$^{flox/flox}$*; Tie2-Cre*)[4], endothelial/platelet-specific cyclo-oxygenase-2 knockout mice (*Ptgs2*$^{flox/flox}$*; Tie2-Cre*)[4] and endothelial/platelet-specific prostacyclin synthase (*Ptgis*$^{flox/flox}$*; Tie2-Cre*)[28] were generated using Tie2 Cre which is thought to have some potential off-target expression in heart valves and hematopoietic cells[32]. Fibroblast-specific cyclo-oxygenase-1 knockout mice (*Ptgs1*$^{flox/flox}$*; Fsp1-Cre*) were generated by crossing floxed *Ptgs1* mice[4] with transgenic mice harbouring *Fsp1/S1004A4-Cre*[35] which is thought to have some potential off-target expression in macrophages[36]. These strains were validated for effective deletion of the floxed gene in target cells and retention in select non-target cells either by our previous work[4,28] or in the current study but we cannot exclude expression in all non-target cells including those highlighted above which can be considered a limitation of these models. These validation data are summarised in Figure S1. Mouse models were maintained on a pure C57Bl/6J (*Ptgis* models), a mixed C57Bl/6J, 129S4/SvJae and BALB/c background (*Ptgs1*$^{flox/flox}$*; Fsp1-Cre* model) or a mixed C57Bl/6J and 129S4/SvJae background (other *Ptgs1*/*Ptgs2* models). EGFP/Cre activity reporter strains were generated by crossing ROSA$^{mT/mG}$ mice (JAX strain: 7676)[37] with transgenic Tie2-Cre[38] or Fsp1/S100A4-Cre mice and were studied as heterozygous animals. For all cell-specific knockout strains, animals were genotyped by genomic PCR to identify Cre-positive knockout animals from the Cre-negative littermates which were used as experimental controls for each strain. Genotyping was performed at weaning only from ear clip material by Charles River Laboratories, UK in a blind manner. Further details of animal strains and ARRIVE reporting are given in the Major Resources Table.

Human tissue studies

All studies using human material were conducted in accordance with the Declaration of Helsinki and samples were donated from volunteers and patients who gave explicit informed consent. Blood was collected into sodium citrate (0.32% final; BD Biosciences, Germany) from healthy male and female volunteers aged 18-40 after ethical approval by the West London & GTAC Research Ethics Committee (approval 15/LO/223) or the St Thomas' Hospital Research Ethics Committee (approval 07/Q0702/24). Lung parenchyma and pulmonary artery was collected from patients undergoing surgical lung resection for treatment adenocarcinoma, small cell carcinoma or squamous carcinoma. Histologically normal areas of the resected tissue was identified for study by a clinical pathologist. 5 male and 5 female patients with an average age 70.2 (range 54-84) donated lung parenchyma samples. Of these, 3 male and 2 female patients with an average age of 67.6 (range 57-78) also donated pulmonary artery. Lung tissue was provided through the Royal Brompton & Harefield NHS

Trust Biomedical Research Unit Advanced Lung Disease Biobank after ethical approval by the South Central – Hampshire B Research Ethics Committee (approval 15/SC/0101) and local project review by the Biomedical Research Unit Heads of Consortia (approval JM04).

Tissue and plasma prostanoid levels

Tissue/vessel segments: Tissue segments (approximately $10mm^3$) or vascular rings (approximately 2mm long) were cleaned of adherent material, transferred to 96-well microplate wells containing A23187 (Ca^{2+} ionophore; Sigma, UK; 30μM) or arachidonic acid (Sigma, UK; 30μM) in DMEM media (Sigma, UK). After 30 mins incubation at 37°C supernatant was collected. Where indicated, before tissue collection the lungs were flushed of blood by perfusion of the pulmonary vasculature with PBS via the right ventricle with effective perfusion confirmed by blanching of the lung tissue. In some experiments, tissues were pre-incubated with the selective COX-1 (SC-560, 1μM; Abcam Laboratories, UK) or COX-2 (rofecoxib; 1μM; Sigma, UK) for 90 mins prior to stimulation. In some experiments, aortic rings were denuded of endothelial cells by rubbing of the luminal surface with fine forceps, then snap frozen for RNA extraction.

Blood: Blood was collected from the inferior vena cava into heparin (10U/ml final; Leo Laboratories, UK) and stimulated with A23187 (30μM) for 30 mins, before centrifugation (8000g, 2 mins) and separation of conditioned plasma.

Homogenates: Segments of lung parenchyma were removed and snap frozen. Lung segments were suspended in 10X volume of ice cold PBS containing cOmplete Mini protease inhibitor cocktail (Roche, Switzerland), 2mM EDTA (Sigma, UK) and an excess of the non-selective cyclo-oxygenase inhibitor, diclofenac (1mM; Sigma, UK). Samples were immediately homogenised using a Precellys24 instrument (Bertin Instruments, France) and the supernatant collected after centrifugation (8000g; 2 mins).

Plasma: Blood was collected from the inferior vena cava into heparin (10U/ml final) immediately post-mortem. Plasma was separated by centrifugation (8000g, 2 mins) and stored.

Perfusates: Immediately post-mortem, the thoracic cavity was opened and the pulmonary vasculature flushed of blood with PBS via the right ventricle. The right atrium of the heart was then cannulated with PE10 tubing, secured with 5-0 silk and the lung and heart were removed intact. The pulmonary vasculature was perfused via the right atrium with DMEM media at 37°C for 20 mins using a peristaltic pump at 50μl/min and the venous outflow collected from the left atrium.

Levels of the prostacyclin breakdown product 6-keto-$PGF_{1\alpha}$, the thromboxane breakdown product TXB_2, PGE_2, $PGF_{2\alpha}$, PGD_2, 12-HETE and/or 15-HETE (Cayman Chemical, USA) were measured in supernatants/plasma by commercial immunoassay. In some cases tissue/vessel segments were weighed and prostanoid levels expressed relative to tissue mass.

qPCR

Tissue segments were snap frozen, then homogenised in ice cold RLT buffer (Qiagen, UK) containing β-mercaptoethanol (1% v/v; Sigma, UK) using a Precellys24 instrument. RNA was extracted using RNeasy mini-prep kits (Qiagen, UK) and gene expression levels measured using a 1-step RT-qPCR master mix (Promega, UK) and a 7500 Fast qPCR instrument (Applied Biosystems, UK) using TaqMan probes (Qiagen, UK) recognising Ptgis (probe ID: Mm00447271_m1), Ptgs1 (probe ID: Mm00477214_m1), Ptgs2 (probe ID: Mm00478374_m1) or the housekeeping genes 18S (probe ID: Mm03928990_g1) and GAPDH (probe ID: Mm99999915_g1). Data were analysed by the comparative Ct method, with relative expression levels normalised to those to 18S and GAPDH and experimental control groups.

Platelet prostacyclin bioassay

A human platelet bioassay was used to measure bioactive prostacyclin levels released from segments of mouse lung parenchyma (approximately $10mm^3$). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were separated from human blood by centrifugation (PRP: 230g, 15 mins; PPP: 8000g, 2 mins). PRP was pre-incubated with aspirin (30μM; 30 mins prior; Sigma, UK) and DEA/NONOate (10μM; 1 min prior; Sigma, UK) to sensitise platelets to prostacyclin. Lung segments were added to individual wells of 96 well microtitre plates containing PRP and pre-incubated for 1 min, before stimulation of platelets and tissues with A23187 (30μM) and vigorous mixing (1200RPM; BioshakeIQ, Q Instruments, Germany). After 5 mins, the tissue segments were removed and the absorbance of each well at 595nm measured by spectrophotometer and the amount of platelet aggregation calculated by reference to the absorbance of unstimulated PRP (0% aggregation) or PPP (100% aggregation).

Mouse lung fibroblast culture

For validation of fibroblast cyclo-oxygenase-1 knockout mice, lung fibroblast cultures were established using the explant method. Finely minced lung tissue was partially digested with collagenase I (5mg/ml; Sigma, UK) in PBS, then cultured in DMEM media for 10 days with regular media changes to remove debris until a homogenous monolayer of fibroblasts grew out. Cells were lysed in RLT buffer (Qiagen, UK) containing β-mercaptoethanol (1% v/v; Sigma, UK) for RNA extraction.

Human lung cell culture

Human primary lung microvascular endothelial cells (1 female, 2 male donors; Lonza, Germany) and human primary lung fibroblasts (3 female donors; Promocell, Germany) from 3 individual donors each were cultured according to suppliers instructions in full endothelial growth factor-2 media (Promocell, Germany) supplemented with 10% fetal calf serum (Biosera, UK) and penicillin/streptomycin (Sigma,

UK). At passage 4-8, cells were plated in 96-well culture plates at a density of 10,000 cells/well in the same media and allowed to settle overnight. The following day, media was replaced and cells stimulated with arachidonic acid (30μM) for 30 mins at 37°C before collection of media for measurement of the stable prostacyclin breakdown product, 6keto-$PGF_{1\alpha}$ by immunoassay. Cells were fixed and counted to confirm the density remained the same between types at the point of stimulation.

Thrombosis

Under isoflurane anaesthesia the left carotid artery was exposed and separated from the attached nerve and vein. $FeCl_3$ (4-6%; Sigma, UK) was applied to the adventitial surface of the vessel for 3 mins, then the vessel irrigated and a Doppler peri-vascular flow probe (Transonic Systems, UK) was secured around the artery. For each batch of experiments the $FeCl_3$ concentration applied was titrated to achieve a threshold injury in the control group (~25% rate of thrombotic occlusion) in order to provide the maximum window to observe a pro-thrombotic modulation of the treatment/genotype. Blood flow was recorded for 30 mins and time to occlusion recorded as the time taken from injury to the first point at which blood flow dropped <10% of baseline. If no occlusion occurred, occlusion time was recorded as 30 mins. In some experiments, after anaesthesia, the cyclo-oxygenase-1 inhibitor, SC-560 (10mg/kg; Cayman Chemical, USA) or vehicle (5% DMSO) were administrated intravenously (tail vein) 15 mins prior to arterial injury.

Cell isolation

Mouse lung, mouse aorta, human lung parenchyma and human pulmonary artery were finely minced with scissors in an enzyme cocktail of collagenase I (5mg/ml; Sigma, UK), DNase I (125U/ml; Sigma; UK) and elastase (100μg/ml; Sigma, UK) in PBS containing $CaCl_2$ (2mM; Sigma, UK) and incubated at 37°C with regular mixing until fully digested. Cycloheximide (3μM; Sigma, UK) was added to this an all solutions to prevent artefactual changes in prostanoid pathways during cell isolation. Erythrocytes were lysed using ammonium-chloride-potassium lysis buffer (Life Technologies, UK) and cells treated with FcR blocking antibodies (Biolegend, UK). Cell suspensions were then labelled and sorted according the specific protocol as below. All antibodies were purchased from Biolegend, UK.

Mouse lung vs aorta endothelial cells: Cells were stained with anti-CD45-PE/Cy7, anti-CD31-AlexaFluor488 and anti-CD41-APC/Cy7 and sorted using a FACSAria III instrument (BD Biosciences, Germany).

Mouse lung cell panel: Cell suspensions were divided and labelled with one of the following antibody cocktails before sorting using a FACSMelody instrument (BD Biosciences, Germany). Antibody mix 1 (endothelial/epithelial/leucocyte): anti-EpCAM-PE, anti-CD41-APC/Cy7, anti-CD45-PE/Cy7, anti-CD31-PerCP/Cy5.5 and anti-Podoplanin-APC. Antibody mix 2 (mesenchymal): anti-EpCAM-PE, anti-

CD41-PE, anti-CD45-PE, anti-Ter119-PE, anti-CD31-PerCP/Cy5.5, anti-Sca1-PE/Cy7, anti-PDGFRα-APC and anti-CD9-APC/Fire750.

Human lung cell panel: Cell suspensions were divided and labelled with one of the following antibody cocktails before sorting using a FACSMelody instrument (BD Biosciences, Germany). Antibody mix 1 (endothelial/epithelial): anti-CD41-APC/Cy7, anti-CD45-PerCP/Cy5.5, anti-CD31-FITC, anti-EpCAM-PE and anti-Podoplanin-APC. Antibody mix 2 (mesenchymal/leucocyte): anti-EpCAM-FITC, anti-CD31-FITC, anti-CD235a-FITC, anti-CD45-PerCP/Cy5.5, anti-CD41-APC/Cy7 and anti-CD146-PE.

Human pulmonary artery endothelial cells: Cells were stained with anti-CD41-APC/Cy7, anti-CD45-PerCP/Cy5.5 and anti-CD31-FITC and sorted using a FACSMelody instrument (BD Biosciences, Germany).

In each case, single live cells were identified from debris and doublets on the basis of scatter properties and negative DAPI staining (Figure S6) and any cells bound to platelets excluded on the basis of CD41 staining. Populations of interest were identified using the gating strategies shown Figures 4 and 5 and approximately 10,000 cells were sorted using 2-way or 4-way purity sort mode using a 100µm nozzle. Fluorescence minus-one (FMO) controls were used to validate staining and defining gating which was further confirmed by qPCR for cell-type marker gene expression in sorted populations (Table S3). After separation, cell populations were re-suspended in DMEM media containing arachidonic acid (30µM; Sigma, UK) and incubated at 37°C for 30 mins. The release reaction was stopped by addition of diclofenac (10µM; Sigma, UK) and levels of the prostanoids measured in the supernatant by immunoassay as above and expressed relative to cell count.

Statistics & data analysis

Data are presented as mean ± standard error for n experiments. n refers to the number of independent biological replicates studied in any experiment – either individual animals, human donors or primary cell lines established from tissue of separate donors. Where technical replicate measurements were made from the same individual, data were averaged prior to analysis. Samples from both male and female mice/donors were used, balanced across experimental groups in keeping with the ethical principles of animal and human research. Unless otherwise stated, data from both sexes were pooled and analysed as a single group because studies were not powered to consider sex as an independent variable. Exploratory analyses (Figure S2) indicated no effect of sex on the underlying biology being studied, however, the combination of both data from male and female samples into a single group may be considered a limitation of our study design.

Statistical analysis was performed using Prism 9 software (GraphPad Software, USA) with tests used indicated in individual figure legends. Normal distribution of each data set was determined using the Shapiro-Wilk test and this was used to dictate the choice of parametric versus non-parametric

statistical approach. Differences were considered significant where $p<0.05$. The corresponding author had full access to all the data in the study. All data are available in the main text or the supplementary material. Supporting material and resources are available from the corresponding author upon reasonable request.

Results

Tissues are major sources of prostacyclin generated through cyclo-oxygenase-1

Using the aorta as a benchmark, we first assayed prostacyclin formation per unit mass from paired mouse tissues to appreciate their relative capacity to generate prostacyclin. Prostacyclin release (stimulated by Ca^{2+} ionophore and measured as 6-keto-$PGF_{1\alpha}$) was observed from all tissues, with the lowest release from the renal cortex and heart and the highest release from the lung and colon (Figure 1A). Lung and colon produced equivalent prostacyclin to aorta on a 'per mg tissue' basis, which, considering their large total mass, suggests they may be major contributors to whole body prostacyclin generation. The relative ability of tissues to generate prostacyclin broadly correlated with prostacyclin synthase gene (*Ptgis*) expression which was enriched in aorta, colon and lung relative to other tissues (Figure 1B; Table S1). By contrast, relative tissue levels of cyclo-oxygenase-1 gene (*Ptgs1*) expression correlated poorly with prostacyclin release; for example, the aorta and lung expressed comparatively little *Ptgs1* gene (Figure 1B; Table S1). Cyclo-oxygeanse-2 (*Ptgs2*) was weakly expressed across all tissues (~10-100-fold less than *Ptgs1*; Figure 1B; Table S1) in keeping with our previous observations of the relative constitutive expression and activity of the two cyclo-oxygenase isoforms[12]. In agreement, global cyclo-oxygenase-1 (*Ptgs1*$^{-/-}$) deletion abolished prostacyclin generation in all tissues studied (Figure 1C). This dominance of cyclo-oxygenase-1 in tissue prostacyclin generation in the systems studied cannot be explained by a loss of shear-maintained vascular cyclo-oxygenase-2 expression ex vivo[15] because assays were completed within 1hr post-mortem and cyclo-oxygenase-2 protein has a half life of >6hrs[12]. Thus, arteries and tissues both generate prostacyclin which is (i) driven by cyclo-oxygenase-1 activity but (ii) at a gene expression level, reflects the relative level of prostacyclin synthase.

Many tissues can produce prostacyclin in the absence of endothelial cell cyclo-oxygenase-1

We next considered whether tissue prostacyclin release is simply a function of the constituent endothelial component, or whether other cell types generate prostacyclin directly. To do this we used mice in which cyclo-oxygenase-1 is specifically deleted from endothelial cells (Ptgs1$^{flox/flox}$; VE-cadherin-Cre^{ERT2}) which have been characterised previously[4]. Aortic rings from these mice had marked reduction (~80%) in prostacyclin release (Figure 2A) as did veins (Figure 2B) and arteries supplying the lung (Figure 2C), kidney (Figure 2D) and gut (Figure 2E). However, the effect of endothelial cyclo-oxygenase-1 deletion on prostacyclin release from isolated tissue segments was variable. In the heart, prostacyclin release when endothelial cyclo-oxygenase 1 is deleted was

reduced ~50% suggesting a major role of endothelial cyclo-oxygnease-1 in prostacyclin in this tissue (Figure 2F). In contrast, in the lung (Figure 2G), colon (Figure 2H), kidney (Figure 2I) and spleen (Figure 2J) endothelial cyclo-oxygenase-1 deletion had no effect on prostacyclin release. The residual 'endothelium-independent' tissue prostacyclin release was not accounted for by vascular smooth muscle cyclo-oxygenase-1 activity; smooth muscle cyclo-oxygenase-1 knockout mice (*Ptgs1*$^{flox/flox}$;Sm22a-Cre)[4], exhibited no change in prostacyclin release in any tissue studied (Figure 2F-J). Thus, within most tissues, bulk prostacyclin production appears to be driven by non-endothelial, non-smooth muscle cyclo-oxygenase-1 activity. This was independent of sex because post-hoc analysis indicated similar prostacyclin release by lung from male and female animals and no interaction between animal sex and the effect of endothelial cyclo-oxygenase-1 deletion (Figure S2). As such, follow on studies used pooled data from both male and female animals/donors.

To validate and explore further these observations we focused on the lung because (1) lung has the highest prostaglandin production amongst tissues (Figure 1A), (2) lung prostacyclin production appeared to be almost completely independent of vascular endothelial/smooth muscle cyclo-oxygenase-1 (Figure 2G) and (3) the anatomical position of the lung means prostacyclin generated here is likely to directly influence the heart and major arteries[22]. We first considered if there may be other sources that can donate prostaglandin H_2 to endothelial cells for conversion to mature prostacyclin, bypassing the effect of endothelial cyclo-oxygenase deletion. This phenomenon of 'transcellular metabolism' has been previously described in platelet/endothelial co-cultures where platelet-derived prostaglandin H_2 can enter endothelial cells to access prostacyclin synthase[39]. However, even after flushing the pulmonary vasculature of blood to remove circulating platelets, endothelial cyclo-oxygenase-1 deletion had no effect on prostacyclin release (Figure 3A). We further confirmed this result by studying dual endothelial/platelet cyclo-oxygenase-1 knockout mouse (*Ptgs1*$^{flox/flox}$;Tie2-Cre) where prostaglandin H_2 cannot be synthesised either by platelets or by endothelial cells[4]. As observed in endothelial-specific cyclo-oxygenase-1 knockout mice, endothelial/platelet cyclo-oxygenase-1 deletion had no effect on lung tissue release of prostacyclin stimulated by Ca^{2+} ionophore (Figure 3B) or exogenous arachidonic acid (Figure 3C). Whilst valuable in determining gross synthetic capacity, *ex vivo* mediator release assays have a potential to produce artefactual results due to loss of the *in vivo* environment, use of exogenous stimuli and removal from normal metabolic/excretion pathways. We therefore considered if these patterns of prostacyclin generation seen in *ex vivo* release assays corresponded to production *in vivo*. Measuring prostanoid formation *in vivo* is complex, with different approaches favoured by different researchers, and no universally agreed upon techniques. We took the approach of measuring prostacyclin levels in snap frozen lung tissue, cold homogenised in an excess of cyclo-oxygenase inhibitor to prevent *ex vivo* prostanoid generation. In this system, lung prostacyclin levels (as 6keto-$PGF_{1\alpha}$) were detected at modest levels of ~100pg/mg tissue (Figure 3D) which compares well with the findings of others using

mass spectrometry-based methods[40]. Using this approach we confirmed no effect of endothelial/platelet cyclo-oxygenase-1 deletion on *in vivo* lung prostacyclin levels (Figure 3D). To exclude a role for other pathways of prostaglandin H_2 generation, we studied lung from endothelial/platelet prostacyclin synthase knockout mice (Figure 3E; *Ptgis*$^{flox/flox}$;Tie2-Cre) and endothelial/platelet cyclo-oxygenase-2 knockout mice (Figure 3F; *Ptgs2*$^{flox/flox}$;Tie2-Cre). Lung from both strains retained a full capacity to generate prostacyclin, confirming that endothelial cells in the lung are neither required to generate prostaglandin H_2 nor to convert prostaglandin H_2 from other sources into prostacyclin. Data from these mouse knockout models also corroborated the relative role of endothelium in prostacyclin release in other tissues – these are presented in Table S2 but not discussed further. To extended this, we established whether non-endothelial prostacyclin release could be mediated by co-operativity or redundancy from cyclo-oxygenase-2 when endothelial cyclo-oxygenase-1 was disrupted. Selective cyclo-oxygenase-2 inhibition by rofecoxib had no impact on prostacyclin release from endothelial/platelet cyclo-oxygenase-1 knockout lung tissue (Figure 3G). By contrast, global cyclo-oxygenase-1 inhibition by SC-560 abolished prostacyclin release (Figure 3G) in agreement with our data from global cyclo-oxygenase-1 knockout mouse tissue (Figure 1C). As such, we focussed on cyclo-oxygenase-1-mediated pathways for subsequent experiments to understand non-endothelial prostacyclin generation.

We next explored whether retention of prostacyclin generation by the lung in these models might be due to incomplete penetrance of Cre-mediated recombination in tissue versus arterial endothelial cells. Previous studies have effectively used Tie2-Cre[41] and VE-cadherin-Cre[42] to delete floxed genes in lung microvasculature. To confirm this we crossed Tie2-Cre mice (used to generated endothelial/platelet cyclo-oxygenase-1, cyclo-oxygenase-2 and prostacyclin synthase knockout mice) and Smmhc-Cre mice (used to generate smooth muscle cyclo-oxygenase-1 knockout mice) with an EGFP reporter strain. Robust recombination occurred throughout the lung (Figure 3H). We went on to isolate live microvascular endothelial cells from lung of endothelial/platelet cyclo-oxygenase-1 knockout mice by FACS and found ~70% reduction in prostacyclin production compared to cells isolated from the lung of floxed littermate control mice; these data confirm effective loss of cyclo-oxygenase-1 activity in lung endothelium (Figure 3I). Finally, to ensure that 6keto-$PGF_{1\alpha}$ detected by immunoassay represented release of genuine bioactive prostacyclin we used a human platelet bioassay[4], analogous to the methodology used in the original identification of prostacyclin[1]. These experiments confirmed that global cyclo-oxygenase-1-deficiency/inhibition, but not endothelium-specific or endothelial/platelet cyclo-oxygenase-1 deletion resulted in loss of prostacyclin-like activity released from the lung in full agreement with idea that lung prostacyclin generation occurs independently of the endothelial cyclo-oxygenase-1/prostacyclin synthase pathway (Figure S3).

Endothelial cells from the lung are deficient in prostacyclin synthesis compared to those from large arteries

The lung is amongst the most highly vascularised organs in the body therefore to understand the presence of a non-endothelial prostacyclin pathway we had to first determine why lung endothelial cells do not meaningfully contribute to prostacyclin production. Lung microvascular endothelial cells have previously been observed to possess an altered prostanoid synthesis profile compared to arterial endothelial cells, particularly elevated PGE_2 formation[29,43]. However, to our knowledge freshly isolated lung microvascular and arterial endothelial cells have not been compared head-to-head for their relative ability to synthesise prostacyclin. Therefore, to address this issue we sorted fresh, matched endothelial cells ($CD31^+$, $CD45^-$, $CD41^-$) from mouse aorta and lung by FACS (Figure 4A) and tested their ability to release prostacyclin immediately after isolation. Endothelial cells from lung released prostacyclin at much lower levels compared to those from the aorta (Figure 4B) in agreement with the broader idea that endothelial cells from the lung microvasculature carry a more immature, stem-like phenotype in comparison to endothelial cells from arteries and veins[44]. Moreover, single cell RNA sequencing studies of endothelial heterogeneity identified prostacyclin synthase (*PTGIS*) in the top 20 transcripts differentiating human lung arterial and capillary endothelial cells[45]. To translate our findings from mice we obtained matched, histologically normal pulmonary artery and lung parenchyma from human donors undergoing lung resection for carcinoma and isolated endothelial cells from each using the same approach (Figure 4C). As observed in mice, endothelial cells from human lung parenchyma showed the same pattern of lesser prostacyclin synthesis when compared to endothelial cells isolated from pulmonary arteries of the same individuals (Figure 4D).

Fibroblasts are the principal contributors to prostacyclin production in the lung

If endothelial cells are not the major source of lung prostacyclin, what cell type(s) account for its production? This question cannot be addressed by immunohistochemical/gene expression approaches, both because of the complex cascade of enzymes and biochemical factors required to support prostacyclin synthesis and because of limitations in the specificity/sensitivity of antibodies. Therefore we again studied cell populations rapidly isolated by FACS from fresh mouse lung tissue. Using endothelial cells ($CD31^+$ $CD45^-$) as a benchmark, we profiled prostacyclin release from epithelial cells ($EpCAM^+$, $Podoplanin^-$), type I alveolar epithelial cells ($EpCAM^+$, $Podoplanin^+$) and leucocytes ($CD45^+$) (Figure 4E) as previously described[46]. We also isolated a mesenchymal cell population by exclusion ($EpCAM^-$, $CD45^-$, $CD31^-$, $Ter119^-$) and, within this, used an approach defined from single cell RNAseq analysis of the lung[47] to select for smooth muscle cells ($CD146^+$), adventitial fibroblasts ($CD146^-$ $Sca1^+$), alveolar fibroblasts ($CD146^-$ $Sca1^-$ $PDGFR\alpha^+$) and peribronchial fibroblasts ($CD146^-$, $Sca1^-$, $PDGFR\alpha^-$, $CD9^+$) (Figure 4E). When stimulated with arachidonic acid, leucocytes, epithelial cells and smooth muscle cells released comparable (and numerically less) prostacyclin to endothelial cells (Figure 4F). However, fibroblasts exhibited significantly greater

prostacyclin production (up to 6-fold greater than endothelial cells; Figure 4F). Importantly this was true of each type of fibroblast studied including peri-bronchial, alveolar and adventitial fibroblasts and indicated that within the lung prostacyclin may be generated by fibroblasts in the lung both within and outside the vascular wall. This ability of fibroblasts to generate prostacyclin was selective, as release of other prostanoids (TXA_2, PGE_2, $PGF_{2\alpha}$ and PGD_2) was low and comparable to endothelial cells (Figure 4G). To exclude the possibility that the prostacyclin generating capacity associated with fibroblast fractions represented contamination with other cell types we performed additional characterisation of FACS sorted fractions by RT-qPCR. We found expression of the endothelial cell marker, VE-cadherin (*Cdh5*), was restricted to the endothelial cell fraction and expression of the smooth muscle cell and pericyte markers, α-smooth muscle actin (*Acta2*), neural/glial antigen-2 (*Cspg4)* and PDGFRβ (*Pdgfrb*) restricted to the SMC fraction (Table S3). The three fibroblast fractions showed little to no expression of any of the above markers, confirming their purity (Table S3). These data therefore support the hypothesis that lung endothelial, smooth muscle, pericytes and leucocytes are poor producers of prostacyclin in comparison to lung fibroblasts (both vascular and non-vascular). It cannot be excluded there may also be roles for other niche vascular/non-vascular cell types that we have not considered.

We replicated these studies with cells isolated from fresh, histologically normal human lung tissue. Endothelial cells ($CD31^+$ $CD45^-$), epithelial cells ($EpCAM^+$, $Podoplanin^-$), type I alveolar epithelial cells ($EpCAM^+$, $Podoplanin^+$) and leucocytes ($CD45^+$) were defined and isolated in the same fashion as from mouse lung (Figure 4H). Because suitable cell surface markers to positively select human lung fibroblast populations have not been defined, we studied only a negatively selected 'mesenchymal cell' population ($EpCAM^-$, $CD45^-$, $CD31^-$, $CD235a^-$) which was subdivided into smooth muscle cells ($CD146^+$) and other cells ($CD146^-$) (Figure 4H). Prostacyclin release from leucocytes, epithelial cells and smooth muscle was low, when compared to endothelial cells (Figure 4I). Prostacyclin levels released from negatively selected mesenchymal cells were similar to those from endothelial cells (Figure 4I). These data are consistent with the suggestion that, in human lung, both fibroblasts and endothelial cells are contributors to total prostacyclin release. However, because this negative selection approach may underestimate fibroblast prostacyclin synthesis as a consequence of contamination by other cell types we performed similar experiments using commercially sourced primary human lung cell cultures (Figure 4J). In carefully matched experiments where primary cells of similar and low passage were grown under identical conditions and stimulated with arachidonic acid, prostacyclin levels released from primary human lung fibroblasts were greater than those from primary lung microvascular endothelial cells (Figure 4K). Although these data are interpreted with some caution given the changes in prostanoid pathways that can occur *in vitro*[48], the results are fully supportive and in agreement with our findings from freshly isolated mouse and human cells that fibroblasts are central contributors to lung prostacyclin production.

Having identified fibroblasts as the major prostacyclin-producing cells in mouse and human lung, we returned to a mouse cell-specific knockout approach to understand the fibroblast contribution to bulk tissue prostacyclin release. We generated a fibroblast cyclo-oxygenase-1 knockout model driven by Cre expression from the fibroblast-specific protein-1 (Fsp1/S100a4) promoter which is expressed lung fibroblasts[49]. Although, this promoter is also active in monocyte/macrophages, these cells have almost no prostacyclin synthetic capacity (Figures 4F, 4I), suggesting there should be little consequence of 'off-target' deletions here. To confirm the suitability of this model, we crossed Fsp1-Cre mice with EGFP reporter animals and observed robust recombination within the lung (Figure 5A). When Fsp1-Cre mice were crossed onto a $Ptgs1^{flox/flox}$ background to generate fibroblast-specific cyclo-oxygenase-1 knockout mice (*$Ptgs1^{flox/flox}$;Fsp1-Cre*) we observed a loss of COX1 mRNA in lung-derived fibroblasts but full retention of COX1 mRNA in aorta (Figure S1). A ~50% reduction in stimulated prostacyclin release was seen from intact segments of lung parenchyma from these mice (Figure 5B) and this was accompanied by a similar reduction in total lung COX1 mRNA (Figure S1). Post-hoc analysis indicated that the effect on prostacyclin release independent of animal sex (Figure S2). In agreement with our data from isolated lung cells, this effect was selective to prostacyclin; release of the other primary prostanoids (PGE_2, TXA_2, PGD_2 and $PGF_{2\alpha}$) as well as the related eicosanoids 12- and 15-HETE were not altered from fibroblast cyclo-oxygenase-1 knockout mouse lung (Figure 5C). Data from these *ex vivo* release experiments reflected production *in vivo* with prostacyclin levels in snap frozen lung homogenates reduced by fibroblast-specific cyclo-oxygenase-1 deletion (Figure 5D). These data support our observations from mouse and human cells and the hypothesis that fibroblasts are major contributors to lung tissue prostacyclin production. Because lung prostacyclin release is abolished by global cyclo-oxygenase-1 deletion (Figure 1C) or inhibition (Figure 3G) the residual prostacyclin synthesis observed in these mice is unlikely to be accounted for by cyclo-oxygenase-2 and instead may be attributed to cyclo-oxygenase-1 in other cell types or sub-sets of fibroblasts which do not express the Fsp1-Cre transgene. Fibroblast-specific cyclo-oxygenase-1-deletion also showed a strong trend to reduce prostacyclin release from gut tissue (colon; p=0.07), which, like the lung, exhibited prostacyclin release essentially independent of the endothelial cyclo-oxygenase-1 (Table S4). No effect of fibroblast-specific cyclo-oxygenase-1 knockout on prostacyclin release was observed from pulmonary artery (Figure 5E), bronchi (Figure 5F), aorta, heart, kidney or spleen (Table S4).

Non-endothelial prostacyclin can act systemically to reduce anti-thrombotic tone

To consider the biological significance of prostacyclin produced outside the vascular wall we explored whether the prostacyclin produced by fibroblasts in tissues acts simply as a local lung mediator and/or can it produce systemic effects either by entering the circulation or acting on circulating cells as they pass through organs. To do this we used an *ex vivo* isolated perfused lung preparation in which

released prostacyclin is collected through the organ's vasculature. In this model, prostacyclin in the perfused tissue effluent (detected as its hydrolysis product 6keto-$PGF_{1\alpha}$) was reduced in both endothelial/platelet cyclo-oxygenase-1 knockout and fibroblast-specific cyclo-oxygenase-1 knockout lung (Figure 5G). To establish whether this also occurs *in vivo* we measured levels of 6keto-$PGF_{1\alpha}$ in plasma. Although some have questioned the validity of immunoassay approaches to measurement of prostacyclin in complex biological fluids[50] we have previously validated our ELISA technique against LC/MS/MS and found direct quantitative equivalence in mouse plasma[8] and separately shown that the immunoreactive 6keto-$PGF_{1\alpha}$ in mouse plasma is completely lost by specific deletion of prostacyclin synthase[51] or cyclo-oxygenase-1[4]. Using this same approach we found plasma 6keto-$PGF_{1\alpha}$ levels to be reduced in plasma from both endothelial/platelet and fibroblast cyclo-oxygenase-1 knockout mice, when compared to their respective control strains (Figure 5H). Thus, prostacyclin derived from both endothelial cells and prostacyclin derived from cyclo-oxygenase-1 in fibroblasts can be detected as 6keto-$PGF_{1\alpha}$ in the vascular compartment. This implies prostacyclin generated in the lung has the potential to exert systemic effects, either by brief circulation of the vasculature in a bioactive form (which is plausible but a controversial idea[52,53]), or by acting locally on platelets and circulating cells as they pass through the lung circulation.

To address this we determined whether non-endothelial prostacyclin depots contribute to cardiovascular protection in the same way we understand for endothelium-derived prostacyclin. This was particularly important given only a partial lung prostacyclin reduction was observed in fibroblast-specific cyclo-oxygenase-1 knockout mice. The biological effects of partial prostacyclin deficiency have not been fully explored but it is important to note that we have found heterozygous deletion of prostacyclin synthase is sufficient to exacerbate renal ischemia perfusion injury in mice[28] and others have found heterozygous prostacyclin receptor mutations in man are associated with increased atherothrombtic risk[5]. Prostacyclin is best understood as an anti-platelet/anti-thrombotic factor, balancing the action of platelet-derived thromboxane. We have previously used an *in vivo* $FeCl_3$ carotid artery injury model to demonstrate a pro-thrombotic phenotype in endothelium-specific cyclo-oxygenase-1 knockout mice[4] and an anti-thrombotic phenotype in platelet-specific or dual endothelial/plateletcyclo-oxygeanse-1 knock out mice[54],[4]. Here we used the same model to show that fibroblast-specific cyclo-oxygenase-1 knockout mice exhibit a modest but significant pro-thrombotic phenotype (Figure 6A,B). This result was not associated with any change in local carotid artery prostacyclin generation (Flox Ctrl: 33.1±9.5ng/ml; Fibro COX1 KO: 34.5±23.3ng/ml; n=6; $p>0.05$ by unpaired t-test), which we have previously shown to be predominately generated by endothelial cells[4]. We next considered if there may be other sources that contribute to the total anti-thrombotic contribution of all non-endothelial cyclo-oxygenase-1-mediated prostacyclin generation in the body that are not accounted for by fibroblast-specific deletion. To address this limitation, we treated endothelial/platelet cyclo-oxygenase-1 knockout mice with the selective cyclo-oxygenase-1 inhibitor,

SC-560, to determine the effect of removal of all residual cyclo-oxygenase-1-derived prostanoids. Because these mice already lack cyclo-oxygenase-1 in platelets and endothelial cells, no effect of SC-560 on carotid artery prostacyclin levels (Figure 6C) or platelet thromboxane levels was noted (Figure S4) but prostacyclin release by lung tissue was reduced ~50% (Figure 6D). This was associated with a marked increase in thrombosis after carotid artery $FeCl_3$ injury (Figure 6E,F). This effect could not be attributed to an off-target SC-560 effect, because SC-560 had no effect on thrombosis in global cyclo-oxygenase-1-deficient mice (Figure S5). These data support the idea that inhibitory prostanoids derived from cyclo-oxygenase-1 in fibroblasts and other non-endothelial sources contribute to systemic anti-thrombotic protection. This may occur either through fibroblast-derived prostanoids directly entering the vascular compartment and circulating to sites of thrombosis, or by 'conditioning' platelets as they pass through the vasculature of the lung and other organs. It should be noted that it cannot be unequivocally proven that this effect is mediated by prostacyclin because models of fibroblast prostacyclin synthase deletion would exhibit shunting of excess PGH_2 into other prostanoid products[51]. However, given that prostacyclin is the major anti-thrombotic prostanoid species, and that prostacyclin is the most abundant prostanoid produced by fibroblasts (Figure 4G) it is logically the most likely candidate to mediate the anti-thrombotic role of fibroblast cyclo-oxygenase-1. Nonetheless, roles for other prostanoids such as PGD_2 in this effect cannot be definitively excluded.

Discussion

The identification of prostacyclin was rapidly followed by the idea that it's manipulation could offer the means to prevent and treat cardiovascular disease[1,2]. Since its discovery, prostacyclin has been mostly commonly associated with the endothelium. Our current findings suggest however a substantive role for fibroblasts in both cyclo-oxygenase-1-mediated prostacyclin synthesis and systemic anti-thrombotic protection. Whilst prostacyclin production by fibroblasts and other non-endothelial sources is not in itself a novel concept, this report demonstrates that these other depots of prostacyclin production make a meaningful and functionally significant contribution to prostacyclin's systemic bioactivity. Indeed, whilst isolated fibroblasts from several tissues have been shown previously to have the ability to produce prostacyclin in vitro[24-26], to our knowledge this is the first demonstration of the extent to which the scale of this contribution impacts on a systemic biological effect *in vivo*. Thus, we must now consider the biological role and therapeutic potential of non-endothelial prostacyclin in the lung and elsewhere:

Firstly, it is clear that pulmonary vascular disease is associated with prostacyclin deficiency[55] and cardiovascular risk. These new findings raise the possibility that diseases of the lung parenchyma may also be associated with a loss of cardioprotective prostacyclin. For example, both chronic obstructive pulmonary disorder and interstitial lung disease feature dysfunction, damage, and

phenotypic alterations to a range of lung parenchymal cells, including fibroblasts. An impairment of the ability of these cells to produce prostacyclin could not only contribute to disease pathogenesis but also explain the increased atherothrombotic risk observed in both conditions[56,57]. Indeed, lung tissue from chronic obstructive pulmonary disorder patients exhibited reduced prostacyclin synthase expression[58] and fibroblasts isolated from pulmonary fibrosis patients show reduced prostacyclin and increased thromboxane formation[59]. These data suggest that a prostacyclin-based therapy may help to mitigate the excess cardiovascular risk and/or treat disease progression in these specific patient groups. In support of this suggestion, it has recently been found that inhaled trepostinil has therapeutic benefits in the treatment of idiopathic pulmonary fibrosis[60].

Secondly, our finding that lung microvascular endothelial cells *in vivo* are relatively deficient in prostacyclin production may also suggest an opportunity to boost its endogenous generation to treat both lung and thrombotic disease. Lung over-expression of prostacyclin synthase is protective including in models of pulmonary hypertension[61] and whilst this non-specific delivery is clearly effective, in light of our findings we suggest that, to maximise efficacy and reduce the systemic side effects, prostacyclin synthase delivery approaches would best be targeted to the pulmonary endothelium. Recently developed simple polymer-based transfection reagents that selectively deliver mRNA cargoes to lung endothelium[62] could provide a practical route to achieving this.

Thirdly, we must also consider the possibility that fibroblast-derived prostacyclin may have important autocrine or paracrine roles. Prostacyclin has well-defined effects on lung cell function including bronchodilation, immunomodulation, and inhibition both of fibrosis and proliferation. For example, we have previously shown that cyclo-oxygenase-1-derived prostanoids regulate airway function[63] and others have shown roles for endogenous prostacyclin in lung fibrosis after bleomycin-induced lung injury[64] and in regulating intravascular thrombosis in pulmonary hypertension[65]. All these responses may be associated, partly or wholly, with prostacyclin derived from non-endothelial sources which should now be considered. Whilst it has not been the focus of the current study, it should be remember that in settings of disease[64] or discrete local niches[66], there may be equally important roles for prostanoids derived from cyclo-oxygenase-2 in fibroblasts or other cell types.

The previously unappreciated degree to which non-endothelial sources contribute to prostacyclin generation presents a new concept. This is not suggested to reduce emphasis on endothelial cyclo-oxygenase-1[4] and cyclo-oxygenase-2-derived prostacyclin[4] and the consequences of these powerful anti-thrombotic pathways – the evidence of their importance is clear. It should also not be forgotten that prostacyclin signalling pathways may be activated by non-traditional ligands including 12-HETrE[67], other prostanoids[68] and molecules derived from other fatty acids substrates[69,70]. Our findings, however, suggest we must now consider an *additional*, parallel cardioprotective and/or local

disease modifying pathway associated with cyclo-oxygenase-1 and prostacyclin outside the vascular endothelium and smooth muscle. In principle, each of these pathways results in activation of the same prostacyclin receptor signalling cascades; but there appears to be a lack of redundancy such that each of these alternative sources of prostacyclin synthesis and receptor activation carries unique biological functions. Consequently, loss of any individual prostacyclin synthesis/activation pathway may have consequences for cardiovascular health. The roles of these, individually and together, should now be evaluated in context, through health and disease and across organ systems. The results suggest new therapeutic opportunities within these new prostacyclin pathways, for the treatment of a range of human diseases, including cardiovascular disease as a co-morbidly of respiratory conditions.

References

1. Moncada S, Gryglewski R, Bunting S, Vane JR. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*. 1976;263:663-665. doi: 10.1038/263663a0
2. Mitchell JA, Kirkby NS. Eicosanoids, prostacyclin and cyclooxygenase in the cardiovascular system. *Br J Pharmacol*. 2019;176:1038-1050. doi: 10.1111/bph.14167
3. Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, et al. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature*. 1997;388:678-682. doi: 10.1038/41780
4. Mitchell JA, Shala F, Elghazouli Y, Warner TD, Gaston-Massuet C, Crescente M, Armstrong PC, Herschman HR, Kirkby NS. Cell-Specific Gene Deletion Reveals the Antithrombotic Function of COX1 and Explains the Vascular COX1/Prostacyclin Paradox. *Circ Res*. 2019;125:847-854. doi: 10.1161/CIRCRESAHA.119.314927
5. Arehart E, Stitham J, Asselbergs FW, Douville K, MacKenzie T, Fetalvero KM, Gleim S, Kasza Z, Rao Y, Martel L, et al. Acceleration of cardiovascular disease by a dysfunctional prostacyclin receptor mutation: potential implications for cyclooxygenase-2 inhibition. *Circ Res*. 2008;102:986-993. doi: 10.1161/CIRCRESAHA.107.165936
6. Kobayashi T, Tahara Y, Matsumoto M, Iguchi M, Sano H, Murayama T, Arai H, Oida H, Yurugi-Kobayashi T, Yamashita JK, et al. Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. *J Clin Invest*. 2004;114:784-794. doi: 10.1172/JCI21446
7. Kirkby NS, Reed DM, Edin ML, Rauzi F, Mataragka S, Vojnovic I, Bishop-Bailey D, Milne GL, Longhurst H, Zeldin DC, et al. Inherited human group IVA cytosolic phospholipase A2 deficiency abolishes platelet, endothelial, and leucocyte eicosanoid generation. *FASEB J*. 2015;29:4568-4578. doi: 10.1096/fj.15-275065

8. Kirkby NS, Zaiss AK, Urquhart P, Jiao J, Austin PJ, Al-Yamani M, Lundberg MH, MacKenzie LS, Warner TD, Nicolaou A, et al. LC-MS/MS confirms that COX-1 drives vascular prostacyclin whilst gene expression pattern reveals non-vascular sites of COX-2 expression. *PLoS One*. 2013;8:e69524. doi: 10.1371/journal.pone.0069524
9. McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci U S A*. 1999;96:272-277. doi: 10.1073/pnas.96.1.272
10. Mitchell JA, Knowles RB, Kirkby NS, Reed DM, Edin ML, White WE, Chan MV, Longhurst H, Yaqoob MM, Milne GL, et al. Kidney Transplantation in a Patient Lacking Cytosolic Phospholipase A2 Proves Renal Origins of Urinary PGI-M and TX-M. *Circ Res*. 2018;122:555-559. doi: 10.1161/CIRCRESAHA.117.312144
11. Liu B, Luo W, Zhang Y, Li H, Zhu N, Huang D, Zhou Y. Involvement of cyclo-oxygenase-1-mediated prostacyclin synthesis in the vasoconstrictor activity evoked by ACh in mouse arteries. *Exp Physiol*. 2012;97:277-289. doi: 10.1113/expphysiol.2011.062034
12. Kirkby NS, Lundberg MH, Harrington LS, Leadbeater PD, Milne GL, Potter CM, Al-Yamani M, Adeyemi O, Warner TD, Mitchell JA. Cyclooxygenase-1, not cyclooxygenase-2, is responsible for physiological production of prostacyclin in the cardiovascular system. *Proc Natl Acad Sci U S A*. 2012;109:17597-17602. doi: 10.1073/pnas.1209192109
13. Mitchell JA, Lucas R, Vojnovic I, Hasan K, Pepper JR, Warner TD. Stronger inhibition by nonsteroid anti-inflammatory drugs of cyclooxygenase-1 in endothelial cells than platelets offers an explanation for increased risk of thrombotic events. *FASEB J*. 2006;20:2468-2475. doi: 10.1096/fj.06-6615com
14. Kirkby NS, Chan MV, Lundberg MH, Massey KA, Edmands WM, MacKenzie LS, Holmes E, Nicolaou A, Warner TD, Mitchell JA. Aspirin-triggered 15-epi-lipoxin A4 predicts cyclooxygenase-2 in the lungs of LPS-treated mice but not in the circulation: implications for a clinical test. *FASEB J*. 2013;27:3938-3946. doi: 10.1096/fj.12-215533
15. Topper JN, Cai J, Falb D, Gimbrone MA, Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci U S A*. 1996;93:10417-10422. doi: 10.1073/pnas.93.19.10417
16. Potter CM, Lundberg MH, Harrington LS, Warboys CM, Warner TD, Berson RE, Moshkov AV, Gorelik J, Weinberg PD, Mitchell JA. Role of shear stress in endothelial cell morphology and expression of cyclooxygenase isoforms. *Arterioscler Thromb Vasc Biol*. 2011;31:384-391. doi: 10.1161/ATVBAHA.110.214031

17. Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A, et al. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med*. 2005;352:1092-1102. doi: 10.1056/NEJMoa050493
18. Yu Y, Ricciotti E, Scalia R, Tang SY, Grant G, Yu Z, Landesberg G, Crichton I, Wu W, Pure E, et al. Vascular COX-2 modulates blood pressure and thrombosis in mice. *Sci Transl Med*. 2012;4:132ra154. doi: 10.1126/scitranslmed.3003787
19. Moncada S, Herman AG, Higgs EA, Vane JR. Differential formation of prostacyclin (PGX or PGI2) by layers of the arterial wall. An explanation for the anti-thrombotic properties of vascular endothelium. *Thromb Res*. 1977;11:323-344. doi: 10.1016/0049-3848(77)90185-2
20. Kennedy MS, Stobo JD, Goldyne ME. In vitro synthesis of prostaglandins and related lipids by populations of human peripheral blood mononuclear cells. *Prostaglandins*. 1980;20:135-145. doi: 10.1016/0090-6980(80)90013-1
21. de Deckere EA, Nugteren DH, Ten Hoor F. Prostacyclin is the major prostaglandin released from the isolated perfused rabbit and rat heart. *Nature*. 1977;268:160-163. doi: 10.1038/268160a0
22. Gryglewski RJ, Korbut R, Ocetkiewicz A. Generation of prostacyclin by lungs in vivo and its release into the arterial circulation. *Nature*. 1978;273:765-767. doi: 10.1038/273765a0
23. Taylor L, Polgar P, McAteer JA, Douglas WH. Prostaglandin production by type II alveolar epithelial cells. *Biochim Biophys Acta*. 1979;572:502-509. doi: 10.1016/0005-2760(79)90157-7
24. Claesson HE, Lindgren JA, Hammarstrom S. Elevation of adenosine 3',5'-monophosphate levels in 3T3 fibroblasts by arachidonic acid: evidence for mediation by prostaglandin I2. *FEBS Lett*. 1977;81:415-418. doi: 10.1016/0014-5793(77)80567-x
25. Yu H, Gallagher AM, Garfin PM, Printz MP. Prostacyclin release by rat cardiac fibroblasts: inhibition of collagen expression. *Hypertension*. 1997;30:1047-1053. doi: 10.1161/01.hyp.30.5.1047
26. Larsson-Callerfelt AK, Hallgren O, Andersson-Sjoland A, Thiman L, Bjorklund J, Kron J, Nihlberg K, Bjermer L, Lofdahl CG, Westergren-Thorsson G. Defective alterations in the collagen network to prostacyclin in COPD lung fibroblasts. *Respir Res*. 2013;14:21. doi: 10.1186/1465-9921-14-21
27. Baenziger NL, Becherer PR, Majerus PW. Characterization of prostacyclin synthesis in cultured human arterial smooth muscle cells, venous endothelial cells and skin fibroblasts. *Cell*. 1979;16:967-974. doi: 10.1016/0092-8674(79)90111-9
28. Cao Y, Guan Y, Xu YY, Hao CM. Endothelial prostacyclin protects the kidney from ischemia-reperfusion injury. *Pflugers Arch*. 2019;471:543-555. doi: 10.1007/s00424-018-2229-6

29. Mitchell JA, Shala F, Pires MEL, Loy RY, Ravendren A, Benson J, Urquhart P, Nicolaou A, Herschman HR, Kirkby NS. Endothelial cyclooxygenase-1 paradoxically drives local vasoconstriction and atherogenesis despite underpinning prostacyclin generation. *Sci Adv*. 2021;7. doi: 10.1126/sciadv.abf6054
30. Kirkby NS, Chan MV, Zaiss AK, Garcia-Vaz E, Jiao J, Berglund LM, Verdu EF, Ahmetaj-Shala B, Wallace JL, Herschman HR, et al. Systematic study of constitutive cyclooxygenase-2 expression: Role of NF-kappaB and NFAT transcriptional pathways. *Proc Natl Acad Sci U S A*. 2016;113:434-439. doi: 10.1073/pnas.1517642113
31. Langenbach R, Morham SG, Tiano HF, Loftin CD, Ghanayem BI, Chulada PC, Mahler JF, Lee CA, Goulding EH, Kluckman KD, et al. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell*. 1995;83:483-492. doi: 10.1016/0092-8674(95)90126-4
32. Payne S, De Val S, Neal A. Endothelial-Specific Cre Mouse Models. *Arterioscler Thromb Vasc Biol*. 2018;38:2550-2561. doi: 10.1161/ATVBAHA.118.309669
33. Holtwick R, Gotthardt M, Skryabin B, Steinmetz M, Potthast R, Zetsche B, Hammer RE, Herz J, Kuhn M. Smooth muscle-selective deletion of guanylyl cyclase-A prevents the acute but not chronic effects of ANP on blood pressure. *Proc Natl Acad Sci U S A*. 2002;99:7142-7147. doi: 10.1073/pnas.102650499
34. Chakraborty R, Saddouk FZ, Carrao AC, Krause DS, Greif DM, Martin KA. Promoters to Study Vascular Smooth Muscle. *Arterioscler Thromb Vasc Biol*. 2019;39:603-612. doi: 10.1161/ATVBAHA.119.312449
35. Tsutsumi R, Xie C, Wei X, Zhang M, Zhang X, Flick LM, Schwarz EM, O'Keefe RJ. PGE2 signaling through the EP4 receptor on fibroblasts upregulates RANKL and stimulates osteolysis. *J Bone Miner Res*. 2009;24:1753-1762. doi: 10.1359/jbmr.090412
36. Kuwabara JT, Tallquist MD. Tracking Adventitial Fibroblast Contribution to Disease: A Review of Current Methods to Identify Resident Fibroblasts. *Arterioscler Thromb Vasc Biol*. 2017;37:1598-1607. doi: 10.1161/ATVBAHA.117.308199
37. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis*. 2007;45:593-605. doi: 10.1002/dvg.20335
38. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol*. 2001;230:230-242. doi: 10.1006/dbio.2000.0106
39. Marcus AJ, Weksler BB, Jaffe EA, Broekman MJ. Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. *J Clin Invest*. 1980;66:979-986. doi: 10.1172/JCI109967

40. Sagliani KD, Dolnikowski GG, Hill NS, Fanburg BL, Levy BD, Preston IR. Differences between basal lung levels of select eicosanoids in rat and mouse. *Pulm Circ*. 2013;3:82-88. doi: 10.4103/2045-8932.109918
41. Boettcher S, Gerosa RC, Radpour R, Bauer J, Ampenberger F, Heikenwalder M, Kopf M, Manz MG. Endothelial cells translate pathogen signals into G-CSF-driven emergency granulopoiesis. *Blood*. 2014;124:1393-1403. doi: 10.1182/blood-2014-04-570762
42. Konishi M, Sakaguchi M, Lockhart SM, Cai W, Li ME, Homan EP, Rask-Madsen C, Kahn CR. Endothelial insulin receptors differentially control insulin signaling kinetics in peripheral tissues and brain of mice. *Proc Natl Acad Sci U S A*. 2017;114:E8478-E8487. doi: 10.1073/pnas.1710625114
43. Gerritsen ME, Cheli CD. Arachidonic acid and prostaglandin endoperoxide metabolism in isolated rabbit and coronary microvessels and isolated and cultivated coronary microvessel endothelial cells. *J Clin Invest*. 1983;72:1658-1671. doi: 10.1172/JCI111125
44. Alvarez DF, Huang L, King JA, ElZarrad MK, Yoder MC, Stevens T. Lung microvascular endothelium is enriched with progenitor cells that exhibit vasculogenic capacity. *Am J Physiol Lung Cell Mol Physiol*. 2008;294:L419-430. doi: 10.1152/ajplung.00314.2007
45. Kalucka J, de Rooij L, Goveia J, Rohlenova K, Dumas SJ, Meta E, Conchinha NV, Taverna F, Teuwen LA, Veys K, et al. Single-Cell Transcriptome Atlas of Murine Endothelial Cells. *Cell*. 2020;180:764-779 e720. doi: 10.1016/j.cell.2020.01.015
46. Fujino N, Kubo H, Ota C, Suzuki T, Suzuki S, Yamada M, Takahashi T, He M, Suzuki T, Kondo T, et al. A novel method for isolating individual cellular components from the adult human distal lung. *Am J Respir Cell Mol Biol*. 2012;46:422-430. doi: 10.1165/rcmb.2011-0172OC
47. Tsukui T, Sun KH, Wetter JB, Wilson-Kanamori JR, Hazelwood LA, Henderson NC, Adams TS, Schupp JC, Poli SD, Rosas IO, et al. Collagen-producing lung cell atlas identifies multiple subsets with distinct localization and relevance to fibrosis. *Nat Commun*. 2020;11:1920. doi: 10.1038/s41467-020-15647-5
48. Ager A, Gordon JL, Moncada S, Pearson JD, Salmon JA, Trevethick MA. Effects of isolation and culture on prostaglandin synthesis by porcine aortic endothelial and smooth muscle cells. *J Cell Physiol*. 1982;110:9-16. doi: 10.1002/jcp.1041100103
49. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest*. 2002;110:341-350. doi: 10.1172/JCI15518
50. Ricciotti E, Yu Y, Grosser T, Fitzgerald GA. COX-2, the dominant source of prostacyclin. *Proc Natl Acad Sci U S A*. 2013;110:E183. doi: 10.1073/pnas.1219073110
51. Kirkby NS, Raouf J, Ahmetaj-Shala B, Liu B, Mazi SI, Edin ML, Chambers MG, Korotkova M, Wang X, Wahli W, et al. Mechanistic definition of the cardiovascular mPGES-1/COX-2/ADMA axis. *Cardiovasc Res*. 2020;116:1972-1980. doi: 10.1093/cvr/cvz290

52. Moncada S, Korbut R, Bunting S, Vane JR. Prostacyclin is a circulating hormone. *Nature*. 1978;273:767-768. doi: 10.1038/273767a0
53. Christ-Hazelhof E, Nugteren DH. Prostacyclin is not a circulating hormone. *Prostaglandins*. 1981;22:739-746. doi: 10.1016/0090-6980(81)90213-6
54. Crescente M, Armstrong PC, Kirkby NS, Edin ML, Chan MV, Lih FB, Jiao J, Maffucci T, Allan HE, Mein CA, et al. Profiling the eicosanoid networks that underlie the anti- and pro-thrombotic effects of aspirin. *FASEB J*. 2020;34:10027-10040. doi: 10.1096/fj.202000312R
55. Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM, Loyd JE. An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Engl J Med*. 1992;327:70-75. doi: 10.1056/NEJM199207093270202
56. Feary JR, Rodrigues LC, Smith CJ, Hubbard RB, Gibson JE. Prevalence of major comorbidities in subjects with COPD and incidence of myocardial infarction and stroke: a comprehensive analysis using data from primary care. *Thorax*. 2010;65:956-962. doi: 10.1136/thx.2009.128082
57. Clarson LE, Bajpai R, Whittle R, Belcher J, Abdul Sultan A, Kwok CS, Welsh V, Mamas M, Mallen CD. Interstitial lung disease is a risk factor for ischaemic heart disease and myocardial infarction. *Heart*. 2020;106:916-922. doi: 10.1136/heartjnl-2019-315511
58. Nana-Sinkam SP, Lee JD, Sotto-Santiago S, Stearman RS, Keith RL, Choudhury Q, Cool C, Parr J, Moore MD, Bull TM, et al. Prostacyclin prevents pulmonary endothelial cell apoptosis induced by cigarette smoke. *Am J Respir Crit Care Med*. 2007;175:676-685. doi: 10.1164/rccm.200605-724OC
59. Wilborn J, Crofford LJ, Burdick MD, Kunkel SL, Strieter RM, Peters-Golden M. Cultured lung fibroblasts isolated from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize prostaglandin E2 and to express cyclooxygenase-2. *J Clin Invest*. 1995;95:1861-1868. doi: 10.1172/JCI117866
60. Harari S, Wells A. Inhaled trepostinil for severe fibrotic interstitial lung disease: grounds for cautious optimism? *Lancet Respir Med*. 2021. doi: 10.1016/S2213-2600(21)00264-2
61. Geraci M, Gao B, Shepherd D, Allard J, Curiel D, Westcott J, Voelkel N. Pulmonary prostacyclin synthase overexpression by adenovirus transfection and in transgenic mice. *Chest*. 1998;114:99S. doi: 10.1378/chest.114.1_supplement.99s
62. Kaczmarek JC, Kauffman KJ, Fenton OS, Sadtler K, Patel AK, Heartlein MW, DeRosa F, Anderson DG. Optimization of a Degradable Polymer-Lipid Nanoparticle for Potent Systemic Delivery of mRNA to the Lung Endothelium and Immune Cells. *Nano Lett*. 2018;18:6449-6454. doi: 10.1021/acs.nanolett.8b02917
63. Harrington LS, Lucas R, McMaster SK, Moreno L, Scadding G, Warner TD, Mitchell JA. COX-1, and not COX-2 activity, regulates airway function: relevance to aspirin-sensitive asthma. *FASEB J*. 2008;22:4005-4010. doi: 10.1096/fj.08-107979

64. Lovgren AK, Jania LA, Hartney JM, Parsons KK, Audoly LP, Fitzgerald GA, Tilley SL, Koller BH. COX-2-derived prostacyclin protects against bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2006;291:L144-156. doi: 10.1152/ajplung.00492.2005
65. Cathcart MC, Tamosiuniene R, Chen G, Neilan TG, Bradford A, O'Byrne KJ, Fitzgerald DJ, Pidgeon GP. Cyclooxygenase-2-linked attenuation of hypoxia-induced pulmonary hypertension and intravascular thrombosis. *J Pharmacol Exp Ther*. 2008;326:51-58. doi: 10.1124/jpet.107.134221
66. Gong Z, Li Q, Shi J, Wei J, Li P, Chang CH, Shultz LD, Ren G. Lung fibroblasts facilitate pre-metastatic niche formation by remodeling the local immune microenvironment. *Immunity*. 2022;55:1483-1500 e1489. doi: 10.1016/j.immuni.2022.07.001
67. Tourdot BE, Adili R, Isingizwe ZR, Ebrahem M, Freedman JC, Holman TR, Holinstat M. 12-HETrE inhibits platelet reactivity and thrombosis in part through the prostacyclin receptor. *Blood Adv*. 2017;1:1124-1131. doi: 10.1182/bloodadvances.2017006155
68. Iyu D, Glenn JR, White AE, Johnson AJ, Fox SC, Heptinstall S. The role of prostanoid receptors in mediating the effects of PGE(2) on human platelet function. *Platelets*. 2010;21:329-342. doi: 10.3109/09537101003718065
69. Bhatt DL, Steg PG, Miller M, Brinton EA, Jacobson TA, Ketchum SB, Doyle RT, Jr., Juliano RA, Jiao L, Granowitz C, et al. Cardiovascular Risk Reduction with Icosapent Ethyl for Hypertriglyceridemia. *N Engl J Med*. 2019;380:11-22. doi: 10.1056/NEJMoa1812792
70. Yamaguchi A, Stanger L, Freedman JC, Prieur A, Thav R, Tena J, Holman TR, Holinstat M. Supplementation with omega-3 or omega-6 fatty acids attenuates platelet reactivity in postmenopausal women. *Clin Transl Sci*. 2022;15:2378-2391. doi: 10.1111/cts.13366

Highlights:

- Prostacyclin, a powerful anti-thrombotic mediator, is abundantly produced by both arteries and in tissues especially the lung.
- In arteries prostacyclin production is produced by vascular endothelial cells but in the lung and other tissues prostacyclin production is essentially independent of the endothelium.
- Lung microvascular endothelial cells are weak producers of prostacyclin by comparison to arterial endothelial cells.
- As such, prostacyclin production in the lung is associated with fibroblasts (both vascular and non-vascular) and contributes to systematic anti-thrombotic protection.
- This previously unappreciated degree to which non-endothelial sources in the lung and elsewhere contribute to prostacyclin production represents a new paradigm in prostacyclin biology and may explain the associations between lung disease and thrombotic risk.

Acknowledgements

The authors acknowledge Dr Jess Rowley, Dr Larissa Zárate García and the Imperial College South Kensington Flow Cytometry Facility for their assistance in FACS, Mr Harshil Bhayani and the Royal Brompton & Harefield NHS Trust Biomedical Research Unit Advanced Lung Disease Biobank for their assistance in obtaining human lung tissue and Prof Timothy Warner for his provision of global cyclo-oxygenase-1-deficient mice.

Sources of Funding

The work was supported by grants from the British Heart Foundation (FS/16/1/31699 to NSK; RG/18/4/33541 to JAM & NSK), Imperial College/Wellcome Trust (to BA-S), the National Institutes of Health/National Cancer Institute (R01-CA084572, R01-CA123055, P50-CA086306; to HRH), the Phelps Family Foundation and the Crump Family Foundation (to HRH), the National Natural Science Foundation of China (81770678 to BL; 31771272 to YZ), and the Natural Science Foundation of Guangdong Province (2019A1515011650 to BL).

Disclosures

JAM is a shareholder of and member of the scientific advisory board for Antibe Therapeutics which develops cyclo-oxygenase inhibitor anti-inflammatory drugs. JAM and NSK hold active research grant funding in the area of cyclo-oxygenase biology. The other authors have no relevant disclosures.

Supplemental Material

Tables S1-S4

Figures S1-S6

Major Resource Table

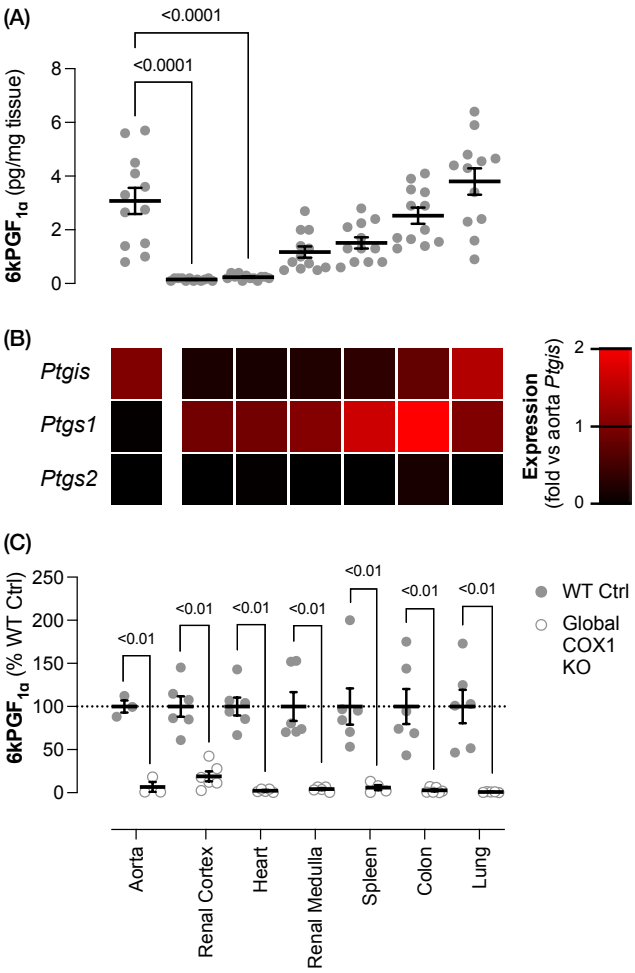
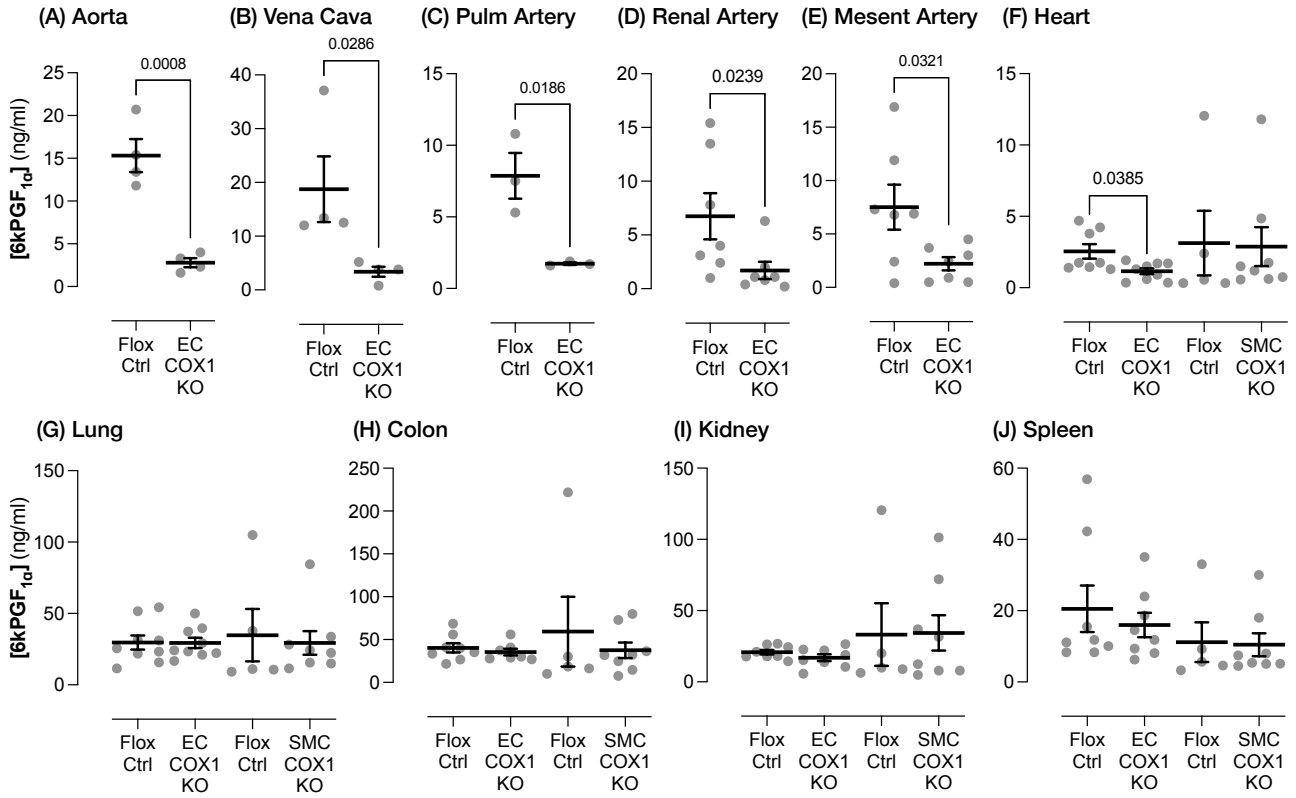


Figure 1 Both tissues and arteries generate prostacyclin which reflects relative prostacyclin synthase expression and requires cyclo-oxygenase-1 (A) Prostacyclin levels (measured as 6kPGF$_{1\alpha}$ after A23187 Ca^{2+} ionophore 30μM stimulation) per unit mass (n=4) and (B) *Ptgis* (prostacyclin synthase), *Ptgs1* (cyclo-oxygenase-1) and *Ptgs2* (cyclo-oxygenase-2) gene expression (n=4) in wild-type mouse aorta and tissue. (C) Prostacyclin release from tissues from global cyclo-oxygenase-1 knockout (global COX-1 KO) and matched wild-type control (WT Ctrl) mice (n=3-6). Data are mean ± SEM with p values by Freidman's test with Dunn's post-test (A) or unpaired t-test (C) indicated where $p<0.05$. n is defined as number individual animals studied.



928

929

930 **Figure 2 Role of vascular cyclo-oxygenase-1 in prostacyclin release from arteries, veins and**
931 **tissues** Prostacyclin release (measured as $6kPGF_{1\alpha}$ after A23187 Ca^{2+} ionophore 30μM stimulation)
932 from (A) isolated aorta (n=4), (B) vena cava (n=4), (C) pulmonary artery (n=3), (D) renal artery (n=7)
933 and (E) mesenteric artery (n=7) from endothelial cyclo-oxygenase-1 knockout (EC COX1 KO) and
934 floxed littermate control animals (Flox Ctrl), and from intact segments of (F) heart (left ventricle; n=5-
935 8), (G) lung (parenchyma; n=5-8), (H) colon (n=5-8), (I) kidney (renal medulla; n=5-8) and (J) spleen
936 (n=5-8) from EC COX1 KO mice, smooth muscle cyclo-oxygenase-1 knockout (SMC COX1 KO) and
937 their respective floxed littermate controls (Flox Ctrl). Data are mean ± SEM with p values by unpaired
938 t-test (A, C, E, G, H, I) or Mann-Whitney U-test (B, D, F, J) indicated where $p<0.05$. n is defined as
939 number individual animals studied.

940

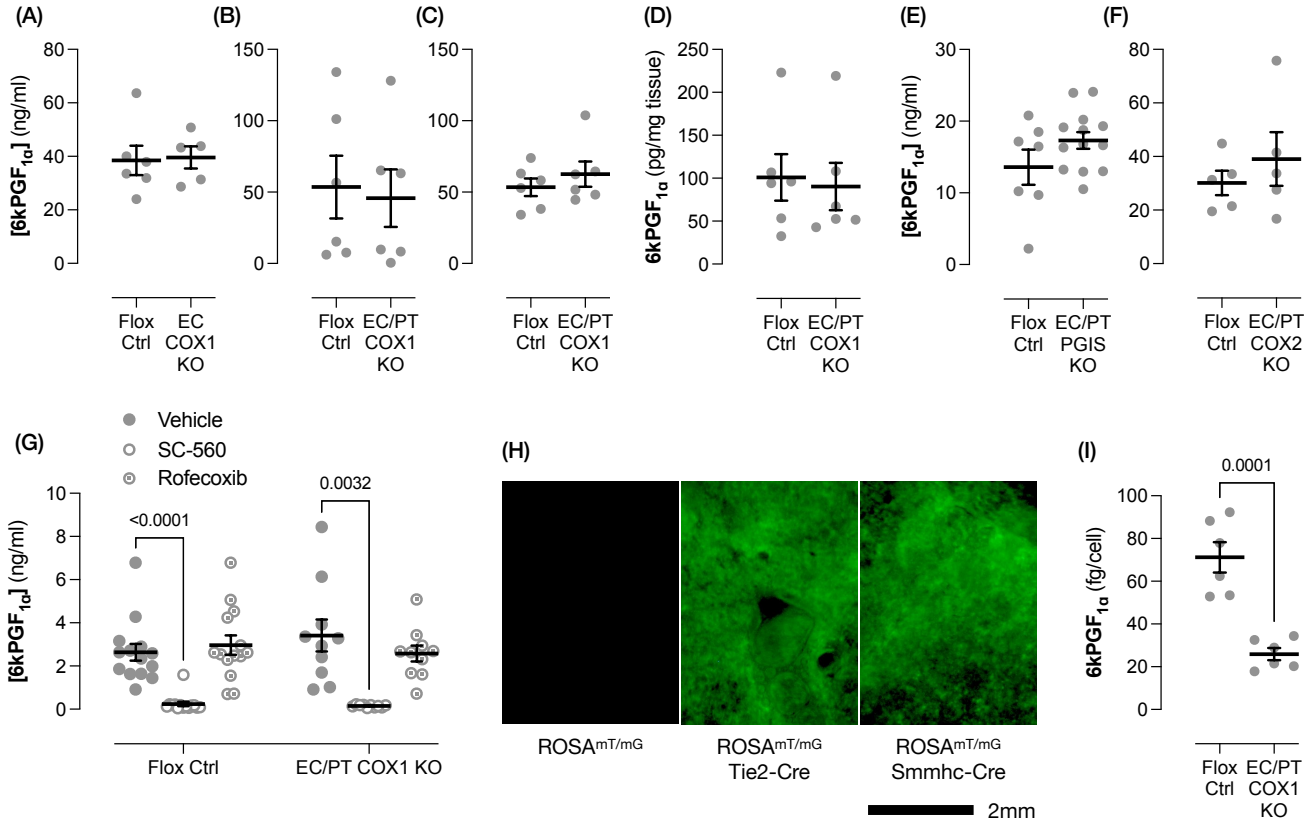
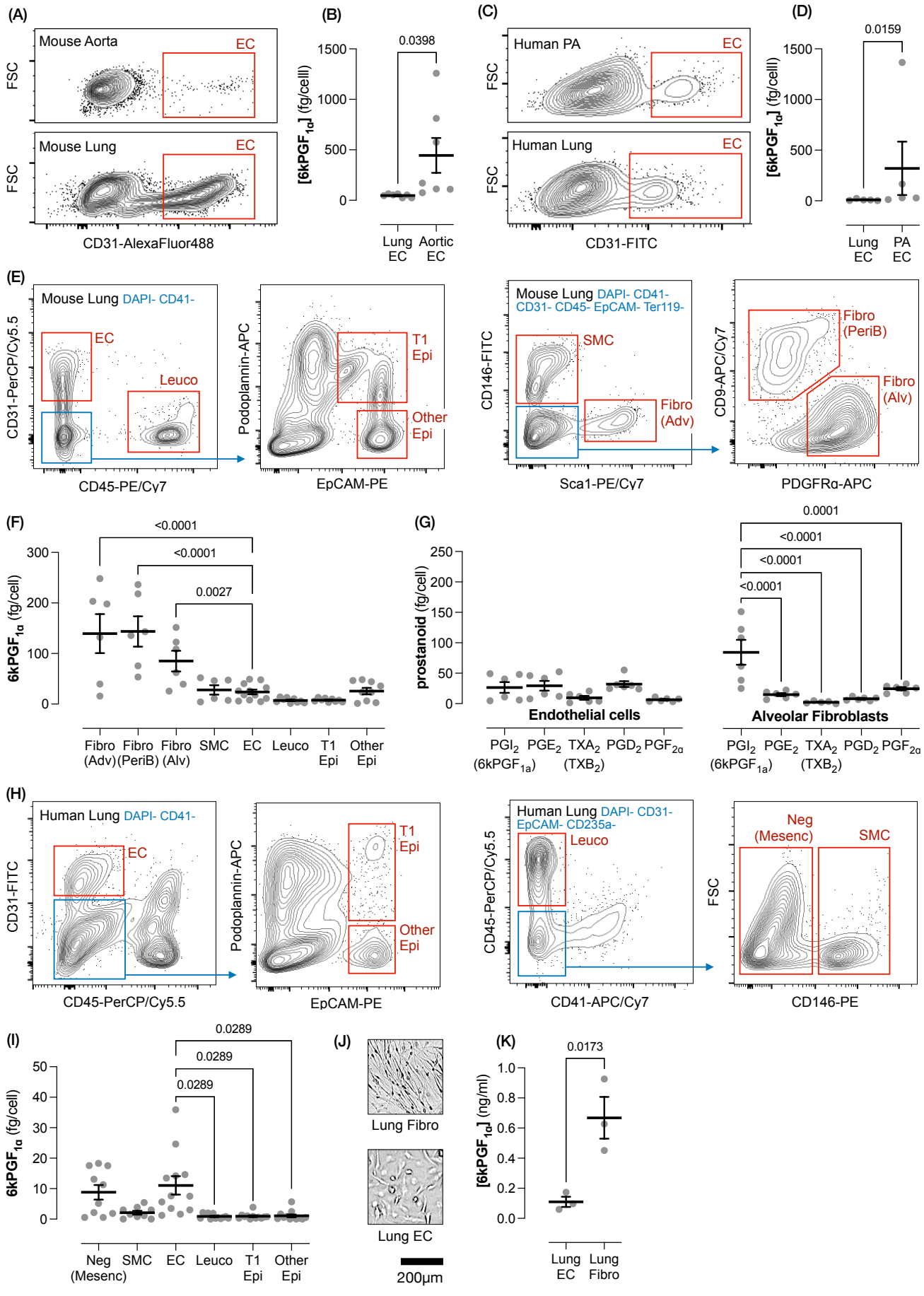


Figure 3 Lung prostacyclin production does not require cyclo-oyxgenase-1, cyclo-oxgenase-2 or prostacyclin synthase in endothelial cells or platelets (A) Prostacyclin release (measured as $6kPGF_{1\alpha}$ after A23187 Ca^{2+} ionophore 30μM stimulation) from lung parenchyma segments from endothelial cyclo-oxygenase-1 knockout (EC COX1 KO) and floxed littermate control animals (Flox Ctrl) in which the lung vasculature has been flushed of blood (n=5-6). (B) Prostacyclin from lung parenchyma segments stimulated after stimulation with A23187 Ca^{2+} ionophore (n=6; 30μM) or (C) arachidonic acid (n=6; 30μM). (D) Prostacyclin levels in unstimulated snap frozen lung homogenates (n=6) from endothelial/platelet cyclo-oxygenase-1 knockout mice (EC/PT COX1 KO). Prostacyclin release (A23187 Ca^{2+} ionophore 30μM stimulation) from lung parenchyma segments from (E) endothelial/platelet prostacyclin synthase knockout mice (EC/PT PGIS KO; n=7-13) and (F) endothelial/platelet cyclo-oxygnease-2 knockout mice (EC/PT COX2 KO; n=5), each compared to respective floxed littermate controls. (G) Prostacyclin release (A23187 Ca^{2+} ionophore 30μM stimulation) from lung parenchyma segments from EC/PT COX1 KO and floxed littermate control animals (n=10-14) in the presence of selective inhibitors of COX-1 (SC-560; 1μM) or COX-2 (rofecoxib; 1μM). (H) EGFP (green) fluorescence in lung segments of $ROSA^{mT/mG}$ mice with/without a Tie2-Cre or Smmhc-Cre transgene (representative of n=3/genotype). (I) Prostacyclin release from endothelial cells ($CD31^+$, $CD45^-$, $CD41^-$) isolated by FACS from EC/PT COX1 KO and Flox Ctrl mouse lung (n=6). Data are mean ± SEM with p values by unpaired t-test (A, B, C, E, F, I), Mann-Whitney U-test (D) or two-way ANOVA with Sidak's post-test (G) indicated where $p<0.05$. n is defined as number individual animals studied.


(A)
Mouse Aorta
EC
FSC
Mouse Lung
EC
FSC
CD31-AlexaFluor488
(B)
0.0398
1500
1000
500
0
[6kPGF1α] (fg/cell))
Lung EC
Aortic EC
(C)
Human PA
EC
FSC
Human Lung
EC
FSC
CD31-FITC
(D)
0.0159
1500
1000
500
0
[6kPGF1α] (fg/cell))
Lung EC
PA EC
(E)
Mouse Lung DAPI- CD41-
EC
Leuco
CD31-PerCP/Cy5.5
CD45-PE/Cy7
Podoplannin-APC
T1 Epi
Other Epi
EpCAM-PE
Mouse Lung DAPI- CD41- CD31- CD45- EpCAM- Ter119-
SMC
Fibro (Adv)
CD146-FITC
Sca1-PE/Cy7
CD9-APC/Cy7
Fibro (PeriB)
Fibro (Alv)
PDGFRα-APC
(F)
<0.0001
<0.0001
0.0027
300
200
100
0
6kPGF1α (fg/cell)
Fibro (Adv)
Fibro (PeriB)
Fibro (Alv)
SMC
EC
Leuco
T1 Epi
Other Epi
(G)
250
200
150
100
50
0
prostanoid (fg/cell)
Endothelial cells
PGI2 (6kPGF1a)
PGE2
TXA2 (TXB2)
PGD2
PGF2α
0.0001
<0.0001
<0.0001
<0.0001
Alveolar Fibroblasts
PGI2 (6kPGF1a)
PGE2
TXA2 (TXB2)
PGD2
PGF2α
(H)
Human Lung DAPI- CD41-
EC
CD31-FITC
CD45-PerCP/Cy5.5
Podoplannin-APC
T1 Epi
Other Epi
EpCAM-PE
Human Lung DAPI- CD31- EpCAM- CD235a-
Leuco
CD45-PerCP/Cy5.5
CD41-APC/Cy7
Neg (Mesenc)
SMC
FSC
CD146-PE
(I)
0.0289
0.0289
0.0289
50
40
30
20
10
0
6kPGF1α (fg/cell)
Neg (Mesenc)
SMC
EC
Leuco
T1 Epi
Other Epi
(J)
Lung Fibro
Lung EC
200µm
(K)
0.0173
1.0
0.8
0.6
0.4
0.2
0.0
[6kPGF1α] (ng/ml)
Lung EC
Lung Fibro

964

Figure 4 Fibroblasts are major contributors to lung prostacyclin generation (A) FACS gating strategy (representative plots) and (B) prostacyclin release (measured as 6kPGF$_{1\alpha}$ after arachidonic acid 30µM stimulation) (n=4) of endothelial cells from matched mouse aorta and lung parenchyma. (C) FACS gating strategy (representative plots) and (D) prostacyclin release (n=5 donors) of endothelial cells from matched human pulmonary artery and lung. (E) FACS gating strategy (representative plots) and (F) prostacyclin release from endothelial cells (EC), leucocytes (Leuco), type 1 (T1 Epi) and other epithelial cells (Other Epi), smooth muscle cells (SMC) and adventitial (Fibro Adv), alveolar (Fibro Alv) and peribronchial fibroblasts (Fibro PeriB) from mouse lung (n=6-12). (G) Relative release of primary prostanoids from freshly FACS isolated lung endothelial and alveolar fibroblasts (n=6). (H) FACS gating strategy (representative plots) and (I) prostacyclin release from EC, Leuco, T1 Epi, Other Epi, SMC and negatively selected mesenchymal cells (Neg Mesenc) from human lung (n=9-12 donors). (J) Representative brightfield images and (K) prostacyclin release (after arachidonic acid 30µM stimulation; n=3 donors) from cultured primary human lung microvascular endothelial cells (Lung EC) and human lung fibroblasts (Lung Fibro). FACS plots show 5% density contours. Data are mean ± SEM with p values by unpaired t-test (B, K), Mann-Whitney U-test (D) or repeated measures one-way ANOVA with Holm-Sidak post-test (F, G, I) indicated where p<0.05. n is defined as number individual animals (B, F, G), human donors (D, I) or independent donor primary cell lines (K) studied.

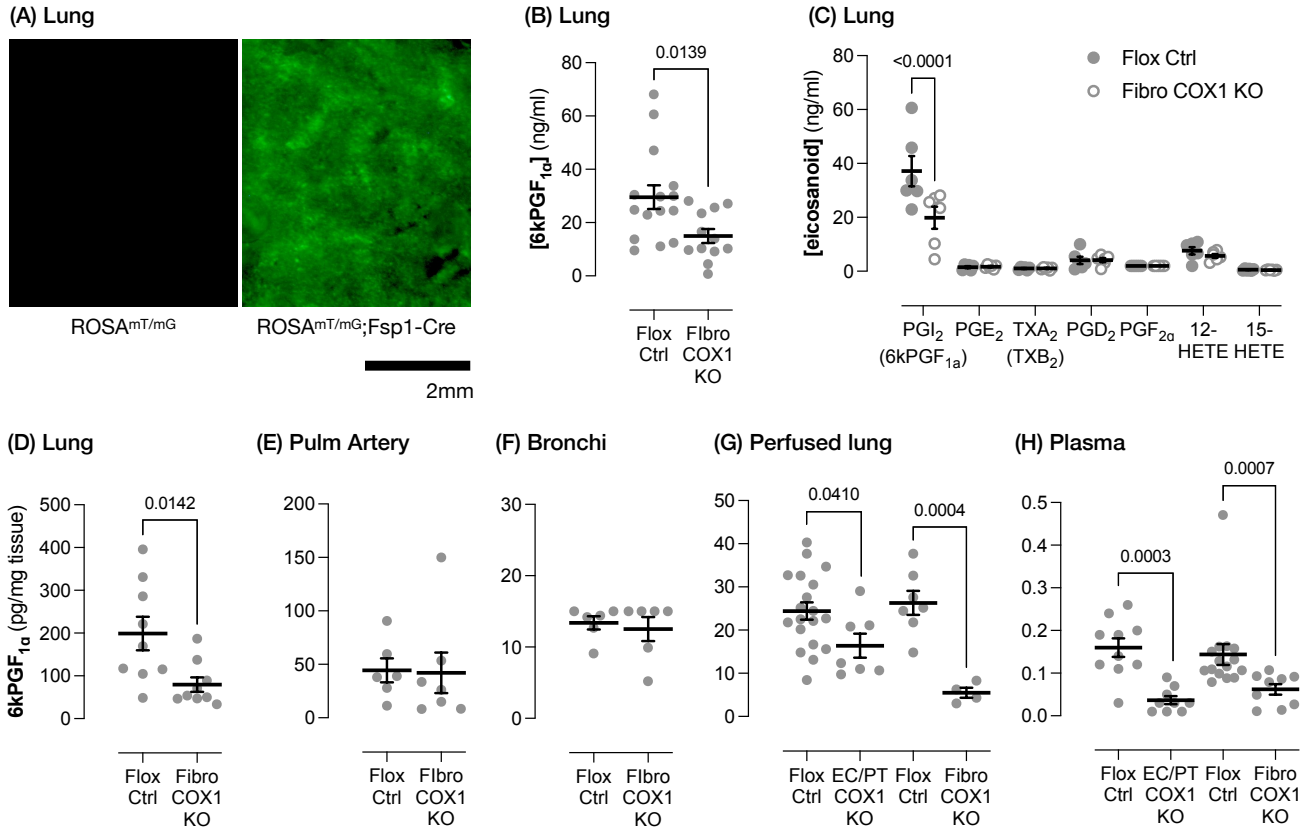


Figure 5 Fibroblasts contribute to total lung prostacyclin production and from where it can enter the systemic circulation (A) EGFP fluorescence (green) in lung segments of $ROSA^{mT/mG}$ mice with/without a Fsp1-Cre transgene (representative of n=3/genotype). Release (A23187 Ca^{2+} ionophore 30µM stimulation) by lung parenchyma segments of (B) prostacyclin (n=12-16) and (C) a panel of primary prostanoids and HETEs (n=6) from fibroblast-specific cyclo-oxygenase-1 knockout (Fibro COX1 KO) and floxed littermate control mice (Flox Ctrl). (D) Prostacyclin levels in unstimulated snap frozen lung homogenates from Fibro COX1 KO knockout mice and Flox Ctrl animals (n=9-10). Prostacyclin release (A23187 Ca^{2+} ionophore 30µM stimulation) from (E) pulmonary artery (n=6-7) and (F) bronchi from Fibro COX1 KO knockout mice and Flox Ctrl animals (n=6-7). Prostacyclin levels (measured as $6kPGF_{1\alpha}$) measured in the outflow from (G) isolated perfused lung (n=4-19) and (H) in plasma (n=9-15) from endothelial/platelet cyclo-oxygenase-1 knockout (EC/PT COX1 KO), Fibro COX1 KO and respective Flox Ctrl mice. Data are mean ± SEM with p values by Mann-Whitney U-test (B, D, E, F, H), or repeated measures two-way ANOVA with Holm-Sidak post test (C) or unpaired t-test (G) indicated where $p<0.05$. n is defined as number individual animals studied.

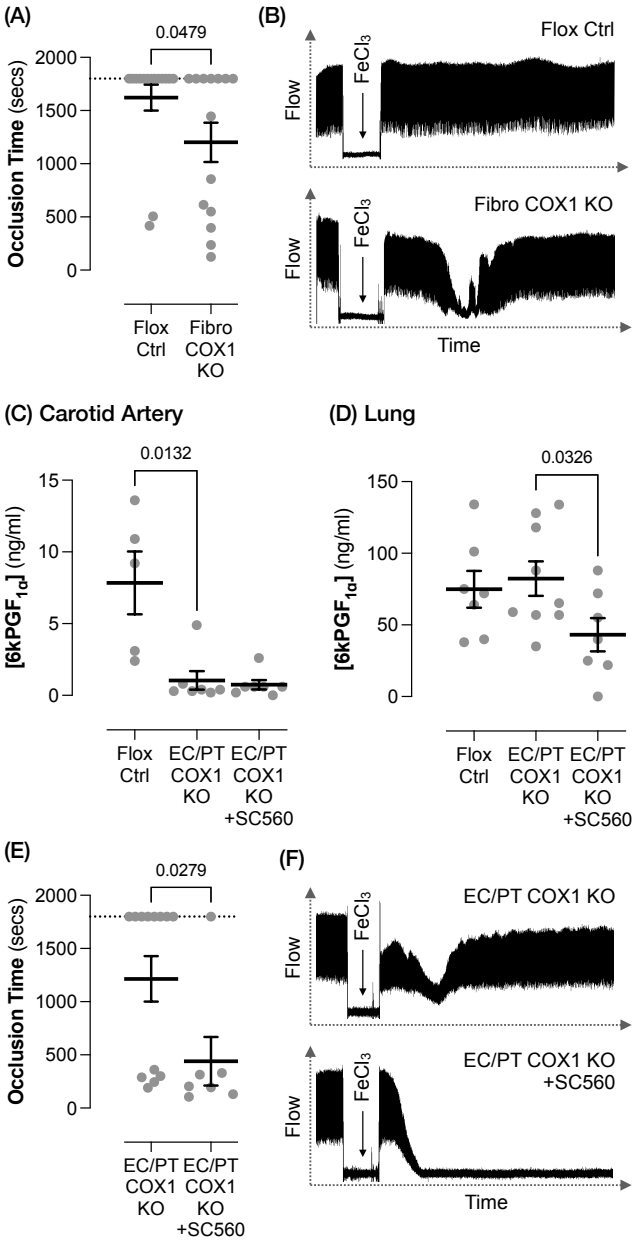


Figure 6 Fibroblasts and other non-endothelial sites of cyclo-oxygenase-1 expression contribute to systemic anti-thrombotic protection (A) Thrombotic occlusion time (n=14-15) and (B) representative blood flow traces after carotid artery $FeCl_3$ injury in vivo in fibroblast cyclo-oxygenase-1 knockout mice (Fibro COX1 KO) and floxed littermate controls (Flox Ctrl). Prostacyclin release (measured as $6kPGF_{1\alpha}$) from (C) carotid artery (n=7) and (D) lung parenchyma (D; n=7-9) *ex vivo* from endothelial/platelet cyclo-oxygenase-1 knockout mice (EC/PT COX1 KO) treated with the cyclo-oxygenase-1 inhibitor, SC-560 (10mg/kg; iv, 15 mins) or vehicle (5% DMSO). Release level from Flox Ctrl tissue is marked on each graph as a dashed line. (E) Thrombotic occlusion time (n=7-13) and (F) representative blood flow traces after carotid artery $FeCl_3$ injury in vivo in EC/PT COX1 KO with treated with SC-560 or vehicle. Data are mean ± SEM with p values by Mann-Whitney U-test (A, E) or Kruskal-Wallis test with Dunn's post-test (C) or one-way ANOVA with Holm-Sidak's post-test (D) indicated where $p<0.05$. n is defined as number individual animals studied.