

Vascular smooth muscle cell-derived osteoprotegerin drives pulmonary arterial hypertension via AKT activation and is attenuated by monoclonal antibody therapy

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Abstract

Aim: Pulmonary arterial hypertension (PAH) is driven by sustained vasoconstriction and structural remodelling of distal pulmonary arteries. We have previously demonstrated that osteoprotegerin (OPG, *tnfrsf11b*) is upregulated in PAH animal models and patient tissues, with circulating levels being prognostic. OPG knockout mice exhibit an attenuated PAH phenotype, and therapeutic inhibition using a human monoclonal anti-OPG antibody reduces disease severity in multiple rodent models. However, the key cellular source of pathogenic OPG and the mechanism underlying therapeutic blockade remain unknown.

Methods and Results: To define the main cellular source of OPG, conditional *Tnfrsf11b* deletion was generated by crossing OPG^{fl/fl} mice with PGKcre^{tg/+} (global), LysMcre^{tg/+} (myeloid), Myl2cre^{tg/+} (cardiac myocytes), Cdh5cre^{tg/+} (endothelial cells, ECs), smMHCcre^{tg/+} (VSMCs), Col1a2cre^{tg/+} (fibroblasts, FBs). Knockouts were induced by tamoxifen injection, and PAH established using Sugen5416/hypoxia (SuHx) model. Cardiopulmonary phenotyping included echocardiography, cardiac catheterisation, and histological analysis. Mechanistic studies were performed in VSMCs using peptide-based kinase activity profiling (PamStation).

PGKcre^{tg/+}/OPG^{fl/fl} mice phenocopied the previous results obtained with global OPG knockout (OPG^{-/-}). Only VSMC-specific deletion (smMHCcre^{tg/0}/OPG^{fl/fl}) resulted in significantly improved haemodynamic parameters, evidenced by reduced RVSP and pulmonary vascular resistance (PVR), and reduced pulmonary vascular remodelling demonstrating that VSMC-derived OPG is the primary driver of PAH pathogenesis.

Kinome profiling of control and IPAH-PASMCs treated with OPG +/- anti-OPG antibody revealed IPAH specific regulation of PI3K-Akt and enrichment of pathways involving autophagy, and longevity regulation

1 linked to cellular stress resistance and growth. Anti-OPG antibody treatment reduced AKT phosphorylation
2 in IPAH PSMCs. Knock-down of AKT1 by siRNA reduced OPG-induced proliferation and migration of
3 human PSMC. These results align with emerging data implicating OPG in cellular senescence and vascular
4 aging.

5 **Conclusion:** Together, these findings highlight VSMC-derived OPG as a critical mediator of pulmonary
6 vascular remodelling in PAH pathogenesis and suggest that targeting OPG via a therapeutic antibody is
7 mediated through PI3K-Akt signalling.

8

9 **Translational perspective**

10 This study identifies vascular smooth muscle cell-derived osteoprotegerin (OPG) as a key determinant of
11 pulmonary vascular remodelling and haemodynamic deterioration in pulmonary arterial hypertension
12 (PAH). By defining the cell-specific contribution of OPG, our findings refine current understanding of its
13 pathogenic role and suggest AKT1-dependent signalling as an important mechanism driving PAH
14 pathogenesis in vascular smooth muscle cells. The validation of our findings and attenuation of disease
15 following monoclonal anti-OPG treatment in experimental models reinforces the translational relevance
16 of these findings.

1. Introduction

Pulmonary arterial hypertension (PAH) is a severe disease characterized by both sustained vasoconstriction and progressive obliteration of small (distal) pulmonary arteries through medial thickening, intimal fibrosis, and the formation of angioproliferative lesions.^{1, 2} This remodelling of the pulmonary vasculature leads to increased resistance and elevated pulmonary artery (PA) pressure, eventually resulting in right ventricular (RV)-mediated heart failure.³

Current treatments primarily target sustained vasoconstriction to relieve symptoms and improve survival^{4, 5}; however, they do not halt the disease progression or reverse vascular pathology. Over the last decade, considerable advancements have been made in understanding the molecular and cellular mechanisms involved in PAH pathogenesis. These include dysregulation of bone morphogenetic protein (BMP) and transforming growth factor-beta (TGF- β) signalling pathways⁶, and the involvement of growth factors such as platelet-derived growth factor (PDGF)⁷. This has led to the development of potential disease modifying therapies such as sotatercept⁸ and imatinib.^{9, 10} However, further research is needed to fully understand the long-term effects of these drugs on PAH pathogenesis to evaluate their sustained efficacy and safety.¹¹ These limitations underscore the need to explore additional therapeutic targets.

Pathologically, PAH is characterised by dysregulated pulmonary arterial endothelial cells (PAECs), which exhibit increased proliferation and resistance to apoptosis.¹²⁻¹⁴ Subsequent proliferation and migration of pulmonary artery smooth muscle cells (PASMCs) and fibroblasts, along with the recruitment of monocytes/macrophages further exacerbate vascular remodelling.¹⁴

Osteoprotegerin (OPG, *tnfrsf11b*) is a secreted glycoprotein within the tumour necrosis factor (TNF) receptor superfamily, most recognised for its role in bone metabolism as a decoy receptor for receptor activator of nuclear factor- κ B ligand (RANKL), where it blocks RANK-mediated osteoclast differentiation and osteoclastogenesis.^{15, 16} OPG has been implicated in vascular aging and stiffness, with the studies

highlighting its expression in vascular smooth muscle cells (VSMC) and its association with arterial
 calcification,^{17, 18} senescence and vascular remodelling.¹⁹ Beyond RANKL, OPG also binds to TNF-related
 apoptosis-inducing ligand (TRAIL, *tnfsf10*), a key regulator of apoptosis with implications in PAH preclinical
 models.²⁰⁻²² In PAH, we have shown that OPG is upregulated in remodelled pulmonary vascular lesions²³
 and serum²⁴ of patients with idiopathic PAH, with elevated serum levels being prognostic. Additionally,
 higher OPG levels were associated with haemodynamic severity and poorer survival outcomes. Both OPG
 and TRAIL can drive PASMC proliferation and migration^{20, 23}, that levels of OPG are elevated in both pre-
 clinical models PAH,^{20, 25} and within the lungs and serum of patients with idiopathic PAH (IPAH).²¹⁻²⁴ We
 have previously demonstrated that deletion or inhibition of either TRAIL²⁰ or OPG²⁵ attenuates and
 reverses PAH progression in pre-clinical models. Using bone marrow transplanted chimeric *Tnfrsf11b*^{-/-}
 mice we identified bone marrow-derived OPG as the main source of OPG promoting PAH in the murine
 SuHx model²⁵. We identified that in human PA-SMC, OPG binds to FAS receptor (*tnfrsf6*) contributing to a
 pro-migratory, pro-proliferative response, increasing TRAIL expression and PASMC proliferation.²⁵
 However, since OPG and FAS are broadly expressed by multiple cell types, and sub-lethal irradiation
 approaches can induce pulmonary endothelial damage, inflammation, and alters immune cell
 reconstitution^{26, 27}, we generated a new conditional knockout *Tnfrsf11b* mouse to address these limitations
 and determine the cellular source of OPG responsible for pulmonary vascular and right ventricular
 remodelling. We generated mice with global (PGK) and lineage-specific deletion of *Opg* (*Tnfrsf11b*^{-/-}) in
 myeloid cells (LysM), endothelial cells (ECs, Chd5-cre), fibroblasts (FBs, Col1a2-cre), cardiomyocytes (CMs,
 Myl2-cre), and vascular smooth muscle cells (VSMCs, smMHC-cre). Whilst most conditional knockouts
 showed limited or no impact on disease phenotype, VSMC-specific OPG deletion robustly attenuated PAH,
 improving both haemodynamics and pulmonary vascular remodelling. To further explore the underlying
 mechanism, we performed kinome profiling in control and IPAH-PASMCs treated with OPG in the presence
 and absence of an anti-OPG antibody. This identified IPAH-specific regulation of the PI3K–Akt pathway and

enrichment of signalling networks involved in autophagy, cellular stress resistance, and longevity regulation.

2. Methods

2.1. Animal Models and Studies

All animal studies were approved by the University of Sheffield Animal Welfare and Ethical Review Body and conducted under the authority of a UK Home Office project licence, in accordance with the UK Animals (Scientific Procedures) Act 1986 and Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Animals were provided unrestricted access to water and a standard diet, housed under a 12-hour light/dark cycle at a controlled room temperature of $22 \pm 2^\circ\text{C}$ and a humidity of 35–60%. Investigators remained blinded to mouse genotype and treatment throughout all catheterization, echocardiography, histological assessments, and immunofluorescence quantifications.

2.1.1. Generation of *Tnfrsf11b* knockout Mouse Line

To create conditional and constitutive knockout (KO) alleles of the *Tnfrsf11b* gene, a targeted strategy was applied based on the NCBI transcript NM_008764.3, located on mouse chromosome 15. Exon 1, which contains the translation initiation codon, was left intact, whilst Exon 2 (approximately 1.5 kb) was flanked by loxP sites to facilitate tissue-specific deletion (**Figure 1a**). A positive selection marker (Puromycin resistance, PuroR) was flanked by FRT sites and inserted into intron 1 to facilitate selection in embryonic stem (ES) cells. The *Tnfrsf11b* floxed mouse is available via <http://www.infrafrontier.eu/emma/strain-search/straindetails/?q=EM:15281>.

The targeting vector was constructed using bacterial artificial chromosome (BAC) clones from the C57BL/6J RPCIB-731 BAC library. This vector was introduced into Taconic Biosciences' C57BL/6N Tac ES cell line via electroporation. Recombinant ES cells were selected using both positive (PuroR) and negative (thymidine kinase, Tk) selection markers to confirm homologous recombination events. Correctly targeted clones were validated through PCR and sequencing.

Following recombination, the conditional KO allele was generated via *in vivo* Flp-mediated excision of the PuroR selection marker to produce the conditional floxed allele 'tm1c'. The constitutive KO allele (tm1d) was subsequently produced by *in vivo* Cre-mediated deletion of Exon 2. This deletion disrupts *Tnfrsf11b* function by removing essential cysteine-rich domains, introducing a frameshift, and resulting in a premature stop codon in Exon 3 (**Figure 1a**), which likely triggers non-sense mediated RNA decay, leading to reduced gene expression. All remaining recombination sites were positioned in non-conserved genomic regions to minimize off-target effects.

2.1.2. Generation of Double-transgenic Mouse Colonies

Transgenic mouse colonies were generated by crossing the indicated mouse genotypes in the **Supplementary Table 1**. Mouse colonies were consequently genotyped for floxed alleles by using the primers given in **Supplementary Table 2**. Cell-specific deletion of *Tnfrsf11b* (*Opg*) was achieved by crossing OPG^{fl/fl} mice with various Cre driver lines to generate double transgenic animals: PGKcre^{tg/+} (ubiquitous), LysMcre^{tg/+} (myeloid cells), Myl2cre^{tg/+} (cardiac myocytes), Cdh5cre^{tg/+} (endothelial cells), smMHCcre^{tg/0} (vascular smooth muscle, VSMC), Col1a2cre^{tg/+} (fibroblasts) (**Figure 1b**). Knock-out of *tnfrsf6* (*Fas*) (JAX Labs, Stain No: 026643) in VSMC was performed by crossing Fas^{fl/fl} mice with smMHCcre^{tg/0}.

The inducible B6.FVB-Tg(Myh11-cre/ERT2)1Soff/J²⁸ (hereafter referred to as SMMHC-Cre-ERT2), which drives tamoxifen-inducible Cre expression under control of the smooth muscle myosin heavy chain 11

(*Myh11*) promoter, was used for targeting VSMCs.²⁹ Since the CreERT2 transgene is linked to Y-chromosome, only male mice were used for breeding and experiments with this transgene. Littermate control mice were generated during breeding (**Figure 1c**), with cre-negative tm1c mice, or corn oil-treated cre-positive mice used depending on the experimental cre line.

2.1.3. PAH Induction in Mice using Sugén/ Hypoxia (SuHx) Model

Following tamoxifen injections for Cre-inducible knockouts (2mg/mouse/day for 5 days) and corn oil injections for littermate controls, SuHx model was used to induce pulmonary arterial hypertension (PAH) in male mice at the age of 10-12 weeks (**Figure 1c**). PAH was induced through weekly subcutaneous injections of Sugén-5416 (Tocris, Bristol, UK) at a dose of 20 mg/kg body weight over a period of three weeks. Mice were maintained in hypoxic chambers, simulating an altitude of approximately 18,000 feet, for three weeks (day 1–21). At the end of this period (day 21), animals were sacrificed for end-point procedures (**Figure 1d**).

2.2. PAH Phenotyping

2.2.1. Transthoracic Echocardiography

Echocardiography was performed as described previously using a linear 30- to 40-MHz transducer (MX-550D) and the Vevo 3100 system (VisualSonics, Toronto, Canada). Animals were placed dorsally on a heated table to maintain the body temperature during the procedure, while ECG and respiration rate were continuously recorded. Anaesthesia was induced and maintained using isoflurane (maintenance at 0.5-1.0% after induction with 2% isoflurane) to sustain heart rates at 450–500 bpm in mice.

1 B-mode and M-mode images were obtained in parasternal long-axis (PSLAX) and short-axis (PSAX) views.
2 Left ventricular function (LVID, LV-EF, LV-FS) was quantified from PSLAX recordings. Right ventricular
3 structure (RVFWT, RVID) was assessed from the right parasternal long-axis view. Pulmonary artery
4 haemodynamics (peak flow, PA AT, PA ET) were measured using PW Doppler. Data analysis was performed
5 in Vevo Lab (v5.5.0). See **Supplemental Methods** for full procedural details.

7 **2.2.2. Haemodynamic Measurement of Right and Left-ventricular Pressure**

8 Left and right ventricular catheterisation was performed under isoflurane-induced anaesthesia
9 (maintenance at 1.5% after induction with 3% isoflurane) by using a closed chest method via the right
10 internal carotid artery and right external jugular vein as described previously. See **Supplemental Methods**
11 for full procedural details. Following haemodynamic measurements, animals were maintained under deep
12 anaesthesia with isoflurane inhalation (4%) and euthanasia was performed by exsanguination with cardiac
13 puncture followed by cervical dislocation in accordance with institutional and national ethical guidelines
14 for animal care and use. Tissues were then harvested for the analyses described below.

16 **2.3. Histological and Immunohistochemical Staining**

17 Paraffin embedded lung tissues were sectioned into 5 μ m slices and stained histologically with Alcian Blue
18 Elastic van Gieson (ABEVG). Immunohistochemical (IHC) staining was performed for α -smooth muscle
19 actin (α -SMA). For IHC, a biotinylated secondary antibody was applied, followed by the addition of an
20 avidin-biotin enzyme complex (Vectastain Kit, Vector Laboratories, USA) and 3,3'-diaminobenzidine
21 tetrahydrochloride (DAB) substrate. Paraffin embedded heart tissues were transversely cut in half and
22 sectioned into 5 μ m slices. Immunofluorescence (IF) staining was performed with wheat-germ agglutinin

(WGA) and Isolectin B4 (IB4) to analyse structural changes in the heart. The antibodies used in IHC and IF staining are listed in **Supplementary Table 3**.

Images of stained sections were captured using a Zeiss Imager Z2 microscope with an Axiocam 506 colour camera (brightfield), and a Zeiss Axio Observer with a Hamamatsu Flash 4.0 camera (fluorescence) (Carl Zeiss, Oberkochen, Germany). Images were analysed using ZEN software 2.6 Blue edition.

2.4. Measurement Serum and Tissue OPG

Serum and lung tissue OPG levels in mice were quantified using the DuoSet ELISA kit (R&D Systems, DY459) according to the manufacturer's instructions. Tissue OPG levels were normalized to total lung protein content, which was determined using the Pierce Micro BCA Protein Assay Kit (Thermo Scientific, 23235).

2.5. Myography

Pulmonary arterial segments were isolated from mice and dissected into 1–2 mm rings. These segments were mounted on a wire myograph system (DMT 620M, Danish MyoTechnology, Denmark) for isometric tension recording. Baseline tension was set to 5 mN for each arterial ring and allowed to equilibrate. To assess contractile function, segments were exposed to 50 mM potassium chloride (KCl) three times, with thorough washing between each application to ensure reproducibility and tissue viability.

For evaluation of vascular smooth muscle cell (VSMC)-dependent relaxation, arterial segments were pre-contracted with 10 μ M phenylephrine (PE) until a stable plateau was achieved. Subsequently, cumulative concentrations of the nitric oxide donor sodium nitroprusside (SNP; 1×10^{-9} to 1×10^{-6} M) were administered to generate concentration–response curves.

2.6. Cell Culture

Human pulmonary arterial smooth muscle cells (HPASMCs) from healthy individuals were obtained from Lonza Ltd (CC-2581, Basel, Switzerland) while HPASMCs from IPAH patients were obtained from the Universities of Giessen and Marburg Lung Center/ German Center for Lung Research (UGMLC/DZL) Giessen Biobank confirming to the principles outlined in Declaration of Helsinki. The collection and use of samples were approved by the ethical committee of Justus Liebig University (approval number: 58/15). All patients provided informed written consent for the use of biomaterials for research purposes. Individual demographic details are provided in **Supplementary Table 4**. Detailed protocols of cell culture experiments can be found in **Supplementary Material**.

2.7. Protein Isolation

Lung tissue protein lysates were prepared from frozen tissue with RIPA lysis buffer with freshly added protease and phosphatase inhibitors. Protein lysates from HPASMCs were prepared using M-PER lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease and phosphatase inhibitor cocktails (Pierce, Rockford, IL, USA). Cell lysates were then transferred into pre-cooled tubes and incubated at 4°C for 1 hour and centrifuged at 16,000 x g for 15 minutes. The supernatant was snap-frozen for further use and stored at -80 °C. Protein quantification was performed by BCA assay (Thermo Fisher Scientific, Pierce BCA Protein Assay Kit, 23225) according to manufacturer's protocol. Protein aliquots were used for peptide-based kinase assays and western-blot analysis.

2.8. Peptide-based kinase activity assay

The peptide-based kinase activity assay was performed using the PamStation platform (PamGene International, s-Hertogenbosch, Netherlands) to analyse the activity of 380 protein kinases (about ~70%

of all protein kinases), i.e. serine/ threonine kinase (STK) and tyrosine kinase (TK), and analysed with Evolve 12 software, as previously described. Briefly, 2 μ g (for STK) and 5 μ g (for TK) of protein lysate was loaded onto the respective PamChip arrays, in the presence of reaction buffer (proprietary information of PamGene) containing 1% (w/v) BSA, STK or TK antibody mix, and ATP. Peptide phosphorylation was detected using fluorescently labelled anti-phospho antibodies (FITC-labelled secondary antibodies) on each microchip.

Data acquisition and analysis were performed according to the manufacturer's instructions. Briefly, fluorescence signals were monitored using a CCD camera over multiple cycles with different exposure times. Software-based image analysis was used to assess the signal intensity, with our analysis focusing only on peptides that exhibit stable signals after filtering for quality control parameters. For each treatment group, six healthy individuals (n=6) and six IPAH patients (n=6) were analysed. Data were represented after logarithmic (Log_2) transformation of the processed raw signal, and the mean of log-transformed values was used for visualization prior upstream kinase prediction.

Additional protein expression analyses for validation of the kinase activity assay were performed using Simple Western and conventional Western blotting, with full protocols and antibody lists (**Supplementary Table 5-6**), and unedited images (**Supplementary Figure 6-7**) were provided in the **Supplemental Methods**.

2.9. Correlation Analysis

To examine the association between circulating and tissue levels of OPG with right ventricular systolic pressure (RVSP) and pulmonary vascular remodelling (medial thickness and cross-sectional area) correlation analyses were conducted in R (version 4.4.2).

Linear regression models were fitted using the *lm()* function with ordinary least squares (OLS) estimation. Model performance was evaluated using coefficients of determination (R^2) and p-values. Monotonic relationships were additionally assessed with Spearman's rank correlation coefficient (ρ). Scatter plots were generated with the *ggplot2* package.

2.10. Statistics

A sample size of at least four animals was used to provide greater than 95% power to detect a 10-mmHg difference in RVSP with a standard deviation (SD) of 3 mmHg with 95% confidence. Additional animals were studied in large group comparisons and to obtain sufficient tissue for biochemical analysis. Data analysis and statistical testing were performed using GraphPad Prism software (Version 10). Data are shown as mean $\pm$ standard error of the mean (SEM). For datasets with adequate sample size, normality (Shapiro–Wilk) and variance homogeneity (Levene/Brown–Forsythe) was assessed. For most animal comparisons, the treatment group sizes are $n < 10$, where normality tests have low power and may not reliably detect non-normality; in these cases, we followed widely used practice and applied non-parametric tests. One-way or two-way ANOVA followed by appropriate *post-hoc* tests for multiple comparisons (as indicated in the figure legends) was used for statistical analysis. For comparisons between only two groups, unpaired t-tests were performed. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cell-specific deletion of *Opg* results in differential serum and tissue levels

OPG^{fl/fl} mice (**Figure 1a**) were crossed with Cre driver lines, including those targeting vascular smooth muscle, endothelium, myeloid cells, fibroblasts, and cardiomyocytes (**Figure 1b**) to generate conditional and constitutive tissue-specific deletion of *Tnfrsf11b* (**Figure 1c**). Following cre-activation via tamoxifen

(corn oil control), double transgenic procedural mice received baseline echocardiography prior to initiation of PAH via weekly injection of Sugen 5416 and exposure to hypoxia (10% O₂) for 3 weeks. After 3 weeks, mice were assessed for PAH, and serum and tissues harvest for downstream analysis (**Figure 1d**). After phenotyping and tissue harvesting, tissue-specific deletion of *Opg* was illustrated by dual immunohistochemistry (IHC) (**Supplementary Figure 1**). Given that OPG is a secreted protein, its levels were additionally measured in serum and whole lung tissue to quantify the efficacy of the cell-specific knockouts. Near-global deletion of OPG driven by PGK-cre resulted in an almost complete reduction in both serum and tissue levels (**Figure 2a, b**). Myeloid-specific (LysM) deletion lowered serum OPG without affecting lung tissue, whereas cardiac myocyte-specific (Myl2) deletion decreased OPG levels in serum and lung tissue (**Figure 2a, b**). Endothelial cell-specific (Cdh5) deletion reduced serum OPG but had no impact on lung levels (**Figure 2c, d**). Vascular smooth muscle cell-specific (smMHC) deletion caused pronounced reductions in both serum and pulmonary tissue, while fibroblast-specific deletion had only minor effects on serum and no effect on tissue OPG (**Figure 2c, d**).

3.2. PGK-Cre—but not LysM-mediated *Opg* deletion Protects Against PAH

PGKcre^{tg/+}/OPG^{fl/fl} mice (PGK; near-global knockout) phenocopied the protective effects of global *Opg* deletion^{25, 30}, demonstrating significantly reduced right-ventricle systolic pressure (RVSP) and pulmonary vascular resistance (ePVR) compared to single transgenic littermate controls (tm1c, SuHx), with no significant changes in left-ventricle end-systolic pressure (LveSP) (**Figure 3a-c, Supplementary Table 7**). Cardiac output (CO, ml/min) in PGKcre^{tg/+}/OPG^{fl/fl} mice was also significantly improved (**Figure 3d, Supplementary Table 7**), however, there were no significant changes observed in pulmonary artery acceleration time (PAAT, ms) and right-ventricle hypertrophy (RVH) between both groups compared to SuHx mice (**Figure 3e-f, Supplementary Table 7**). Immunohistochemical analysis of lungs with ABEVG and

SMA, to assess pulmonary vascular remodelling revealed that PGKcre^{tg/+}/OPG^{fl/fl} mice showed a significant reduction in both the percentage of muscularized arteries, and the degree of muscularization (media/CSA) in small arteries (<50 μ m) (**Figure 3g-i**). Considering the involvement of monocytes and macrophages in pulmonary vascular during the early onset of disease, and based on our previous findings on bone-marrow chimeric OPG^{-/-} mice suggesting bone-marrow derived OPG driving PAH development²⁵, we investigated whether myeloid-specific deletion of *Opg* (LysM; LysMcre^{tg/+}/OPG^{fl/fl}) could protect against PAH progression. However, LysMcre^{tg/+}/OPG^{fl/fl} mice did not exhibit any considerable improvements in pulmonary vascular remodelling or haemodynamic parameters (**Figure 3a-d**), suggesting non-myeloid OPG sources drive PAH progression.

3.3. Cardiomyocyte-specific deletion of *Opg* does not prevent PAH progression or cardiac remodelling

Since we also previously reported that OPG is highly expressed in RV of IPAH patients²⁵, we next investigated the role of OPG in cardiac myocytes by generating cardiac myocyte-specific deletion of *Opg* by crossing OPG^{fl/fl} mice with Myl2cre^{tg/+} mice. There were no significant haemodynamic differences between Myl2cre^{tg/+}/OPG^{fl/fl} (Myl2) and OPG^{fl/fl} (tm1c) animals following SuHx exposure (**Figure 4a-d, Supplementary Table 7**). Analysis of pulmonary vascular remodelling indicated a modest reduction in the percentage of muscularized arteries in Myl2cre^{tg/+}/OPG^{fl/fl} mice compared to OPG^{fl/fl} mice after SuHx exposure, however, the degree of remodelling in small arteries remain unchanged between groups (**Figure 4e-g**), and these changes had no impact on haemodynamics. Cardiac function and remodelling were assessed with echocardiography and immunofluorescence analysis to confirm the changes at the cellular level, respectively. Cardiomyocyte cross-sectional area (CSA, μ m²) was similar between Myl2cre^{tg/+}/OPG^{fl/fl} and OPG^{fl/fl} mice with no considerable changes (**Figure 4h, i**). Additionally, isolectin B4 (IB4) staining of cardiac capillaries reveal no significant changes in capillary density across experimental groups (**Figure 4j**)

suggesting that myocyte-specific deletion of *Opg* has impact on pulmonary vascular and cardiac remodelling during PAH progression induced by SuHx model.

3.4. Smooth muscle cell-specific deletion of *Opg* predominantly modulates PAH pathogenesis

We next sought to investigate the contribution of endothelial cell (EC, *Cdh5*), vascular smooth muscle cell (VSMC, *smMHC*), and fibroblast (FB, *Col1a2*) derived *Opg* in PAH pathophysiology. There were no significant haemodynamic differences between corn oil and tamoxifen treated *Cdh5cre^{tg/+}/OPG^{fl/fl}* or *Col1a2cre^{tg/+}/OPG^{fl/fl}* following induction of PAH (**Figure 5a-c, Supplementary Table 7**). Mice with VSMC-specific knockout of *Opg*, *smMHCcre^{tg/0}/OPG^{fl/fl}* (TAM), demonstrated a significant reduction in RVSP (27.57 ± 4.71 vs. 36.64 ± 1.98 mmHg, $p < 0.001$, respectively) but no difference in LVeSP (**Figure 5a, b, Supplementary Table 7**). There was a marked reduction in PVR (80.59 ± 33.86 vs. 145 ± 43.82 mmHg ml⁻¹ min, $p < 0.05$, respectively) but no significant effect on cardiac output when compared to corn oil treated controls following SuHx (**Figure 5c, Supplementary Table 7**). EC-specific *Opg* knockouts, *Cdh5cre^{tg/+}/OPG^{fl/fl}* mice, exhibited no significant reduction in RVSP, or PVR (**Figure 5a, c, Supplementary Table 7**).

Assessment of pulmonary vascular remodelling revealed that both EC- and VSMC-specific deletion of *Opg* resulted in a significant reduction in the proportion of muscularized arteries (%) compared to their respective controls (**Figure 5e, f**). In contrast, FB-specific *Opg* knockout mice (*Col1a2cre^{tg/+}/OPG^{fl/fl}*) were not protected from pulmonary vascular remodelling following SuHx exposure. Notably, only tamoxifen-treated *smMHCcre^{tg/0}/OPG^{fl/fl}* mice exhibited a reduction in vessel wall thickness (Media/CSA) (**Figure 5e, g**). Since only VSMC-specific *Opg* deletion led to improvements in both pulmonary vascular remodelling and haemodynamics, we next assessed whether vasoreactivity was altered in those animals compared to

corn-oil treated controls. We confirmed that *Opg* deletion did not significantly affect PASMC vasoreactivity (Figure 5h).

3.5. Smooth muscle cell-derived OPG does not act predominantly via Fas in PAH

We previously identified Fas as a novel receptor for OPG on human PASMCs.²⁵ To test whether VSMC-derived OPG signals via the Fas receptor, we generated a mouse line by crossing smMHCcre^{tg/0} mice with Fas^{tm1Cgn} mice to achieve VSMC-specific deletion of *Fas*. These mice were then assessed using the SuHx model to investigate the pathobiological relevance of OPG-Fas signalling in PAH (Supplementary Figure 2).

Haemodynamic analysis revealed that VSMC-specific deletion of *Fas* did not confer any protective effect in the murine SuHx model, with no significant changes observed in haemodynamics or pulmonary vascular remodelling (Supplementary Figure 2). These findings suggest that OPG-Fas binding is not a contributing mechanism by which OPG modulated PASMCs in the mouse SuHx model, indicating the involvement of alternative molecular pathways.

3.6. Kinome profiling reveals OPG disruption dysregulates activity of AKT in IPAH PASMCs

Since only smMHCcre^{tg/0}/OPG^{fl/fl} mice exhibited improvements in both pulmonary vasculature remodelling and haemodynamic parameters, we analysed OPG-mediated kinase activity in PASMCs to gain some mechanistic insight. To this end, kinome profiling was performed with primary PASMCs isolated from the lungs of disease-control individuals (donors) and patients with IPAH after lung transplantation. Cells were then stimulated with human recombinant OPG protein, with and without the addition of human anti-OPG monoclonal antibody (Supplementary Figure 3a).

We assessed the activation status of serine/threonine (STK) kinases and tyrosine kinases (TK) using the PamGene platform based on the phosphorylation of 144 different substrates peptides for STKs or 196 substrates peptides for TKs, respectively (**Supplementary Figure 3a**). We observed significant differences in phosphorylation levels between PSMCs from donors and IPAH patients, as well as among treatment groups: IgG1-only control (IgG1), OPG-stimulated (OPG+IgG1) and OPG-stimulated with anti-OPG antibody treatment (OPG+mAB:OPG). Heatmaps illustrate the alterations in the phosphorylation levels of TK and STK substrate peptides, respectively (**Figure 6a**), which were used to predict the upstream kinase activity of up to 380 protein kinases.

To determine the overall effect of OPG on the kinome, two-group comparisons between OPG-stimulated PSMCs compared to controls (IgG1) were analysed. Across both IPAH- and control-PSMCs OPG stimulation resulted in an overall lower peptide phosphorylation pattern in IPAH-derived compared to control-derived PSMC (**Figure 6a**). We filtered the predicted upstream kinases based on two important parameters: Firstly, the mean specificity score is expressed as $-\log_{10} p$ -value, where $p < 0.05$ refers to the statistical significance for the changes of the phosphorylation of substrate peptide sets between the two selected experimental conditions. Secondly, the predicted differential kinase activity, i.e., median kinase statistic, depicts the overall change of the peptide set that represents the group of substrates for the given kinase (**Supplementary Figure 3b, c**). We subsequently identified 25 kinases, (14 in donor- and 11 in IPAH- PSMC, **Supplementary File 1**) that significantly alter their substrate peptide phosphorylation levels following OPG stimulation (e.g Janus Kinase 1 (JAK1), MAPK Activated Protein Kinase 2/3 (MAPKAPK2/3), atrial natriuretic peptide (ANP alpha)), and generated a protein-protein interaction (PPI) network to visualize predicted interactions (**Supplementary Figure 3b- 4a**). Further analysis was conducted to identify signalling pathways affected by the kinases with significantly altered activation levels. Most notably, pathways related with Programmed Cell Death Protein-1 (PD-1) and Programmed Cell Death Ligand-1 (PD-L1) checkpoint, osteoclast differentiation and platelet and chemokine activation were altered following

OPG stimulation (**Supplementary Figure 4b**). The kinases that were only altered in IPAH patients compared to donor PSMCs, were mainly affecting VEGF and MAPK signalling pathways, osteoclast differentiation and cellular senescence (**Supplementary Figure 4b**).

Subsequent analysis identified 17 kinases (3 in donor, and 14 in IPAH-derived PSMC, **Supplementary File 2**) with altered activation following anti-OPG treatment in PSMCs from IPAH patients (e.g. AKT1, Death-associated protein kinase 2 (DAPK2)) and donors (e.g. Casein kinase I isoform epsilon (CK1), Protein kinase C (PKC)) revealed specific regulation of the FoxO, mTOR, and PI3K-Akt signalling pathways (**Figure 6b**). These changes were accompanied by enrichment in pathways related to autophagy and longevity regulation, both of which are linked to cellular stress resistance and growth (**Figure 6b**).

The seventeen kinases with altered activation levels post-anti-OPG treatment were used to construct a PPI network (**Figure 6c**). To explore potential downstream signalling, FAS, OPG, TRAIL, and their receptors were also mapped onto this network. The resulting model suggests that OPG interact with these kinases indirectly through TRAIL and FAS (**Figure 6c**). Among the affected kinases, AKT1 appeared as a central node. Importantly, AKT1 kinase activity was predicted to be significantly reduced in IPAH PSMCs, but not in disease control samples, which was validated by Simple Western analysis using PSMCs from IPAH patients and disease control donors (**Figure 6d, e**).

3.7. Anti-OPG Treatment Reduces Akt Signalling in Rodent Models

We previously demonstrated that a human monoclonal anti-OPG antibody treatment reverses PAH in both prophylactic and therapeutic settings in rat SuHx model of PAH.²⁵ To validate our findings following kinome analysis and to further confirm results observed in patient-derived PSMCs, we analysed Akt phosphorylation in lung tissue from SuHx rats (3 weeks hypoxia followed by up to 9 weeks), treated with anti-OPG antibody (KymAb3) (3 mg/kg, 3 doses) (**Figure 7a**). Western blot analysis revealed that anti-OPG

1 treatment significantly reduced p-Akt expression compared to both SuHx and IgG4-treated controls
 2 **(Figure 7b, c)**, indicating that OPG contributes to Akt pathway activation *in vivo*. Additional, analysis of
 3 p-AKT (Ser473) levels was performed in lung homogenates from VSMC-specific *Opg* knockout mice
 4 (smMHC-Tam) and corn-oil-treated littermate controls (smMHC-CO). Western blotting and quantification
 5 **(Figure 7d, e)** showed a reduction in p-AKT levels in smMHC-Tam mice compared with smMHC-CO
 6 controls; however, this difference did not reach statistical significance ($p = 0.0635$). Total AKT levels were
 7 unchanged between groups.

8 To examine functional relevance of Akt1 in OPG induced cell phenotype we utilised an siRNA approach.
 9 Human PASMCs stimulated with OPG recombinant protein following AKT siRNA treatment confirmed a
 10 significant downregulation of AKT in the mRNA levels **(Figure 7f)**. Following functional assays indicated
 11 that pro-migratory **(Figure 7g-h)** and pro-proliferative **(Figure 7i)** effects of OPG stimulation on PASMCs
 12 were suppressed after siAKT treatment. Overall, these results indicates that OPG modulation on PASMCs
 13 are AKT-dependent.

14 15 **3.8. Tissue OPG Levels Correlate with RVSP and Pulmonary Vascular Remodelling in** 16 **VSMC-specific Knockout mice**

17 To further investigate the relationship between OPG levels and disease severity, we performed correlation
 18 analyses in VSMC-specific *Opg* knockout mice **(Figure 7f, Supplementary Figure 5)**. Tissue OPG levels
 19 showed strong positive correlations with both RVSP ($R^2 = 0.411$, Spearman $r=0.749$, $p=0.00029$) and
 20 vascular remodelling ($R^2 = 0.373$, Spearman $r=0.682$, $p=0.00072$) **(Figure 7f)**. In contrast, serum OPG levels
 21 did not correlate significantly with either parameter **(Supplementary Figure 5)**. These findings highlight
 22 the importance of local OPG expression in driving pulmonary vascular pathology.

4. Discussion

While OPG has previously been implicated in the pathogenesis of PAH, the precise cellular source and the disease specific downstream mechanisms of OPG signalling have remained poorly defined. Here, we utilised a combination of cell-specific conditional *Opg* knockout mouse models, primary human (PAH patient-derived) pulmonary artery smooth muscle cells (PASMCs), and kinome-wide profiling to systematically understand the role of OPG in PAH progression and pulmonary vascular remodelling. We have shown among all cell-type specific conditional knockouts generated, only VSMC-specific *Opg* deletion significantly attenuated all markers of disease. In contrast, deletion of *Opg* in myeloid cells, cardiomyocytes and fibroblasts did not confer any haemodynamic or vascular benefit. Interestingly, while endothelial-specific *Opg* deletion attenuated the number of remodelled pulmonary arteries, it did not reduce the degree of remodelling in affected vessels and was not sufficient to improve haemodynamics. This could be due to the smaller number of ECs in comparison to SMC, and thereby lower local concentrations. Collectively, our findings suggest that VSMC-derived OPG is the predominant contributor to vascular remodelling and haemodynamic deterioration in PAH. These data fit well with our previous work showing high levels of OPG within the media of remodelled pulmonary vascular lesions²³ and serum,²⁴ further emphasising its utility as a biomarker for PAH development and prognosis.

While our findings provide new insight into the cell-specific roles of OPG in PAH, several limitations should be acknowledged. First, since OPG is naturally secreted, its functional regulation across autocrine and paracrine signalling may not be captured by our model since deleting *Opg* in individual cell populations may be compensated by OPG secreted from other cell

1 types. The use of constitutive cell-specific *Opg* knockout models limits our ability to assess the
2 temporal role of OPG during disease progression.

3 Although we previously proposed OPG and FAS interaction as a main mechanism of action to
4 mediate PASMCM proliferation and migration²⁵, our VSMC-specific *Fas* knockout model did not
5 replicate the protective phenotype observed with *Opg* deletion. This suggests that FAS might not
6 be the principal mediator of OPG activity in VSMC within the mouse model studies, and that there
7 are functional differences in Fas signalling, or in regulation between human and mouse as has
8 been previously reported.^{31, 32} Given that VSMC-specific *Fas* deletion was not protective, these
9 data point to alternative upstream mediators of OPG-driven signalling. While our earlier work had
10 identified Fas as the leading candidate receptor, other putative receptors were also described in
11 that study.²⁵ Moreover, additional reports show that OPG can interact with integrins (e.g., $\alpha\beta3$)
12 to activate FAK-AKT signalling³³, and $\alpha5\beta1$ has recently been implicated in vascular remodelling,
13 aligning with the current findings.

14 Through an unbiased kinome investigation to elucidate downstream signalling networks in
15 primary HPASMCs from IPAH patients, we identified marked alterations in phosphorylation
16 activity across multiple signalling pathways with OPG stimulation followed with antibody
17 blockade. Notably, anti-OPG treatment reduced activity in PI3K-AKT pathway, and autophagy-
18 related pathways that are linked to cell survival and stress resistance. Among these, AKT1
19 emerged as a central hub for our analysis when we generated a network of OPG, FAS, TRAIL and
20 TRAIL receptors (TRAIL-R1, R2, R3, R4) with differentially phosphorylated kinases after anti-OPG
21 treatment following OPG stimulation. Based on kinome profiling, AKT1 kinase activity was
22 significantly suppressed in IPAH-derived PASMCMs treated with anti-OPG antibody. Functionally, we

1 demonstrated the dependence of OPG-induced proliferation and migration on AKT. These effects
2 are likely to be more pronounced in hyper-proliferative IPAH-derived PASMC where AKT
3 phosphorylation is increased. These findings are consistent with prior work implicating
4 dysregulated AKT signalling in PAH, in which AKT1 is involved in cell proliferation, survival, and
5 migration³⁴, and has been shown to be overactive in PAH.³⁵ In a previous study, Jia and colleagues
6 demonstrated that OPG induce cell proliferation via interacting with integrin $\alpha_v\beta_3$ leading to
7 downstream focal adhesion kinase (FAK) and AKT pathway activation in SuHx mouse model of
8 PAH.³⁰ A recent study from Lemay et al. also studied the role of integrin $\alpha_5\beta_1$ as a potential
9 therapeutic target for PAH, in which their data suggests integrin $\alpha_5\beta_1$ promotes vascular
10 remodelling via Forkhead box protein M1 (FOXM1) activation.³³ Although this kinome profiling in
11 PASMCs provided valuable mechanistic insights into OPG-associated signalling, it is important to
12 note that the methodology only captures approximately 70% of known protein kinases. Other
13 signalling mechanisms may also have a role in OPG signalling, limiting our ability to fully identify
14 the full complexity of OPG and its cellular interactions within the pulmonary vasculature. While
15 AKT1 emerged as a key downstream target, broader signalling networks and compensatory
16 pathways remain to be explored.

17 Our results in the *in vivo* experimental model of SuHx rats treated with monoclonal anti-OPG
18 antibody (KymAb3) further confirms the findings in kinome analysis. This intervention significantly
19 reduced AKT phosphorylation in lung tissue, confirming that OPG contributes to PI3K–AKT
20 pathway activation *in vivo*. Although there was a reduction in pAKT levels in VSMC-specific *Opg*
21 mice this did not reach statistical significance. This is likely due to exogenous OPG secreted from
22 other cell types, which could still activate AKT within VSMCs.

OPG is primarily defined by its role as a secreted glycoprotein in bone metabolism, where it functions as a decoy receptor for receptor activator of nuclear factor- κ B ligand (RANKL), thereby inhibiting osteoclastogenesis.¹⁵ Beyond the skeletal system, OPG has increasingly been recognized for its function in vascular biology, where elevated OPG levels are associated with vascular calcification, arterial stiffness, and increased cardiovascular risk in aging and chronic inflammatory conditions.^{17, 18, 23, 24, 36} Recent studies have identified OPG as a key mediator linking premature VSMC senescence to their phenotypic modulation, in which senescent VSMCs upregulate OPG as a part of the senescence-associated secretory phenotype (SASP),¹⁹ contributing to the loss of contractile identity and reinforces a synthetic, pro-inflammatory state that impairs their capacity to re-differentiate.¹⁹ Based on our kinome-wide analysis, blockade of OPG using a monoclonal antibody led to reduced activity of kinases involved in apoptosis, cellular stress and senescence. These findings align with the growing evidence of positioning OPG as a component of the SASP and suggest a potential role in regulating vascular plasticity.

Additionally, we assessed vascular reactivity using wire myography in pulmonary arteries from wild-type and *smMHC-Opg* knockout mice. Interestingly, we did not observe significant differences in vasorelaxation responses, suggesting that the protective effects of VSMC-specific *Opg* deletion in PAH are unlikely mediated through changes in contractile function. Instead, our findings support a mechanism whereby VSMC-derived OPG primarily contributes to disease progression via its effects on smooth muscle proliferation, migration, and phenotypic modulation, rather than altering vascular tone directly. Overall, our results indicate deletion of *Opg* from VSMCs reduce vascular thickening and restore vascular architecture and may shift VSMCs to a less synthetic or proliferative phenotype. However, it should be noted that our studies were limited

1 to the use of male mice due to the Y-chromosome integration of the *smMHC*-Cre transgene. This
2 approach precludes evaluation of potential sex-specific differences in OPG signalling and vascular
3 remodelling.

4 Interestingly, correlation analyses performed in VSMC-specific *Opg* knockout mice revealed that
5 tissue levels of OPG strongly correlated with both RVSP and pulmonary vascular remodelling,
6 whereas serum levels of OPG did not show any significant association. This suggests that local
7 OPG expression in vasculature, although more challenging to measure, may be a more relevant
8 biomarker of disease severity than circulating levels, and further support the notion that VSMC-
9 derived OPG drives disease progression.

10 **5. Conclusion**

11 In summary, our study identifies vascular smooth muscle cell-derived OPG as a predominant
12 contributor to pulmonary vascular remodelling and haemodynamic deterioration in experimental
13 PAH. These findings refine prior knowledge derived from global knockout models and
14 demonstrate that OPG can promote pathological remodelling via AKT1-dependent signalling
15 rather than Fas in VSMCs. Furthermore, our results support an emerging paradigm in which OPG
16 acts not only as a vascular remodelling mediator but also as a SASP component that links
17 premature senescence to pulmonary vascular pathology. Collectively, these findings provide
18 mechanistic insight and further emphasis to exploring OPG as a translational target in PAH and
19 age-related vascular disease.

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5

ACCEPTED MANUSCRIPT

1 Figures

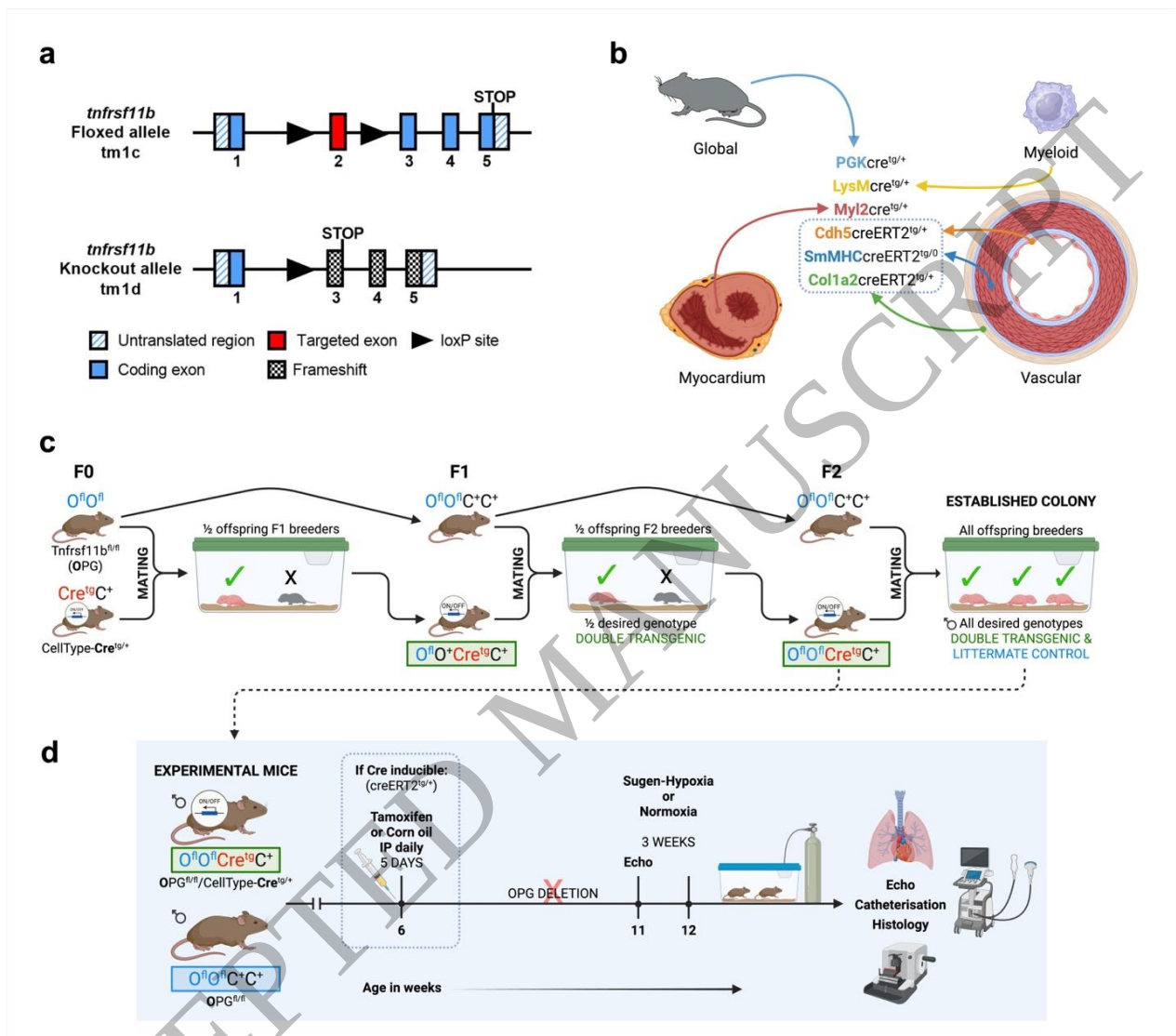


Figure 1: Transgenic mice breeding strategy and experimental setup for Sugen/Hypoxia (SuHx) induced PAH.

a. Schematic representation of the targeting strategy to generate knockout of the *Tnfrsf11b* gene (not drawn to scale). The upper panel showing *Tnfrsf11b* allele after flp recombination, while lower panel shows the knockout allele following Cre recombination. **b.** Diagram illustrating cell-specific OPG deletion strategy which mice with floxed OPG^{fl/fl} alleles were crossed with Cre-driver lines targeting specific cell types to

1 achieve targeted OPG deletion. Global KO (PGKcre^{tg/+}), myeloid (LysMcre^{tg/+}), cardiac myocytes
2 (Myl2cre^{tg/+}), endothelial cells (Cdh5creERT2^{tg/+}), vascular smooth muscle cells (SmMHCcreERT2^{tg/0}) and
3 fibroblast (Col1a2creERT2^{tg/+}). **c.** Diagram illustrating the colony establishment process from the F0 to F2
4 generations to obtain mice with the desired genotypes. Successful breeding led to F2 offspring with cell-
5 specific OPG knockouts, which were used in PAH studies. **d.** Schematic of the Sugeng/hypoxia protocol,
6 including tamoxifen injections for Cre-inducible knockouts and corn oil injections for littermate controls.
7 Mice received Sugeng5416 injection (20 mg/kg), followed by three weeks of hypoxia (10% O₂), with
8 subsequent evaluation using echocardiography, catheterisation, and histological analysis.

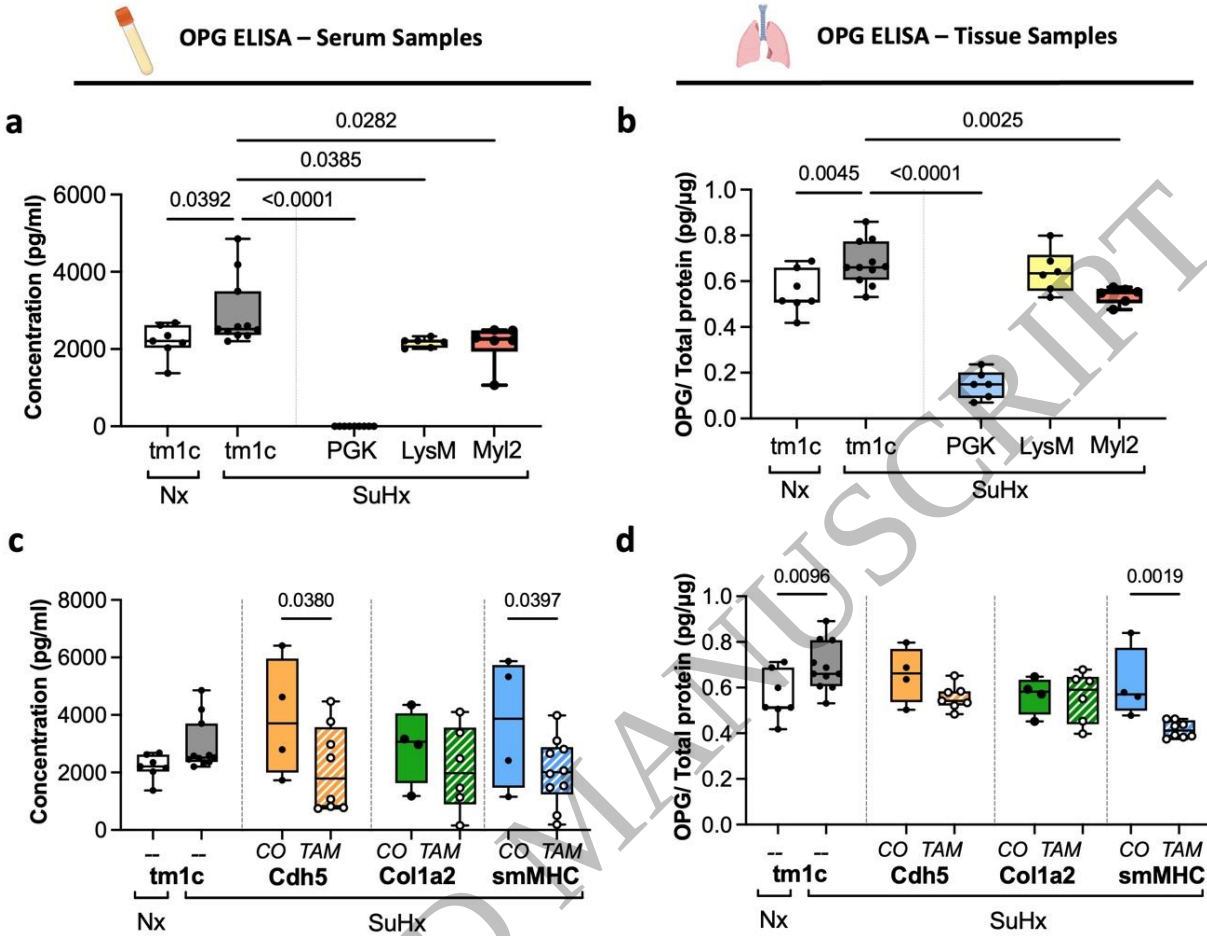


Figure 2: Cell-specific deletion of *Opg* leads to differential OPG levels in serum and lung tissue.

a. Circulating OPG levels measured by ELISA in serum collected from control (Nx, n=7), global knockout (PGK, n=6-9), myeloid-specific (LysM-Cre, n=6), and cardiomyocyte-specific (Myl2-Cre, n=6) *Opg* deletion mice after 3 weeks of Sugren-hypoxia (SuHx, n=11). **b.** OPG protein levels in lung tissue homogenates from the same animals, quantified by ELISA and normalized to total protein concentration measured via BCA assay (expressed as pg OPG per μg total protein). **c-d.** Circulating and tissue OPG levels in conditional *Opg* knockout mice with cell-specific deletions in endothelial cells (Cdh5-Cre (CO, n=4 and TAM, n=7-8)), smooth muscle cells (smMHC-Cre (CO, n=4, TAM, n=10)), and fibroblasts (Col1a2-Cre (CO, n=4, TAM, n=6)). Data from tm1c control mice in panel a and c, and panel b and d are reused, with plots separated only for representation purposes. Each dot (n) represents an individual animal. Data are presented as mean ± SEM

1 and were analysed using one-way ANOVA followed with Fisher's LSD for multiple comparisons. Statistical
2 significance is indicated by p-values.

3

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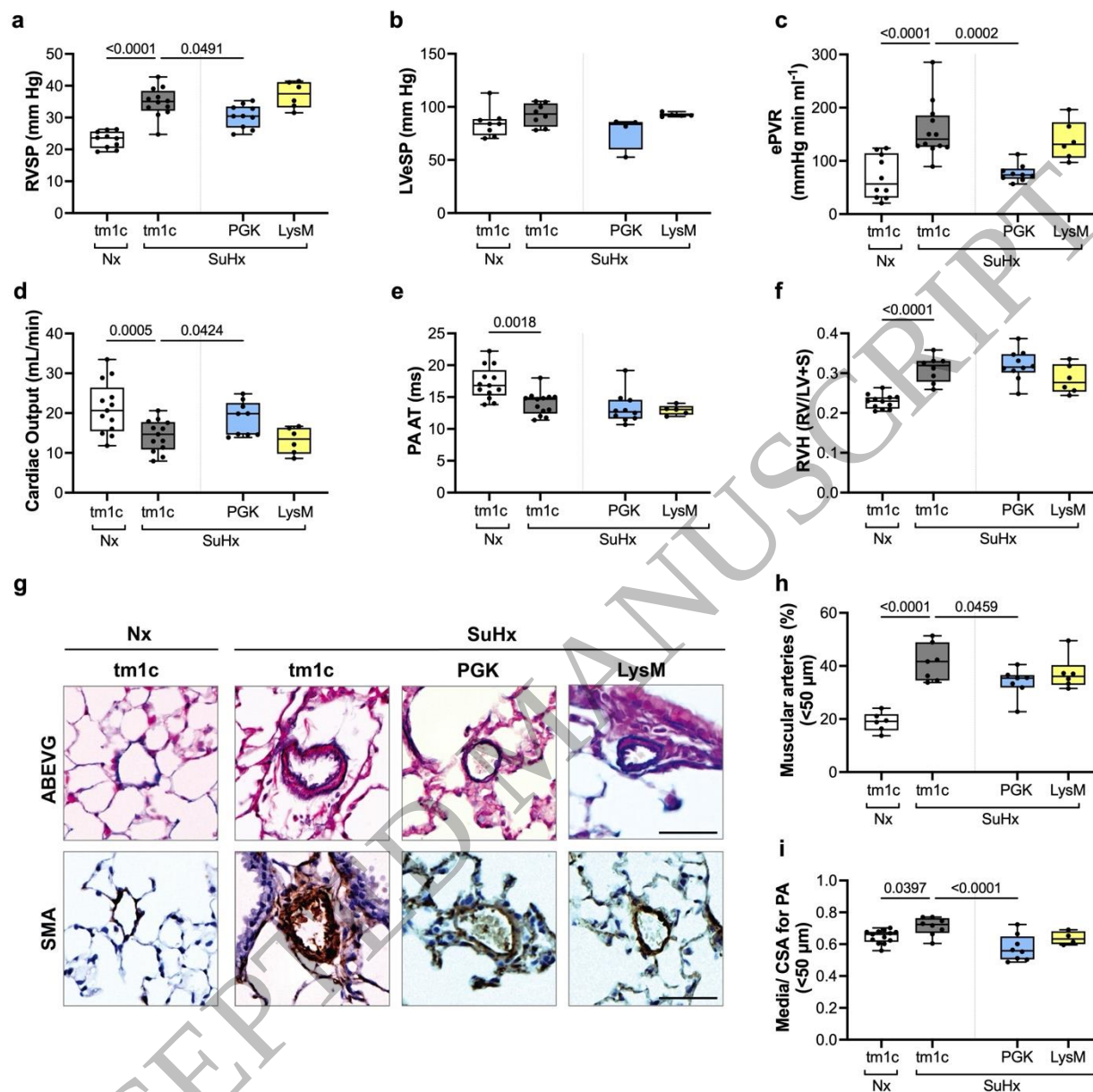


Figure 3: PGKcre^{tg/+}/OPG^{fl/fl} mice phenocopied previous results obtained from OPG^{-/-} mice and were protected from developing PAH.

Box plots showing **a.** right ventricular systolic pressure (RVSP, mm Hg), **b.** left ventricular end-systolic pressure (LVEsP, mm Hg), **c.** estimated pulmonary vascular resistance (ePVR, mmHg ml⁻¹ min⁻¹), **d.** cardiac output (CO, ml/min), **e.** pulmonary artery acceleration time (PA AT, ms), **f.** right ventricular hypertrophy (RVH). Representative images **g.** of lung sections stained with Alcian Blue Elastic van Gieson (ABEVG) and

1 immunostained for α -smooth muscle actin (α -SMA). **Tamoxifen-induced**. Box plots showing the
2 quantification of **h.** the relative percentage of muscularised pulmonary arteries less than 50 μ m, and **i.** the
3 degree of medial wall thickness as a ratio of total vessel size (Media/CSA) in small pulmonary arteries
4 pulmonary arteries less than 50 μ m. (Each dot represents an individual animal (n). Nx: n= 6-13, SuHx: n=
5 7-13, PGK: n=4-10, LysM-Cre: n=6) Data are shown as mean $\pm$ SEM. *P*-values were calculated with one-way
6 ANOVA followed by Dunnett's test for multiple comparisons and indicated on the plots.

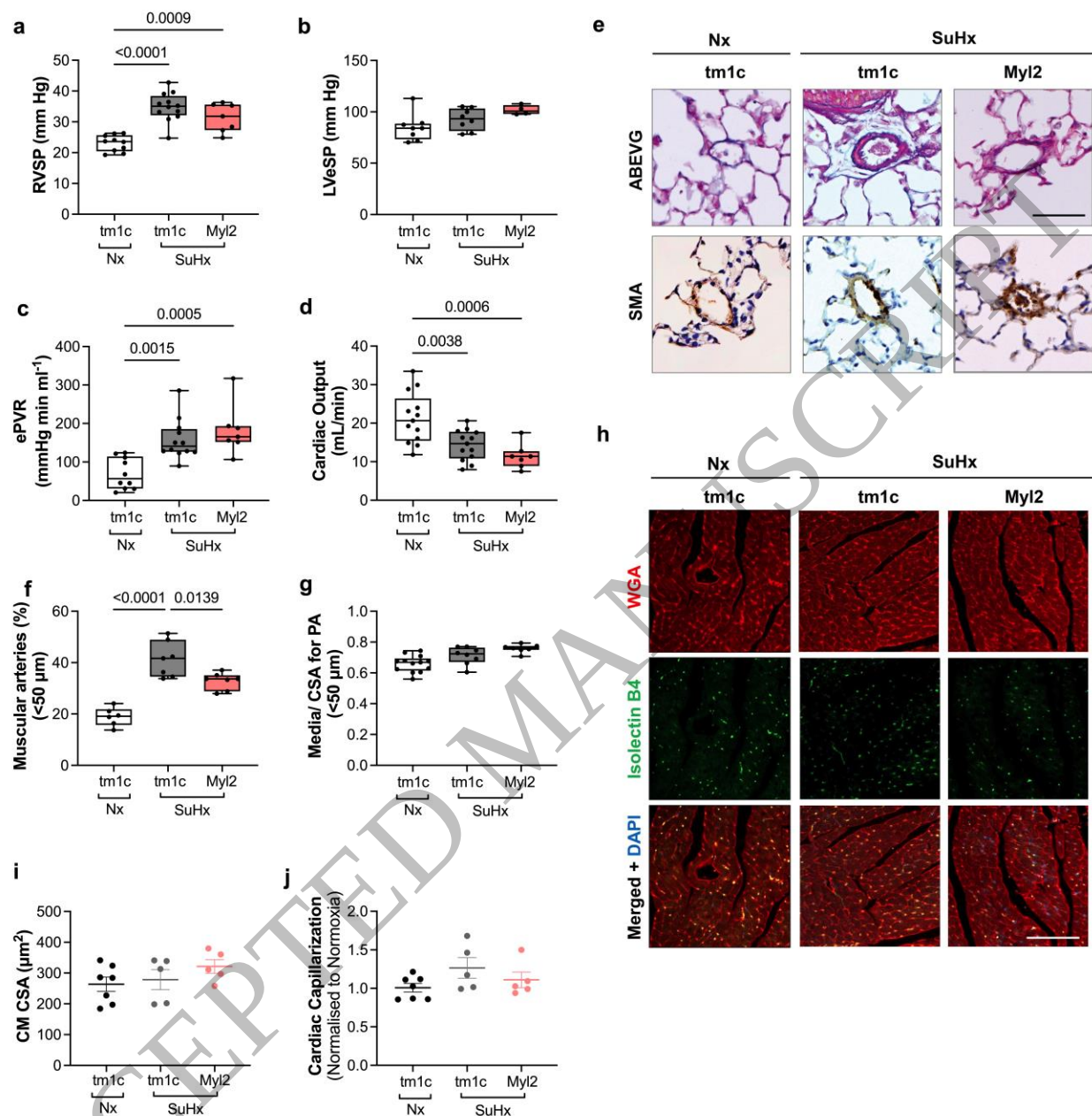


Figure 4: Deletion of OPG in myocardium did not prevent PAH progression or cardiac remodelling

Box plots showing **a.** right ventricular systolic pressure (RVSP, mm Hg), **b.** left ventricular end-systolic pressure (LVESP, mm Hg), **c.** pulmonary artery acceleration time (PA AT, ms) and **d.** cardiac output (CO, ml/min). Representative images **e.** of lung sections stained with Alcian Blue Elastic van Gieson (ABEVG) and immunostained for α -smooth muscle actin (α -SMA). Scale bar represents 50 μm . Box plots showing

1 the quantification of **f.** the relative percentage of muscularised pulmonary arteries less than 50 μm , and
2 **g.** the degree of medial wall thickness as a ratio of total vessel size (Media/CSA) in small pulmonary arteries
3 pulmonary arteries less than 50 μm . (Panels a-g: Nx: n= 8-10, SuHx: n= 8-12, Myl2-Cre: n=4-7) **h.**
4 Representative immunofluorescence images of cardiac tissue samples stained with WGA (wheat-germ
5 agglutinin coupled with Alexa Fluor 555) and IB4 (Isolectin B4 coupled with Alexa Fluor 488). Scale bar
6 represents 100 μm . Each dot represents an individual animal (n) showing the quantification of
7 **i.** cardiomyocyte cross-sectional area (CSA, μm^2) and **j.** myocardial capillary density as normalized to
8 normoxia condition. (Panel h-j: Nx: n= 7, SuHx: n= 5, Myl2-Cre: n=5) Data are shown as mean $\pm$ SEM. *P*-
9 values were calculated with one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons
10 and indicated on the plots.

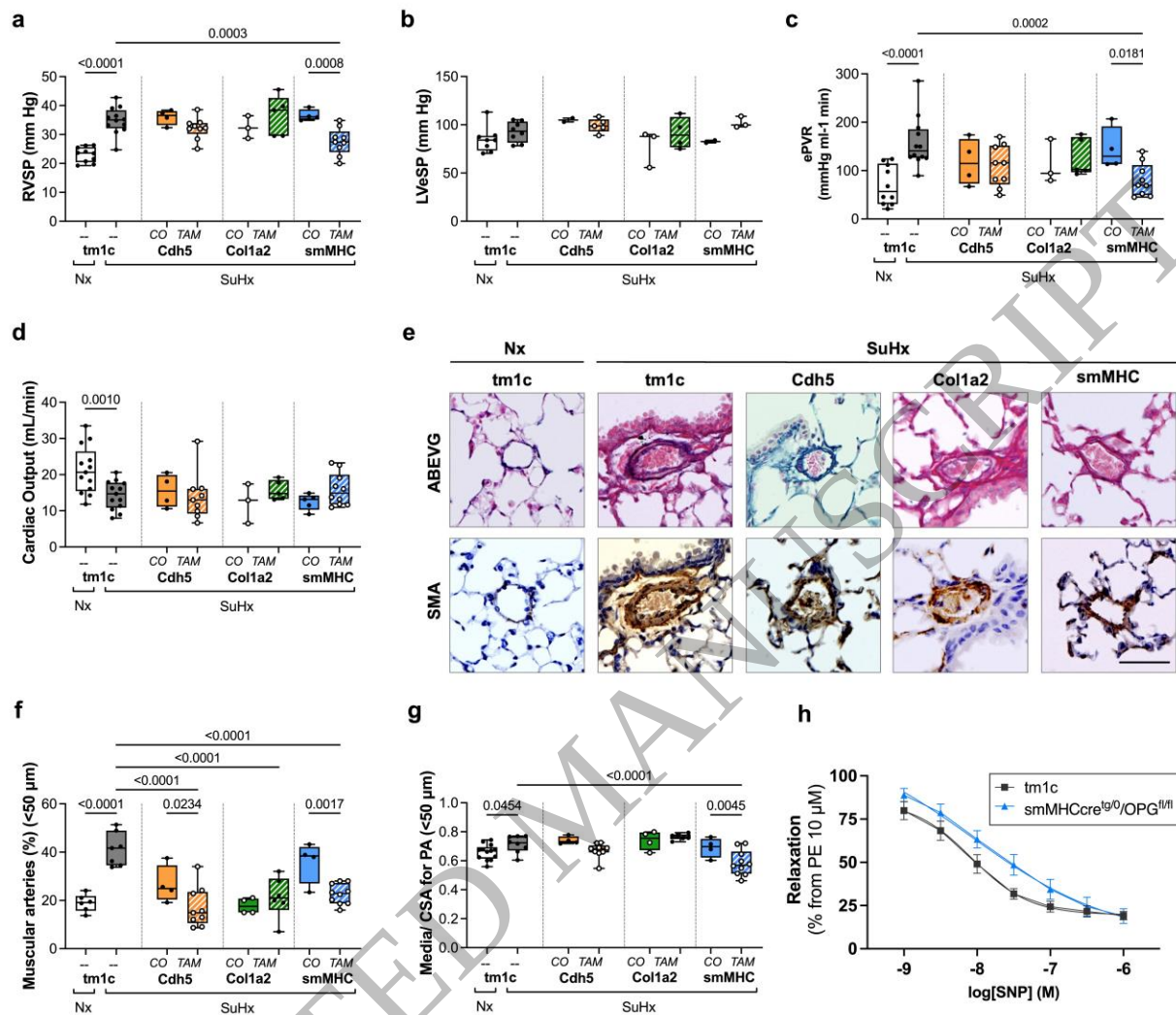


Figure 5: Smooth muscle cell-derived OPG predominantly modulates PAH pathogenesis

Box plots showing **a.** right ventricular systolic pressure (RVSP, mm Hg), **b.** left ventricular end-systolic pressure (LVESP, mm Hg), **c.** estimated pulmonary vascular resistance (ePVR, mmHg ml⁻¹ min⁻¹), **d.** cardiac output (CO, ml/min). Representative images **e.** of lung sections stained with Alcian Blue Elastic van Gieson (ABEVG) and immunostained for α-smooth muscle actin (α-SMA). Scale bar represents 50 μm. Box plots showing the quantification of **f.** the relative percentage of muscularised pulmonary arteries less than 50 μm, and **g.** the degree of medial wall thickness as a ratio of total vessel size (Media/CSA) in small pulmonary arteries pulmonary arteries less than 50 μm. Each dot represents an individual animal (n), data

are shown as mean $\pm$ SEM. *P*-values were calculated with one-way ANOVA followed by Fisher's LSD post-hoc test and indicated on the plots. **h.** Vascular reactivity assessed by wire myography. Relaxation-response curve showing contraction force (as % of PE-induced maximum) on the y-axis and log-transformed sodium nitroprusside (SNP) concentration (M) on the x-axis. Vessels were pre-contracted with phenylephrine (PE), and relaxation in response to SNP was measured to evaluate endothelium-independent vasodilation. (Nx: n= 6-13, SuHx: n=6-13, (Cdh5creERT2^{tg/+}:CO, n=4, TAM, n=9), (SmMHCcreERT2^{tg/0}: CO, n=4, TAM, n=10), (Col1a2creERT2^{tg/+}: (CO, n=4, TAM, n=5)). Data shown as mean $\pm$ SEM. *P*-values were calculated with one-way ANOVA followed by Fisher's LSD test.

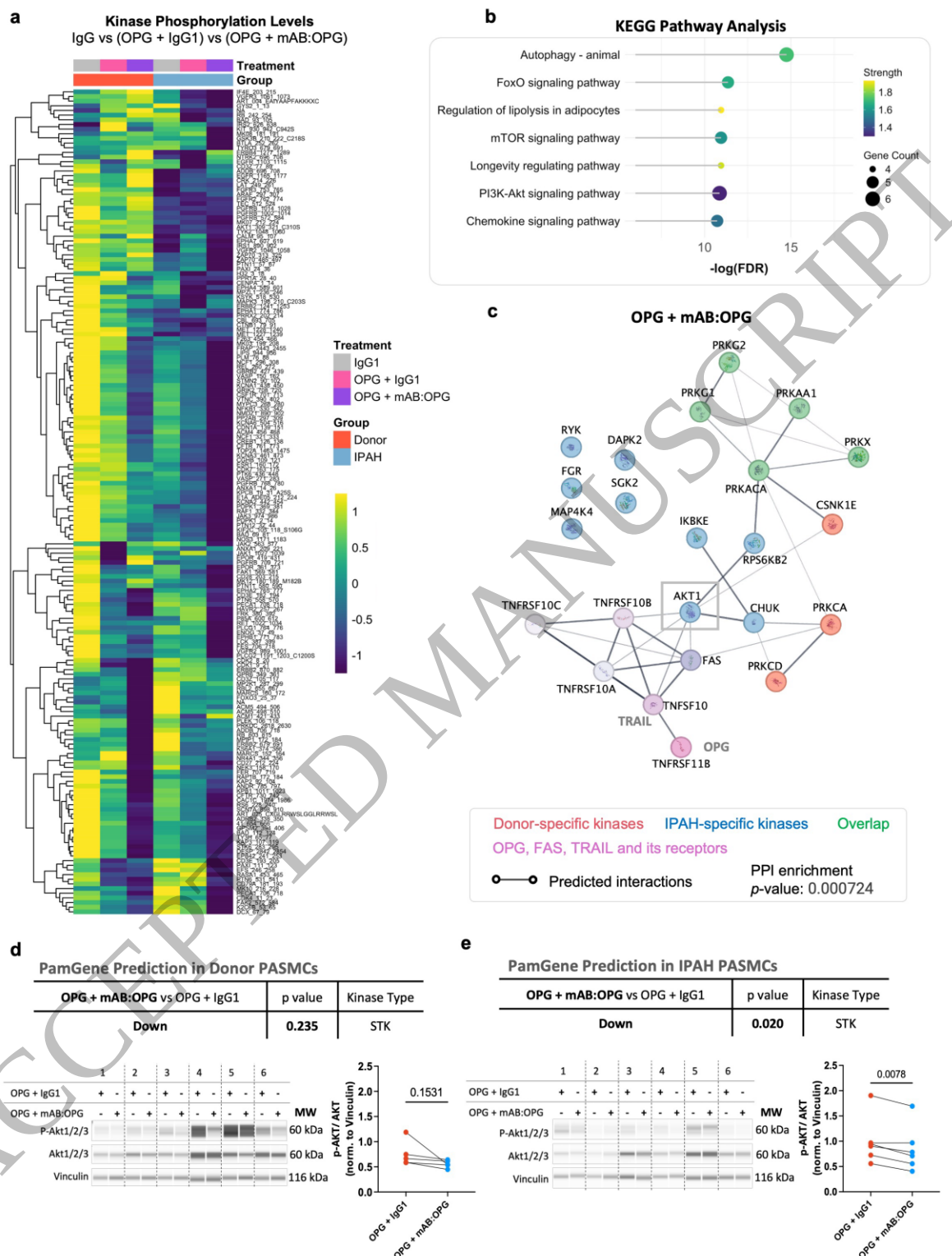


Figure 6: Kinome profiling reveals decreased AKT activity in IPAH HPASMCs following anti-OPG antibody treatment.

1 **a.** Heatmap representing serine/ threonine and tyrosine phosphorylation pattern of the substrates. Mean
2 log-transformed fluorescence signals are shown following OPG stimulation and anti+OPG antibody
3 treatment in PSMCs from disease controls individuals (Donor, n = 6) and from IPAH patients (n = 6). **b.**
4 Bubble plots showing KEGG pathway enrichment analysis of 17 kinases significantly differentially activated
5 after OPG stimulation followed with anti-OPG treatment (OPG + mAB:OPG). **c.** Protein-protein interaction
6 (PPI) map constructed using Cytoscape. Kinases with significantly altered activation specific to donors
7 (red), IPAH patients (blue) and shared between both (green) were integrated with FAS, OPG, TRAIL and
8 TRAIL-receptors (TRAILR1-3) (shades of purple). **d-e.** Validation of AKT1 phosphorylation levels using the
9 Simple Western system. Donor and IPAH samples were run on the same gel and processed in parallel;
10 panels 6d and 6e are displayed separately for visual clarity only. Predicted AKT1 activity after OPG
11 stimulation followed anti-OPG treatment (compared to IgG1 controls) were listed in the tables, with
12 quantification was shown as a ratio to total protein. Vinculin was used as a protein loading control. Results
13 are shown as mean $\pm$ SEM (n = 6) and were compared using a paired t-test.

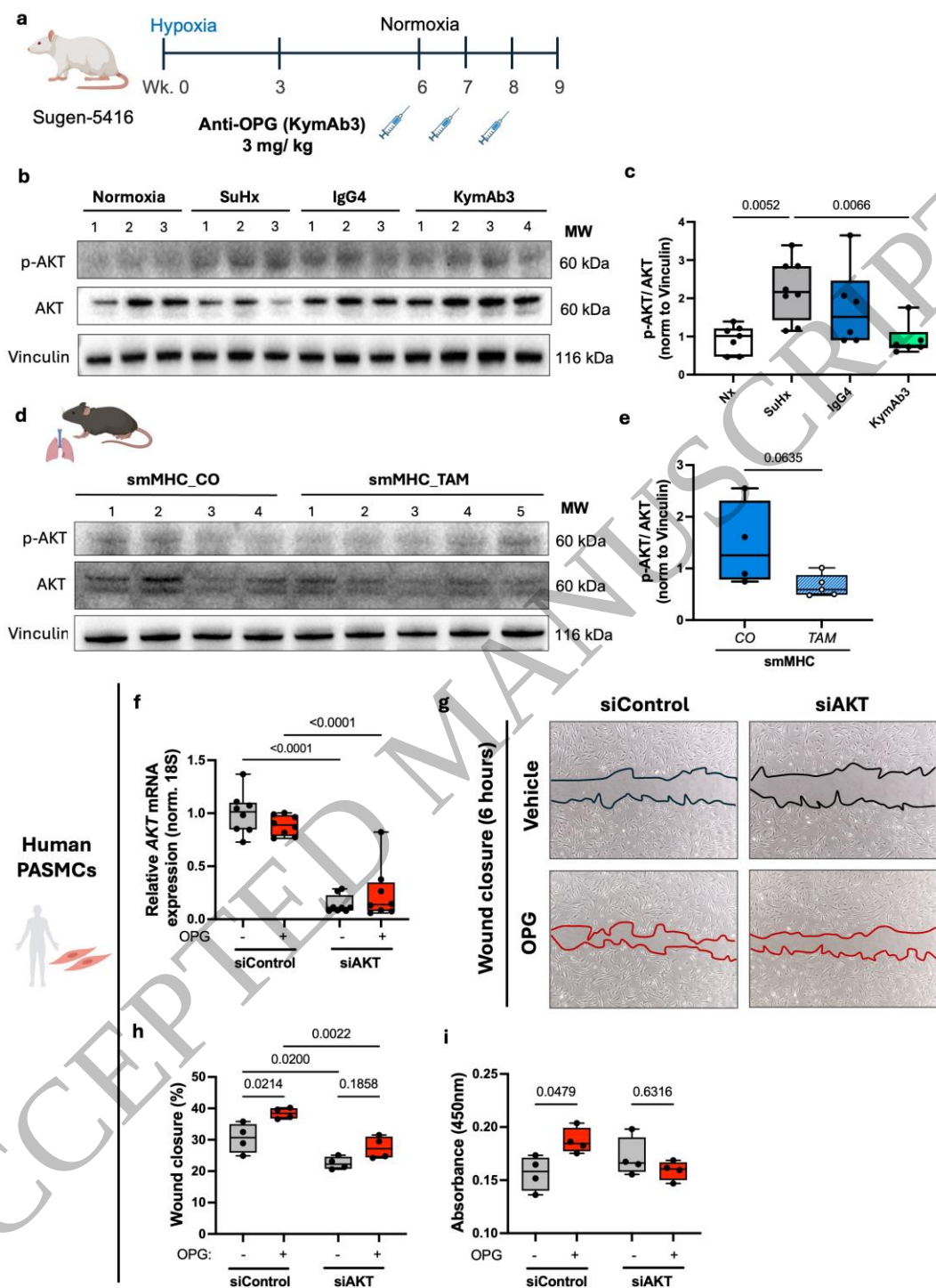
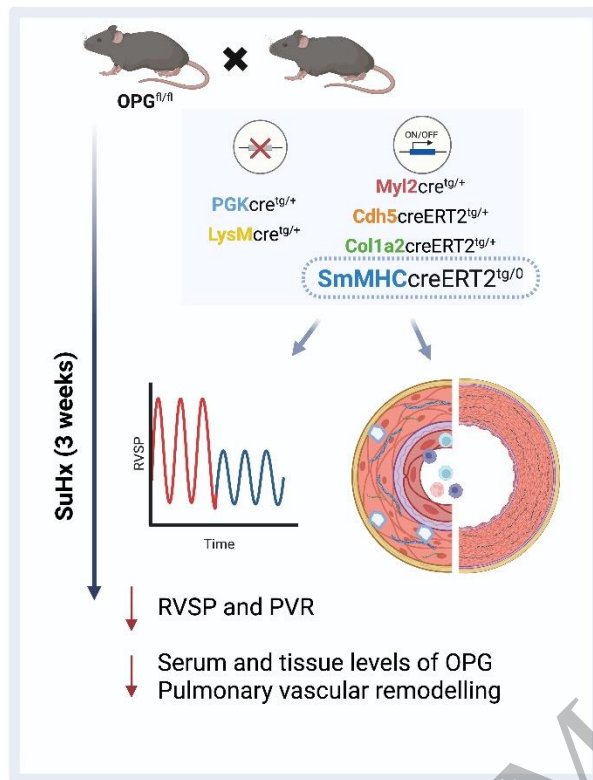


Figure 7: OPG modulates AKT signalling to regulate smooth muscle cell migration and proliferation.

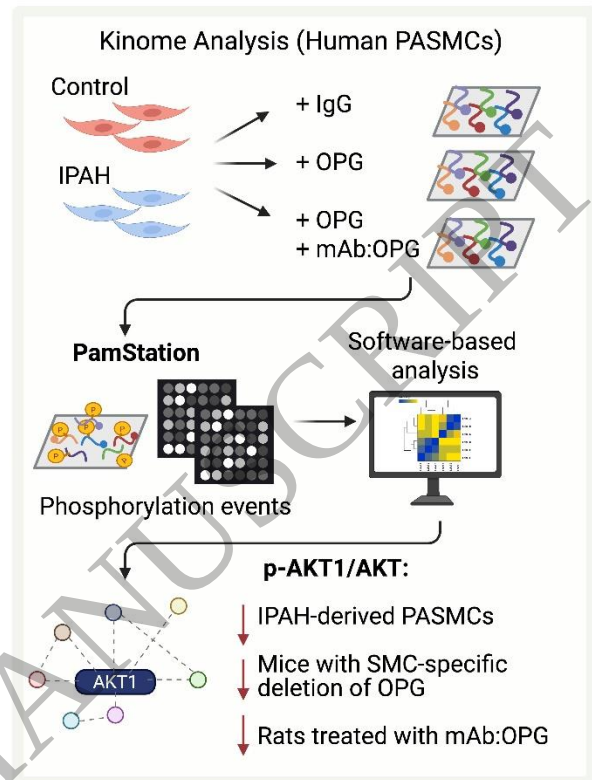
a. Schematic of the experimental timeline showing Sugen-5416 and hypoxia (SuHx) exposure in rat, with anti-OPG antibody (KymAb3) treatment administered (3 mg/kg). **b.** Representative Western blot images

1 of phosphorylated Akt (p-Akt), total Akt, and Vinculin in lung tissue from rats under normoxia (n=5), SuHx
2 (3), IgG4 control (n=5), and KymAb3 (n=6) treatment conditions. **c.** Quantification of p-Akt/Akt ratios
3 normalized to Vinculin across experimental groups. Each dot represents an individual animal (n), data are
4 presented as mean $\pm$ SEM; statistical significance is indicated. **d.** Representative Western blot images of
5 phosphorylated Akt (p-Akt), total Akt, and Vinculin in lung tissue from SmMHCcreERT2^{tg/0} mice (CO n=4,
6 Tamoxifen (Tam) n=5) after SuHx. **e.** Quantification of p-Akt/Akt ratios normalized to Vinculin across
7 experimental groups. Each dot represents an individual animal (n), data are presented as mean $\pm$ SEM. **f.**
8 Relative AKT mRNA expression in siControl- and siAKT-transfected human pulmonary artery smooth
9 muscle cells (PASMCs) treated with vehicle or OPG. **g.** Representative images of wound-healing assay at 6-
10 hours after OPG (or vehicle) stimulation following siControl and siAKT treatments. **h.** Quantification of
11 wound closure (%). **i.** Cell proliferation assessed using MTS assay and measured at absorbance (450 nm).
12 For cell culture experiments, each dot represents a biological replicate with three technical replicates. Data
13 shown as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's
14 post-hoc test.

Animal Model and Phenotyping



Molecular and Functional Analysis



Graphical Abstract