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ORIGINAL RESEARCH

# Platelet-Derived Growth Factor Receptor Type α Activation Drives Pulmonary Vascular Remodeling Via Progenitor Cell Proliferation and Induces Pulmonary Hypertension

Julien Solinc, PhD*; Jessica Raimbault-Machado, BS*; France Dierick, PhD; Lamiaa El Bernoussi, PharmD; Ly Tu, PhD; Raphaël Thuillet, MS; Nathalie Mougenot, PhD; Bénédicte Hoareau-Coudert, MS; Virginie Monceau, PhD; Catherine Pavoine, PhD; Fabrice Atassi, BS; David Sassoon, PhD; Giovanna Marazzi, MD, PhD; Richard P. Harvey, PhD; Peter Schofield, PhD; Daniel Christ, PhD; Marc Humbert, MD, PhD; Christophe Guignabert, PhD; Florent Soubrier, MD, PhD; Sophie Nadaud, PhD

**BACKGROUND:** Platelet-derived growth factor is a major regulator of the vascular remodeling associated with pulmonary arterial hypertension. We previously showed that protein widely 1 (PW1$^+$) vascular progenitor cells participate in early vessel neomuscularization during experimental pulmonary hypertension (PH) and we addressed the role of the platelet-derived growth factor receptor type α (PDGFRα) pathway in progenitor cell-dependent vascular remodeling and in PH development.

**METHODS AND RESULTS:** Remodeled pulmonary arteries from patients with idiopathic pulmonary arterial hypertension showed an increased number of perivascular and vascular PW1$^+$ cells expressing PDGFRα. PW1$^{nLacZ}$ reporter mice were used to follow the fate of pulmonary PW1$^+$ progenitor cells in a model of chronic hypoxia–induced PH development. Under chronic hypoxia, PDGFRα inhibition prevented the increase in PW1$^+$ progenitor cell proliferation and differentiation into vascular smooth muscle cells and reduced pulmonary vessel neomuscularization, but did not prevent an increased right ventricular systolic pressure or the development of right ventricular hypertrophy. Conversely, constitutive PDGFRα activation led to neomuscularization via PW1$^+$ progenitor cell differentiation into new smooth muscle cells and to PH development in male mice without fibrosis. In vitro, PW1$^+$ progenitor cell proliferation, but not differentiation, was dependent on PDGFRα activity.

**CONCLUSIONS:** These results demonstrate a major role of PDGFRα signaling in progenitor cell–dependent lung vessel neomuscularization and vascular remodeling contributing to PH development, including in idiopathic pulmonary arterial hypertension patients. Our findings suggest that PDGFRα blockers may offer a therapeutic add-on strategy to combine with current pulmonary arterial hypertension treatments to reduce vascular remodeling. Furthermore, our study highlights constitutive PDGFRα activation as a novel experimental PH model.

**Key Words:** fibrosis ■ pulmonary hypertension ■ platelet-derived growth factor receptor alpha ■ stem cells ■ vascular remodeling

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Pulmonary arterial hypertension (PAH), a severe rare disease, is histologically defined by pulmonary vascular remodeling characterized by endothelial cell proliferation, media thickening, neointima formation, and neomuscularization of small pulmonary vessels. This vascular remodeling leads to

Correspondence to: Sophie Nadaud, UMR_S 1166 ICAN, Faculté de Médecine Pitié-Salpêtrière, 91 Boulevard de l'Hôpital, 75013 Paris, France. E-mail sophie.nadaud@sorbonne-universite.fr

†J. Solinc and J. Raimbault-Machado are co-first authors.

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## CLINICAL PERSPECTIVE

### What Is New?

- Platelet-derived growth factor receptor type α (PDGFRα)$^+$ vascular progenitor cells accumulate in the perivascular area in lungs of patients with idiopathic pulmonary arterial hypertension.
- The PDGFRα pathway regulates progenitor cells proliferation and their participation in pulmonary vessels neomuscularization in a pulmonary hypertension experimental mouse model.
- Activating the PDGFRα pathway leads to pulmonary vessels neomuscularization but to moderate pulmonary hypertension only in male mice.

### What Are the Clinical Implications?

- PDGFRα activation of PDGFRα$^+$ progenitor cells may participate in pulmonary vascular remodeling during pulmonary arterial hypertension development.
- Blocking PDGFRα could help reduce pulmonary vessel muscularization in patients with idiopathic pulmonary arterial hypertension.

### Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **α-SMA** | alpha smooth muscle actin |
| **CH** | chronic hypoxia |
| **iPAH** | idiopathic pulmonary arterial hypertension |
| **PAH** | pulmonary arterial hypertension |
| **PDGFR** | platelet-derived growth factor receptor |
| **PH** | pulmonary hypertension |
| **PW1** | protein widely 1 |
| **RVSP** | right ventricular systolic pressure |
| **SMC** | smooth muscle cell |

increased pulmonary vascular resistance and to PAH, subsequently causing right ventricular hypertrophy and ultimately right heart failure and death. During the development of PAH, pulmonary arterioles, which are predominantly nonmuscularized, become covered by smooth muscle cells (SMCs). This neomuscularization, observed in patients with PAH and rodent models of PAH, is driven by both SMC proliferation and the recruitment of pericytes and other SMC progenitor cells.[1–4] We have recently identified new perivascular pulmonary progenitor cells characterized by the expression of stem cell markers PW1 (protein widely 1), platelet-derived growth factor receptor type α (PDGFRα), and CD34 in rodent and human lungs.[3] During the development of early chronic hypoxia (CH) in an experimental pulmonary hypertension (PH) model, these PW1$^+$ progenitor cells proliferate and differentiate into new SMCs, contributing to small pulmonary vessel neomuscularization. We demonstrated that their differentiation into SMCs was under the control of the C-X-C chemokine receptor type 4/C-X-C motif chemokine 12 pathway, but the specific factors regulating their proliferation remained unknown.

Studies from the past 25 years have highlighted the platelet-derived growth factor (PDGF) pathway as a major regulator of pulmonary vascular remodeling and PAH development.[5–7] PDGF receptors (PDGFRs) are transmembrane tyrosine kinase receptors with 2 isoforms, PDGFRα and PDGFRβ, which assemble into homo- or heterodimers. Five PDGF ligand dimers (PDGF-AA, -BB, -AB, -CC, -DD) differentially activate these receptors and play an important paracrine role during embryonic and vascular development (for review see Andrae et al[8]). All PDGFR forms can activate phosphoinositide 3-kinase, phospholipase Cγ, and Ras–mitogen-activated protein kinase signaling pathways but trigger distinct cellular responses. Expression of PDGF-A and -B ligands and PDGFRα and β was found to be significantly increased in the lungs of patients with PAH compared with control lungs.[5] Imatinib (STI 571), which targets both the PDGFRα and β subtypes as well as Bcr/Abl and c-kit, protects against PH development in rodent experimental models.[9] These protective effects were attributed to the PDGFRβ pathway as constitutively activating or inhibiting PDGFRβ signaling, respectively, increased or reduced CH-induced PH development in mice.[10,11] In contrast, it remains unknown whether PDGFRα also plays a role during PAH development. This receptor is a marker for mesenchymal stem cells in various organs (heart, skeletal muscle, adipose tissue, and bone marrow) and of pulmonary vascular progenitor cells.[3,12] The PDGF-A/PDGFRα pathway controls stem cell proliferation and differentiation[13–16] and is involved in myofibroblast formation during lung development in particular.[17]

Here, we provide evidence that PW1$^+$/PDGFRα$^+$ progenitor cells are actively recruited in human idiopathic pulmonary arterial hypertension (iPAH) remodeled arteries. We establish that PDGFRα activation regulates pulmonary vascular remodeling during experimental chronic hypoxia-induced PH. We demonstrate in vitro and in vivo that this receptor controls the proliferation of PW1$^+$/PDGFRα$^+$ pulmonary vascular progenitor cells that form new SMCs during CH. Furthermore, we highlight that PDGFRα activation leads to PH development in both a constitutive PDGFRα activation mouse model or after chronic PDGF-AA treatment.

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## METHODS

The data, protocols, and materials that support the findings of this study are available from the corresponding author upon reasonable request. Detailed methods are provided in Data S1 and Tables S1 and S2.

Studies with patients complied with the Declaration of Helsinki and are part of the French Network on Pulmonary Hypertension, a program approved by our institutional Ethics Committee (2018-A01252-53). Written informed consent was received from participants before inclusion in the study. Animal experiments for the project were approved by our institutional review board (APAFIS#3566bis-2016022617318129 v8).

$Pw1^{IRESnLacZ}$ transgenic mice ($Pw1^{nlacZ/+}$) bear a nuclear operon lactose gene expressed under the control of the Pw1 gene locus.[18] The stability (perdurance) of the β-galactosidase reporter facilitates short-term lineage tracing of cells derived from PW1$^+$/β-galactosidase$^+$ cells. These mice were crossed with $RosaCre^{ERT2}$x$PDGFR\alpha^{+/(S)K}$ mice, which, when administered tamoxifen, express a constitutively activated form of PDGFRα (D842V) in PDGFRα-expressing cells.[19] Mice (7- to 10-week-old male and female littermates) were exposed to normobaric room air (normoxia) or chronic normobaric hypoxia (10% $O_2$) for 4 or 21 days. PDGFRα activity was inhibited in vivo using either imatinib or the specific blocking antibody, APA5. Imatinib was injected daily (50 mg/kg of body weight intraperitoneally), and the blocking antibody or a control IgG was injected intraperitoneally once (500 µg/mouse) 3 days before starting CH, then every 3 to 4 days throughout the CH periods (100 µg/mouse). PDGF-AA (or vehicle) was injected daily (150 ng/mouse, intraperitoneally) for 3 weeks in $Pw1^{nlacZ/+}$mice. Following the different treatments, mice were anesthetized to measure hemodynamic parameters, right ventricular hypertrophy, and to collect lung samples for tissue analysis. Fluorescence-activated cell sorting was used to isolate PDGFRα$^+$/PW1$^+$ progenitor cell populations from $Pw1^{nlacZ/+}$mice based on their β-galactosidase activity and a combination of progenitor cell markers, to study them in culture. Fluorescence-activated cell sorting–isolated CD45$^-$/CD34$^+$/β-galactosidase$^+$/c-kit$^-$ cells were cultured for 5 days in the presence of PDGF ligands (20 ng/mL), control IgG (150 ng/mL), or APA5 blocking antibody (150 ng/mL) before immunofluorescence analysis.

## RESULTS

### Perivascular PW1$^+$/PDGFRα$^+$ Cells Are Increased in the Lungs of Patients With iPAH

Activation of the PDGFRα pathway in human iPAH has been suggested by the increased expression of PDGF-A in patient lungs.[5] We studied the presence and regulation of PW1$^+$ and PW1$^+$/PDGFRα$^+$ progenitor cells adjacent to or within pulmonary arteries of control patients and patients with iPAH. Patient characteristics are indicated in Table S3. Immunofluorescence studies (Figure 1A) confirmed the presence of PW1$^+$ cells organized in clusters in the perivascular zone and their absence in the media of control patient lungs, as seen previously.[3] Importantly, these PW1$^+$ cells were positive for PDGFRα expression. In patients with iPAH, perivascular PW1$^+$/PDGFRα$^+$/alpha smooth muscle actin (α-SMA$^-$) cells were found to be significantly more numerous around remodeled arteries ($P$=0.016; Figure 1A and 1B), as observed in murine experimental models.[3] The number of PW1$^+$/PDGFRα$^+$ cells within the media and neointima of small or large arteries was equivalent in control and patients with iPAH, whereas PDGFRα$^+$/PW1$^+$/α-SMA$^+$ cells were significantly increased ($P$=0.008; Figure 1C). Complex lesions did not contain PW1$^+$ cells (as illustrated in Figure S1) but showed numerous PDGFRα$^+$ cells. We then used the mouse chronic hypoxia PH model to study the regulation of these PW1+/PDGFRα$^+$ progenitor cells during pulmonary vascular remodeling and to decipher their role.

### Chronic Hypoxia Induces the PDGFRα Pathway and Increases the Number of Perivascular PDGFRα$^+$/PW1$^+$ Progenitor Cells in Mouse Lungs

We reported previously that early neomuscularization of pulmonary arteries observed in the chronic hypoxic PH experimental model relies on the recruitment of pulmonary resident CD34$^+$/PDGFRα$^+$/PW1$^+$ progenitor cells.[3] To study the role of the PDGFRα pathway in the activation of these progenitors and associated vascular remodeling, we first analyzed the expression of the 4 PDGF ligands and the 2 PDGFR isoforms in total mouse lung samples extracted during the first 4 days of CH with reverse transcriptase polymerase chain reaction (Figure S2A). PDGF-A, -B, and -D and PDGFRα and β demonstrated increased expression (×1.3 to ×2, with $P$=0.000, $P$=0.002, $P$=0.001, $P$=0.012, and $P$=0.002, respectively) after 1 day of CHs. It is noteworthy that PDGF-A, a specific PDGFRα ligand, remained upregulated after 4 days of CH compared with normoxia. PDGFRα protein expression also showed a tendency to increase after 1 day of CH (Figure S2B). Concomitantly with the increased expression of PDGFRα ligands, we observed an early and significant increase in the number of pulmonary vessels associated with perivascular PDGFRα$^+$/PW1$^+$ progenitor cells ($P$=0.024; Figure S3A and B). On the contrary, there was no difference in the number of medial PDGFRα$^+$/α-SMA$^+$ SMC during CH (Figure S4A and S4B).

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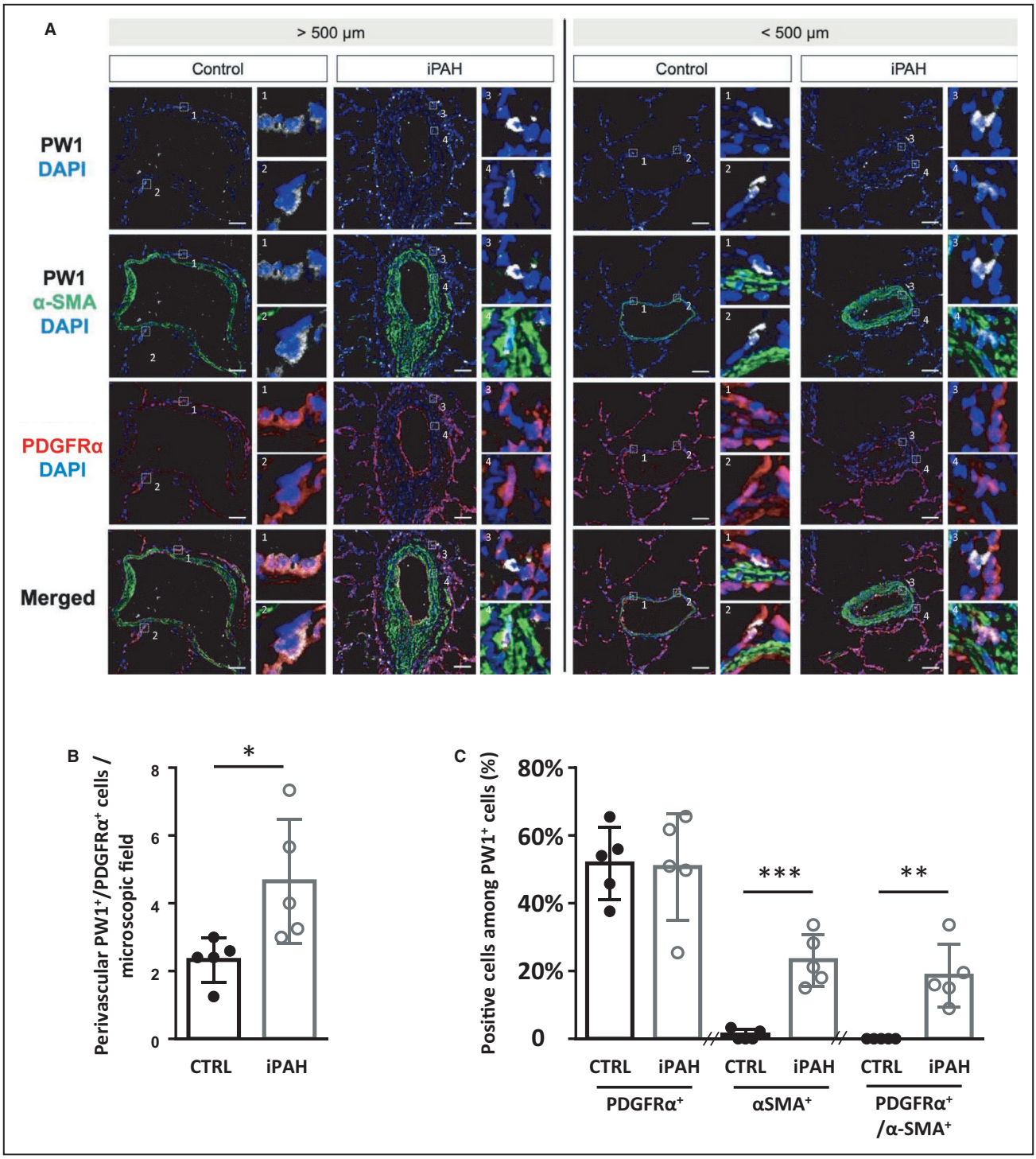



**Figure 1. Perivascular PW1⁺/PDGFRα⁺ cells are increased in the perivascular zone and within the vascular wall of remodeled arteries in patients with iPAH.**

Lung sections from control (CTRL) or patients with idiopathic pulmonary arterial hypertension (iPAH) were labeled for PW1 (white), PDGFRα (red), and α-SMA (green). **A**, Representative confocal images of pulmonary vessels (>500 µm diameter, left panels; <500 µm diameter, right panels) from control patients (n=5) and patients with iPAH (n=5). Panels 1, 2, and 3 show details of perivascular PW1⁺/PDFGFRα⁺ cells. Panel 4 shows details of PW1⁺/PDFGFRα⁺/α-SMA⁺ cells found within arteries in patients with iPAH. **B**, quantification of perivascular PW1⁺/PDGFRα⁺ cells. **C**, quantification of PDGFRα⁺ or α-SMA⁺ or PDGFRα⁺/α-SMA⁺ cells among perivascular and vascular PW1⁺. Scale bar, 50 µm. Bars represent means and whiskers represent SD. **P*<0.05, ***P*<0.01, ****P*<0.001; ns indicates not significant (2-tailed Mann-Whitney); PDGFRα indicates platelet-derived growth factor receptor type α; PW1, protein widely 1; and SMA, smooth muscle actin.

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## PDGFRα Inhibition During Chronic Hypoxia Inhibits PW1+ Progenitor Cells Proliferation and Differentiation as Well as Pulmonary Vascular Remodeling In Vivo

To explore whether PDGFRα influences PW1+ progenitor cell participation in vascular remodeling during CH, we inhibited PDGFRα using 2 approaches: (1) intraperitoneal injection of imatinib, a pharmacological inhibitor that also targets PDGFRβ, c-kit, and c-abl, and has been used in PH models[9,11]; and (2) intraperitoneal injection of the PDFGRα-specific blocking antibody APA5.[20]

Experiments were performed in the PW1$^{nLacz}$ transgenic mouse model, in which the innate stability of the β-galactosidase reporter enables short-term lineage tracing of PW1+-derived cells. As such, PW1+ progenitor cell proliferation and their differentiation into SMCs can be assessed. Mice were maintained under normoxia or CH for 4 days and treated daily with DMSO or imatinib. Pulmonary vessel neomuscularization (the total percentage of partially and fully muscularized vessels), as observed by α-SMA and vWF immunofluorescence colabeling (Figure 2A), was increased by CH (83%±4.7% of muscularized arterioles versus 36%±6.7% in normoxia, $P$<0.0001; Figure 2B). As previously shown,[3] this neomuscularization was at least partially attributable to an increased number of β-galactosidase+ vascular SMC derived from PW1+ progenitor cells (72%±4% per vessel versus 42%±7% in normoxia, $P$<0.0001; Figure 2C and 2D) associated with a greater number of pulmonary PW1+ progenitor cells (19%±2% of parenchymal cells versus 8%±1% in normoxia, $P$<0.0001; Figure 2E and 2F). Imatinib treatment had no effect on these parameters under normoxia but significantly reduced CH-induced neomuscularization by 40% (from 83%±4.7% in CH+DMSO to 65%±4.5% in CH+IMA, $P$<0.0001; Figure 2B). Imatinib completely abolished PW1+ progenitor cell participation in neomuscularization under CH, reducing the number of PW1+ progenitor-derived SMCs to basal levels (from 72%±4% per vessel in CH+DMSO to 47%±8% in CH+IMA; $P$<0.0001 versus DMSO; Figure 2D). PW1+ cell numbers also returned to baseline with imatinib treatment (from 19%±2% of parenchymal cells in CH+DMSO to 10%±1% in CH+IMA, $P$<0.0001; Figure 2F). Imatinib treatment did not modify PDGFRα mRNA or protein expression (Figure S5). As we demonstrated previously,[3] PW1+ progenitor cells involved in early CH-induced neomuscularization are PDGFRα+, suggesting that PDGFRα activation could be involved in increased levels of PW1+ cells and their differentiation into SMCs.

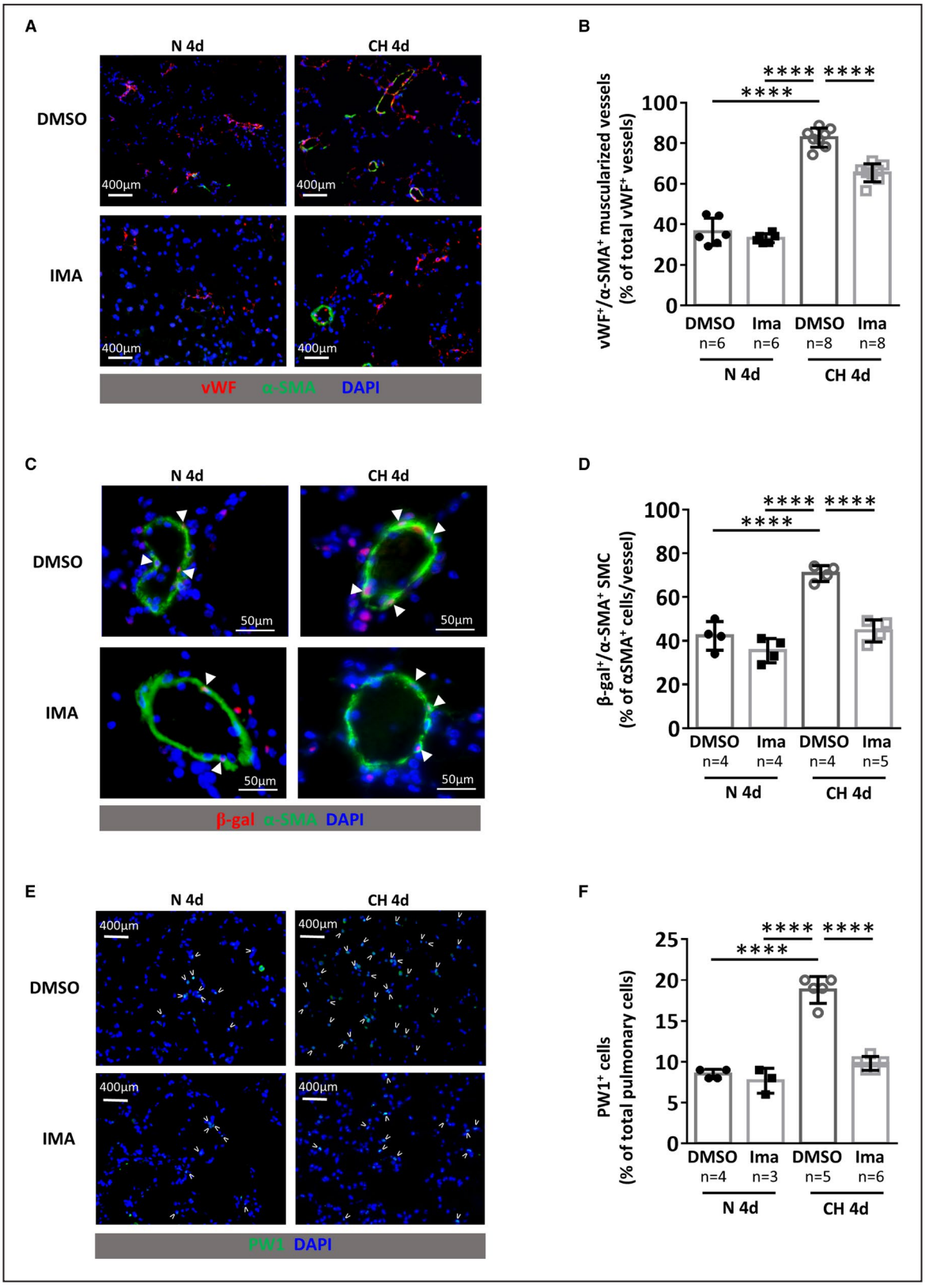
To understand the role of PDGFRα in the proliferation and differentiation of PW1+ cells during CH, PW1$^{nLacz}$ transgenic mice were maintained under CH for 4 days and treated with control IgG or the PDGFRα-blocking antibody APA5. Specific inhibition of PDGFRα during CH reproduced the results obtained with imatinib: Early CH-induced pulmonary arterial neomuscularization was significantly reduced from 86%±2.3% to 63%±4% ($P$=0.0006; Figure 3A). In parallel, the CH-induced increase in PW1+ progenitor-derived β-galactosidase+ vascular SMC was significantly inhibited (from 80.1%±4.2% to 50.3%±2.4%, $P$=0.0006; Figure 3B). We previously established that the increase in PW1+ progenitor cells observed during early CH was explained by induced proliferation.[3] The proportion of proliferating ethynyldeoxyuridine+ PW1+ cells was reduced by 66% from 46.0%±7.0% to 15.6%±9.1% ($P$=0.016; Figure 3C and 3D), whereas pulmonary cell apoptosis, as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling, was very low and did not differ between control IgG- and APA5-treated mice (Figure 3E).

Together, these data show that PDGFRα activation is essential for CH-induced proliferation and differentiation of PW1+ progenitor cells into vascular SMCs, and that PDGFRα participates in the induction of early pulmonary arterial neomuscularization during CH.

## PDGFRα Activation Elicits Neomuscularization of Pulmonary Arterioles Through PW1+ Progenitor Cell Differentiation Into New Smooth Muscle Cells Under Normoxic Conditions In Vivo

We developed a novel PW1$^{nLacz/+}$/PDGFRα$^{+/(S)K}$/Rosa-CRE$^{ERT2}$ mouse model to directly examine the role of PDGFRα in the regulation of pulmonary progenitor cells and vascular remodeling. We crossed PDGFRα$^{+/(S)K}$ mice with (1) Rosa-CRE$^{ERT2}$ mice for tamoxifen induction and generation of an inducible model of a mutated constitutively-activated form of PDGFRα[19] and (2) with PW1$^{nLacz}$ mice to follow PW1+ progenitor cell fates (Figure 4A). Two weeks after the final tamoxifen injection, normoxic male and female PW1$^{nLacz/+}$/PDGFRα$^{+/(S)K}$/Rosa-CRE$^{ERT2}$ mice demonstrated significant neomuscularization with a 50% augmentation in the number of muscularized vessels (67%±13.9% of muscularized arteries in induced mice versus 35.5%±10.3% in noninduced littermates, $P$=0.004; Figure 4B). This was associated with increased numbers of PW1+ progenitor-derived vascular SMC (65.8%±11.3% per vessel versus 48.8%±9.8% in noninduced littermates, $P$=0.026; Figure 4C). Both the vascular remodeling and increased number of PW1+ cells was similar to observations after 4 days of chronic hypoxia[3] and was not associated with PH development at this early time point (Figure S6).

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**Figure 2. Imatinib treatment prevents early chronic hypoxia (CH)-induced neomuscularization and PW1$^+$ progenitor cell recruitment and differentiation in SMCs.**
PW1$^{nLacz}$ mice were maintained under normoxia (N 4d) or chronic hypoxia (CH 4d) for 4 days and treated daily with DMSO or imatinib (IMA). **A**, Representative images of von Willebrand factor (vWF, red) and α-smooth muscle actin (α-SMA, green) staining in lungs. **B**, Quantification of muscularized vessels (fully+partially) (n=6–8 mice/group). **C** Representative images of pulmonary muscularized vessel stained with α-SMA (green) and β-galactosidase (red) in lungs (double positive are marked by arrowheads). **D**, Quantification of lung β-galactosidase (β-gal)-expressing SMC in fully-muscularized vessels (n=4–5 mice/group). **E**, Representative images of lung parenchyma stained for PW1 (PW1 in green, positive cells are marked by arrowheads). **F**, Quantification of PW1$^+$ cells in lung parenchyma (n=3–6 mice/group). Bars represent means and whiskers represent SD. ****$P$<0.0001, 2-way ANOVA and Tukey. PW1 indicates protein widely 1; and SMCs, smooth muscle cells.

Altogether, these results demonstrate that PDGFRα activation leads to pulmonary vessel neomuscularization through PW1$^+$ progenitor cell proliferation.

## PDGFRα Controls PW1$^+$ Progenitor Cell Proliferation In Vitro

To confirm the direct role of the PDGFRα pathway on PW1$^+$ progenitor cell proliferation or differentiation, we studied fluorescence-activated cell sorting–purified pulmonary CD34$^+$/PDGFRα$^+$/PW1$^+$ progenitor cells, which are amplified after chronic hypoxia.[3] These cells can be separated into 2 subpopulations according to PDGF-dependent c-kit expression.[3] As c-kit$^+$ cells could not be cultured at low serum concentrations, we were unable to determine whether PDFG-AA activation affected this cell population. We therefore focused on the role of the PDGFRα pathway in the c-kit$^-$ cell population, which undergoes spontaneous differentiation into α-SMA-expressing SMCs under normal culture conditions.[3] CD34$^+$/PDGFRα$^+$/β-galactosidase$^+$/c-kit$^-$ cells were sorted and cultured using the strategy previously published[3] (Figure S7). Incubation with PDGFRα-blocking antibody APA5 significantly inhibited their proliferation by 50% as compared with control IgG ($P$=0.016, Figure 5A) and reduced their differentiation by 40% ($P$=0.016, Figure 5B) without inducing apoptosis as measured by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay (Figure 5C). While treatment of these progenitor cells with PDGF-AA (a specific PDGFRα ligand) did not induce their differentiation into α-SMA$^+$ SMC (Figure 5D), it significantly increased their proliferation by 2-fold ($P$=0.0625, Figure 5E). This effect was also observed with PDGF-AB and PDGF-BB, which bind both PDGFRα and β. These data demonstrate that PDGFRα activation directly controls the proliferation of PW1$^+$/PDGFRα$^+$/ckit$^-$ progenitor cells.

## PDGFRα Inhibition Limits Vascular Remodeling After Long-Term Chronic Hypoxia but Does Not Reduce Right Ventricular Systolic Pressure, Whereas PDGFRα Activation Induces PH

Our in vitro and in vivo data show that the PDGFRα pathway controls pulmonary neomuscularization by regulating PW1$^+$/PDGFRα$^+$ progenitor cell proliferation. We therefore investigated the long-term effects of PDGFRα inhibition or activation on PH development.

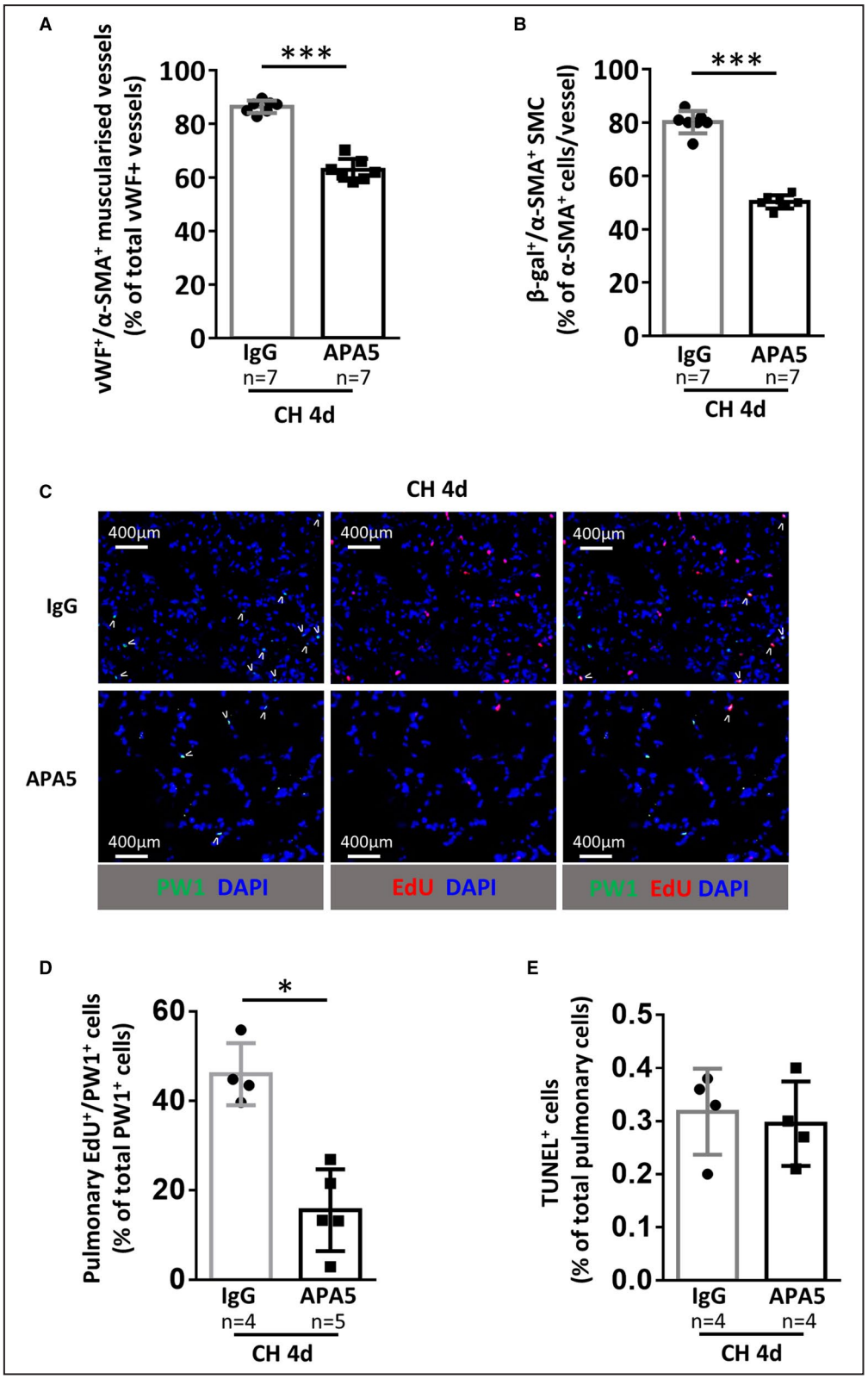
PDGFRα was inhibited during CH by treating mice (7 males and 1 female per group) with APA5 blocking antibody. Chronic hypoxia was confirmed by increased blood hemoglobin concentration (Figure S8). After 21 days of CH, PDGFRα inhibition significantly reduced the number of fully muscularized vessels (27.2%±9.7% with APA5 treatment versus 39.8%±6.2% with IgG treatment; $P$=0.039) and increased the number of partially muscularized vessels (53.8%±8% with APA5 treatment versus 39.7%±7.1% with IgG treatment; $P$=0.008) as compared with IgG-treated control mice (Figure 6A). However, APA5 treatment did not significantly modify right ventricular systolic pressure (RVSP) or the Fulton index (Figure 6B and 6C) as compared with IgG-treated control mice, and both values were increased as compared with normoxic mice. This result demonstrates that PDGFRα inhibition for 21 days reduces CH-induced pulmonary vessel neomuscularization but does not prevent either increased RVSP or right ventricular hypertrophy.

The effect of PDGFRα activation was first studied in tamoxifen-induced PW1$^{nLacz/+}$/PDGFRα$^{+/(S)K}$/Rosa-CRE$^{ERT2}$ mice (Figure 7A). After 5 weeks of constitutive PDGFRα activation, male mice displayed both a significant RVSP increase (22.5±5.2 mm Hg, Figure 7B) and right ventricular hypertrophy (Fulton index of 0.31±0.02; Figure 7C) as compared with noninduced littermates (17.1±3.5 mm Hg, $P$=0.0185, Figure 7B; and 0.24±0.03, $P$=0.0043, Figure 7C, respectively). Interestingly, female mice did not develop PH by this time point, with no demonstrable increase in RVSP and no right ventricular hypertrophy (Figure 7D and 7E), although they did present with increased neomuscularization 15 days after tamoxifen induction, similar to males (data not shown). Total weight, heart rate, and left ventricle weight were not modified in tamoxifen-induced mice (Figure S9). These results show that PDGFRα activation first triggers vascular remodeling and eventually PH. To further confirm that PDGFRα activation induces PH, we treated male and female mice with PDGF-AA (150 ng/mouse daily) for 5 weeks (Figure 7F). In male mice, this treatment increased RVSP (19.7±2.6 mm Hg versus 14.7±1.8 mm Hg in the

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control group, *P*=0.03; Figure 7G) but not to the same extent as observed after a similar duration of constitutive PDGFRα activation (22.5±5.2 mm Hg; Figure 7B). Five weeks of PDGF-AA treatment also did not lead to right ventricular hypertrophy (Figure 7H). In female mice, the RVSP or Fulton index were not significantly modified by PDGF-AA treatment in contrast to the constitutive PDGFRα activation model (Figure 7I and 7J).

Altogether, these results establish that constitutive PDGFRα activation is a novel mouse model of mild




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**Figure 3. PDGFRα inhibition with the specific blocking antibody APA5 prevents early chronic hypoxia (CH)-induced neomuscularization and PW1⁺ progenitor cell proliferation and differentiation in SMCs.**
PW1$^{nLacz}$ mice were maintained under CH for 4 days and treated with control antibody (IgG) or PDGFRα blocking antibody (APA5). **A**, Quantification of pulmonary muscularized vessels (fully+partially). For each animal (n=7 mice/group), at least 100 vWF⁺ vessels (<100 μm) were analyzed for muscularization (α-SMA⁺). **B**, Quantification of lung β-galactosidase (β-gal)-expressing PW1-derived SMCs in fully-muscularized vessels (<100 μm) (n=7 mice/group). **C**, Representative images of lung parenchyma from CH mice treated with control IgG or APA5 and stained for PW1 (green) and EdU (red), double-positive cells are marked by arrowheads. **D**, Quantification of EdU⁺/PW1⁺ cells in lung parenchyma (n=4–5 mice/group). **E**, Quantification of pulmonary apoptotic cells. The percentage of TUNEL⁺ cells in lung parenchyma was determined by immunofluorescence (n=4 mice/group). Bars represent means and whiskers represent SD. **P*<0.05, ***P*<0.01, ****P*<0.001. 2-tailed Mann-Whitney. Scale bar 400 μm. EdU indicates ethynyl-deoxyuridine; PDGFRα, platelet-derived growth factor receptor type α; PW1, protein widely 1; SMA, smooth muscle actin; SMC, smooth muscle cell; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; and vWF, von Willebrand factor.

pulmonary hypertension in males, recapitulating the pulmonary vascular alterations observed during chronic hypoxia.

## Lung Fibrosis Is Not Induced by PDGFRα Activation nor Reduced by PDGFRα Inhibition During Chronic Hypoxia

Long-term constitutive PDGFRα activation was reported to induce fibrosis in multiple organs including the lung.[19] We therefore sought to determine whether lung fibrosis was induced by PDGFRα activation in our model or if it was inhibited by blocking PDGFRα during CH. We measured the mRNA expression of fibrosis factors (collagens 1 and 3 and transforming growth factor-β) and assessed changes in collagen levels by quantifying picrosirius red histological staining in lung samples from male PW1$^{nLacz/+}$/PDGFRα$^{+/(S)K}$/Rosa-CRE$^{ERT2}$ mice. After 5 weeks of constitutive PDGFRα activation, collagen 1a1 and 3a1 mRNA levels remained unchanged, but transforming growth factor-β mRNA expression increased in lungs (Figure 8A). Histological

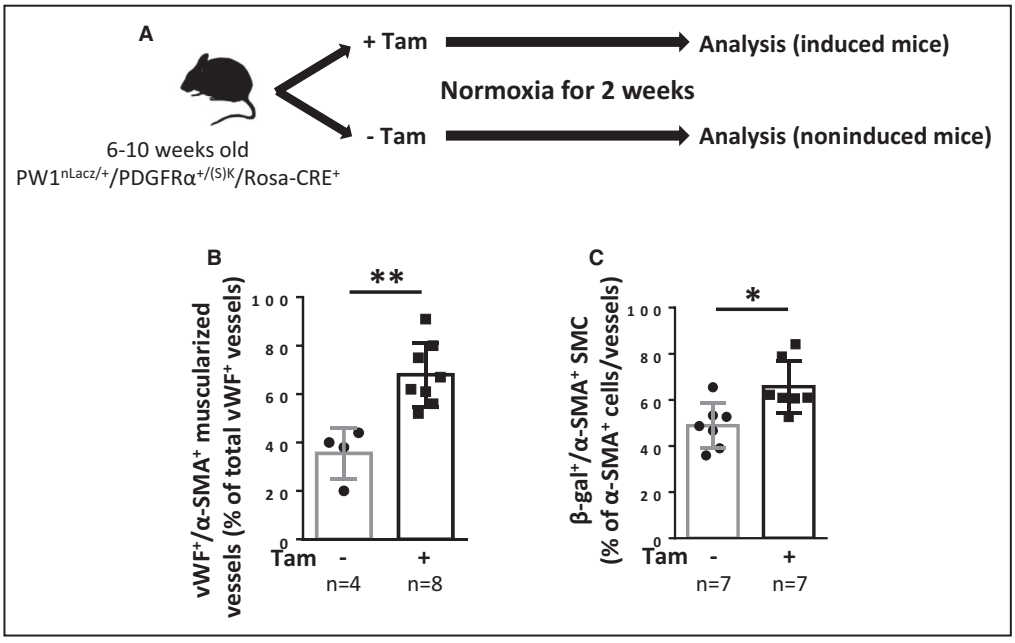
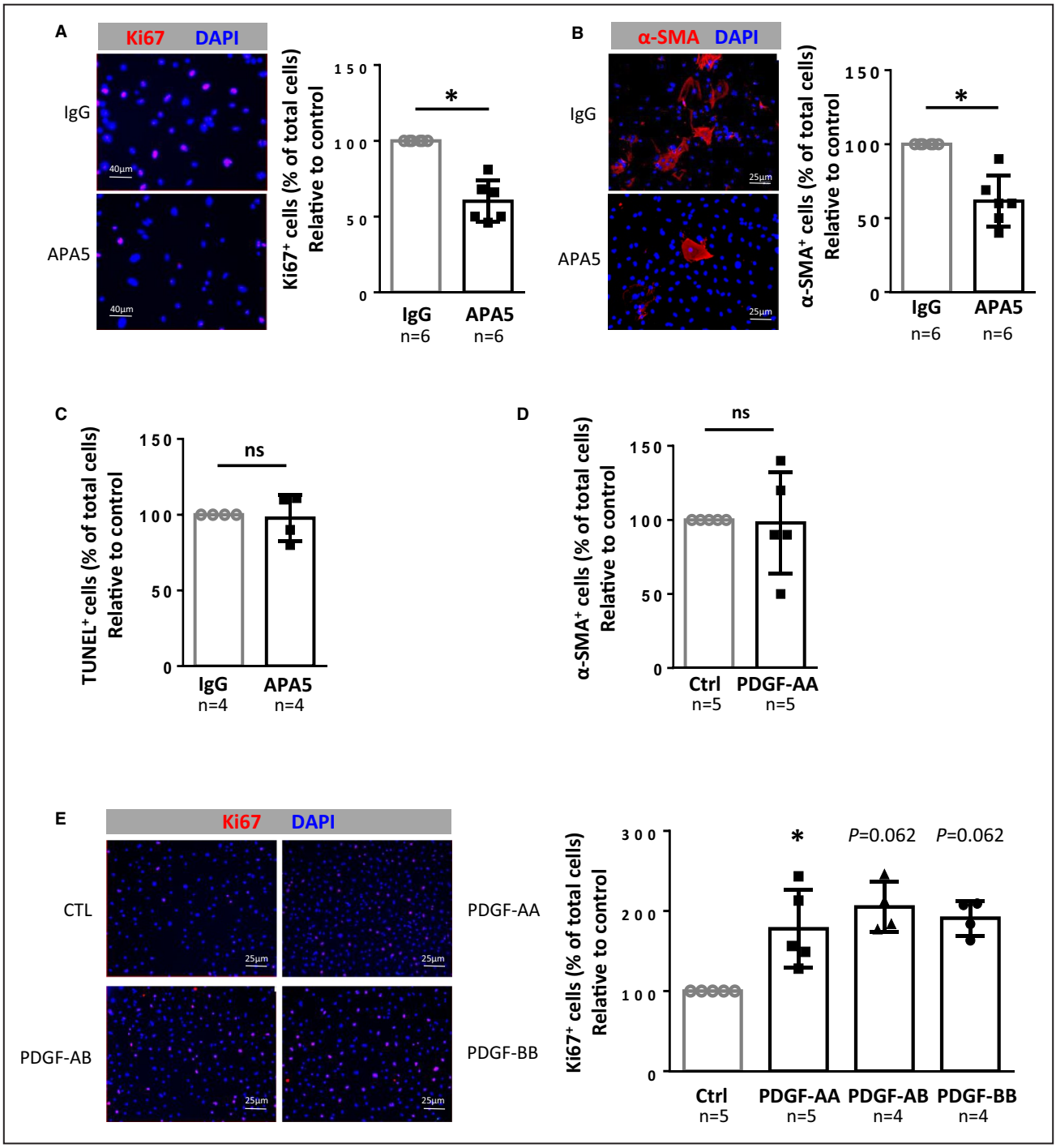



**Figure 4. Constitutive PDGFRα activation leads to pulmonary vessel neomuscularization because of increased numbers of PW1⁺ progenitor-derived SMCs.**
**A**, Timeline of tamoxifen (Tam) induction and analysis of PW1$^{nLacz/+}$/PDGFRα$^{+/(S)K}$/Rosa-CRE⁺ mice (males + females). **B** and **C**, Analysis of mice lungs 2 weeks after tamoxifen induction. **B,** Quantification of muscularized vessels in lungs from control or tamoxifen-treated mice. Pulmonary vessel muscularization was determined by immunofluorescence using anti–α-smooth muscle actin (α-SMA) and anti-von Willebrand factor (vWF) antibodies (n=4–8). For each animal, ≈100 vWF⁺ vessels (<100 μm) were analyzed for muscularization (α-SMA⁺). **C,** Quantification of lung β-galactosidase (β-gal)-expressing SMC in fully-muscularized pulmonary vessels. The percentage of lung β-gal⁺/α-SMA⁺ cells among α-SMA⁺ cells in fully-muscularized vessels (<100 μm) was determined by immunofluorescence (n=7 for each condition). Bars represent means and whiskers represent SD. **P*<0.05, ***P*<0.01 (2-tailed Mann-Whitney). PDGFRα indicates platelet-derived growth factor receptor type α; PW1, protein widely 1; SMA, smooth muscle actin; and SMC, smooth muscle cell.

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analyses and quantification of picrosirius red-stained lung sections did not indicate any significant differences in fibrotic area size between tamoxifen-induced mice and noninduced littermates (Figure 8B and 8C and Figure S10). Samples did, however, demonstrate areas of thickened alveolar walls in tamoxifen-induced mice indicating local tissue alterations (Figure S10). Constitutive PDGFRα activation for 5 to 6 weeks was therefore not sufficient to elicit pulmonary fibrosis at this stage. We also investigated the effect of PDGFRα inhibition on late CH-induced pulmonary fibrosis with the APA5 blocking antibody[19,20] (7 males and 1 female per group). As expected, CH significantly increased transforming growth factor-β and collagen 3a1 and 1a1 mRNA expression (Figure 8D). No difference was observed between control IgG and APA5-treated mice exposed to CH. Histological analyses and quantification of fibrotic areas by picrosirius red staining (Figure 8E and 8F and Figure S11) confirmed similar CH-induced increases in collagen-rich areas in both control IgG- and APA5-treated mice (20.1%±4% and 17.5%±5.1%, respectively) as compared with control




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**Figure 5. PDGFRα regulates PW1⁺ progenitor cell proliferation in vitro.**
**A**, Effect of PDGFRα inhibition on PW1⁺ progenitor cell proliferation. Pulmonary PW1⁺ progenitor cells were sorted by FACS, incubated for 5 days with IgG control or APA5 and proliferative cells were then labeled with anti-Ki67 antibody (red). (Left) Representative immunofluorescence images and (right) quantification of PW1⁺ progenitor cell proliferation as a ratio of Ki67⁺ cells over total cells reported to control (n=6). **B**, Effect of PDGFRα inhibition on PW1⁺ progenitor cell differentiation into SMC. FACS-sorted pulmonary PW1⁺ progenitor cells were incubated for 5 days with IgG control or APA5 and then labeled with anti-α-SMA antibody (red). (Left) Representative immunofluorescence images and (right) quantification of PW1⁺ progenitor cell differentiation as a ratio of α-SMA⁺ cells over total cells reported to control (n=6). **C**, Effect of PDGFRα inhibition on PW1⁺ progenitor cell apoptosis. FACS-sorted pulmonary PW1⁺ progenitor cells were incubated for 5 days with IgG control or APA5 and then labeled following the TUNEL method. The ratio of TUNEL⁺ cells over total cells was determined and reported to control (n=4). **D**, Effect of PDGFRα activation on PW1⁺ progenitor cell differentiation into SMCs. FACS-sorted pulmonary PW1⁺ progenitor cells were incubated for 5 days with IgG control or PDGF-AA, -AB, or -BB (20 ng/mL) and labeled with anti–α-SMA antibody (red). The ratio of α-SMA⁺ cells over total cells was reported to control (n=5). **E**, Effect of PDGFRα activation on PW1⁺ progenitor cell proliferation. FACS-sorted pulmonary PW1⁺ progenitor cells were incubated for 5 days with either vehicle, PDGF-AA, PDGF-AB, or PDGF-BB (20 ng/mL) and then labeled with anti-Ki67 antibody (red). (Left) Representative immunofluorescence images and (right) quantification of PW1⁺ progenitor cell proliferation as a ratio of Ki67⁺ cells over total cells reported to control (n=4–5). Bars represent means and whiskers represent SD. *$P$<0.05, ns indicates not significant (1-tailed Wilcoxon signed-ranked test). FACS indicates fluorescence-activated cell sorting; PDGF, platelet-derived growth factor; PDGFRα, platelet-derived growth factor receptor type α; PW1, protein widely 1; SMA, smooth muscle actin; and SMC, smooth muscle cell; and TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; and vWF, von Willebrand factor.

normoxic mice (6.3%±1.7%; $P$=0.001 and $P$=0.004, respectively). Taken together, our results concerning PDGFRα inhibition under chronic hypoxia or constitutive PDGFRα activation under normoxia demonstrate that PDGFRα signaling does not affect lung fibrosis in our models.

## DISCUSSION

To identify new therapeutic targets for this life-threatening pulmonary vascular disease, it is crucial that we understand the molecular mechanisms leading to new SMC production, which contributes to pulmonary vascular remodeling and ultimately PAH. In this study, we describe, for the first time, the precise role of PDGFRα in progenitor cell proliferation, in neomuscularization, and in PH development. Using a novel genetic model and a specific blocking antibody, our results provide evidence that PDGFRα is a pivotal player regulating neomuscularization, and thus participates in PH development by inducing CD34⁺/PDGFRα⁺/PW1⁺ vascular progenitor cell proliferation and their differentiation into SMCs. Importantly, our data suggest that this pathway intervenes during vascular remodeling of human patients with iPAH.

PDGF pathway activation has been associated with pulmonary hypertension development and related vascular remodeling in both experimental PH models and in humans.[5,9] Our observation of increased expression of PDGF-A, -B, and -D after 1 day of CH suggest an early activation of both PDGFR pathways. However, most studies have examined the role of PDGFRβ signaling in experimental PH models, in particular during CH,[10,11] and the role of the PDGFRα pathway has never been directly investigated. In the present study, and using several experimental models, we found that this pathway induces pulmonary vascular remodeling. While PDGFRβ activation was shown to stimulate pulmonary pericyte and SMC proliferation, our data demonstrate that PDGFRα induces pulmonary vascular progenitor cell expansion, leading to their differentiation into vascular SMCs in the adult mouse lung. Related observations have been reported during pulmonary development where PDGFRα signaling is necessary to form alveolar α-SMA⁺ SMCs (also known as alveolar myofibroblasts) during secondary septation.[17,21] Indeed, PDGFRα⁺ cells harvested shortly after birth express α-SMA, but postnatal cells have lost this expression.[22,23] Our results (herein and in Dierick et al[3]) are consistent with these observations and are the first—to our knowledge—to link PDGFRα activation of progenitor cells and pulmonary vascular SMC production in adult mice.

Multiple cellular sources provide new SMCs during PH development. CH-activated SMCs have been shown to proliferate and lead to neomuscularization of downstream arterioles.[2] Pulmonary pericytes are also a source of periendothelial α-SMA⁺ cells in newly muscularized vessels.[1,4] Our previous data[3] and our observation that CH induces an early increase in perivascular PDGFRα⁺/PW1⁺ progenitor cells argue for an important role played by PDGFRα⁺/PW1⁺ progenitor cells during PH remodeling in experimental models. We demonstrate here that PDGFRα inhibition reduces early CH-induced neomuscularization and limits neomuscularization in late CH. Using a novel mouse model of inducible constitutive PDGFRα activation enabling PW1⁺ progenitor cell detection and tracing, we further establish a major role for PDGFRα signaling in inducing vascular progenitor cells to form new SMCs. Interestingly, constitutive PDGFRβ activation did not induce neomuscularization per se but slightly increased the number of fully muscularized vessels after CH.[11] These contrasting results suggest that PDGFRβ-expressing SMCs and pericytes—which participate in

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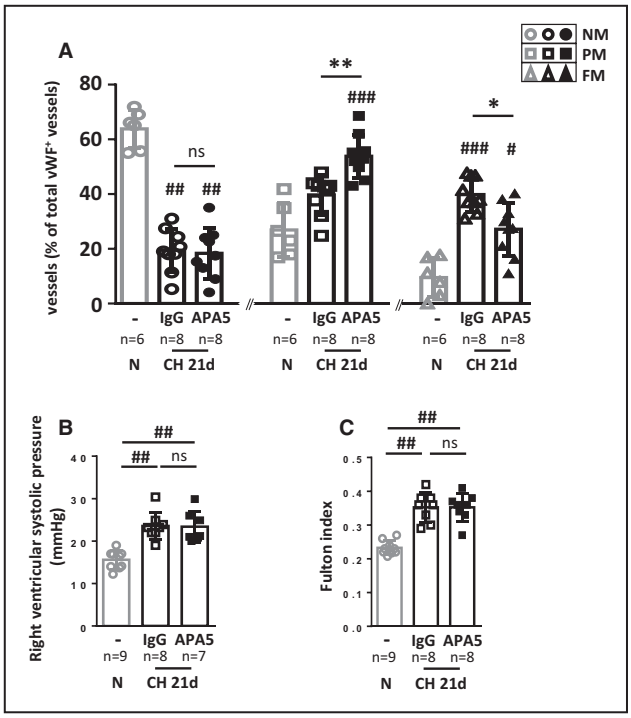



**Figure 6. PDGFRα inhibition with APA5 limits vascular neomuscularization but does not prevent chronic hypoxia (CH)-induced PH.**

PW1$^{nLacz}$ mice were maintained untreated under normoxia (N) or under CH for 21 days and treated with control IgG or PDGFRα blocking antibody APA5. **A**, Pulmonary vessel muscularization was determined by immunofluorescence in lungs of control untreated normoxic mice (n=6) or of IgG- and APA5-treated mice after 21 days of chronic hypoxia (CH 21d, n=9) using anti–α-smooth muscle actin (α-SMA) and anti–von Willebrand factor (vWF) antibodies. For each animal, >100 vWF$^+$ vessels (<100 μm) were analyzed for muscularization (α-SMA$^+$). Vessels were identified as nonmuscularized (NM), partially muscularized (PM) or fully muscularized (FM). **B**, Right ventricular systolic pressure (RVSP, mm Hg) was measured in untreated normoxic mice (n=9) and in IgG- and APA5-treated mice after 21 days of CH (CH 21d, n=7–8). **C**, Right ventricle hypertrophy was measured as the Fulton index in normoxic untreated mice (n=9) and in IgG- and APA5-treated mice in CH (CH 21d, n=8). Bars represent means and whiskers represent SD. *$P$<0.05, **$P$<0.01, ns indicates not significant for APA5 vs IgG within CH 21d group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 vs normoxic; ns indicates not significant (Kruskal-Wallis and Dunn). PDGFRα, platelet-derived growth factor receptor type α; PH, pulmonary hypertension; PW1, protein widely 1; and SMA, smooth muscle actin.

CH-induced vascular remodeling processes[1,2,24]—may first need to be activated by other pathways to become responsive to PDGFRβ activation. Strikingly, imatinib and APA5 only partially inhibited early CH-induced neomuscularization, suggesting that pathways other than PDGF also regulate production of new SMCs. Indeed, previous studies demonstrate that factors such as interleukin-6 and fibroblast growth factor-2 participate in PH-associated vascular remodeling processes.[1,25] These other pathways may predominate when PDGFRα$^+$/PW1$^+$ progenitor cell recruitment is inhibited. Further studies in other rodent PH experimental models (eg, monocrotaline or Sugen-CH rat) will be necessary to confirm the importance of the PDGFRα pathway in vascular remodeling.

Our study establishes constitutive PDGFRα activation as a novel PH experimental model independent of CH. The pathological mechanisms leading to increased pulmonary pressure in this model are not clearly elucidated. Vessel neomuscularization might participate in disease development by inducing arteriolar stiffening and increasing the vascular resistance, thus leading to altered SMC or endothelial cell function. This result underlines the major role of PDGFRα in controlling pulmonary vascular structure and suggests that the beneficial effects observed using imatinib in PH experimental models are partly dependent on inhibiting PDGFRα activity.[9] We observed that in this transgenic model, progenitor cells differentiate into new SMCs. This observation contrasts with our in vitro results showing that PDGFRα activation alone does not induce progenitor cell differentiation. One hypothesis is that other factors are present in the local environment of these pulmonary progenitor cells in vivo that are lacking in our in vitro system. stromal cell–derived factor-1 (C-X-C motif chemokine 12) could be one of these factors, as we already established that in vivo blocking of C-X-C chemokine receptor type 4—the stromal cell–derived factor-1 receptor—inhibits their differentiation into vascular SMC.[3] Female mice appear to be protected against PDGFRα activation-induced PH development, despite an induction of neomuscularization. The mechanisms leading to this sex difference are unclear but may involve estrogen-dependent pathways, which are known to attenuate PH in several rodent models.[26] Estrogens are potent vasodilators in particular through activation of NO and prostacyclin production and inhibition of endothelin synthesis by endothelial cells.[26] One hypothesis could hence be that estrogen stimulation may favor vasodilation reducing pulmonary vascular resistance in females. Studying these protective mechanisms will help decipher the relationship between pulmonary hypertension and vascular remodeling.

PDGFRα activation has been associated with fibrosis development, where mice with a constitutively activated form of this receptor ultimately develop multiple organs fibrosis.[19] The production of collagens 1 and 3 and total collagen deposition remained unchanged in mouse lungs after 5 weeks of constitutive PDGFRα activation. Indeed, studies using this genetic model report fibrosis after 3 to 6 months.[13,19] Thus, in this model of constitutive PDGFRα activation, PH is induced much earlier than fibrosis development, indicating that fibrotic remodeling is not

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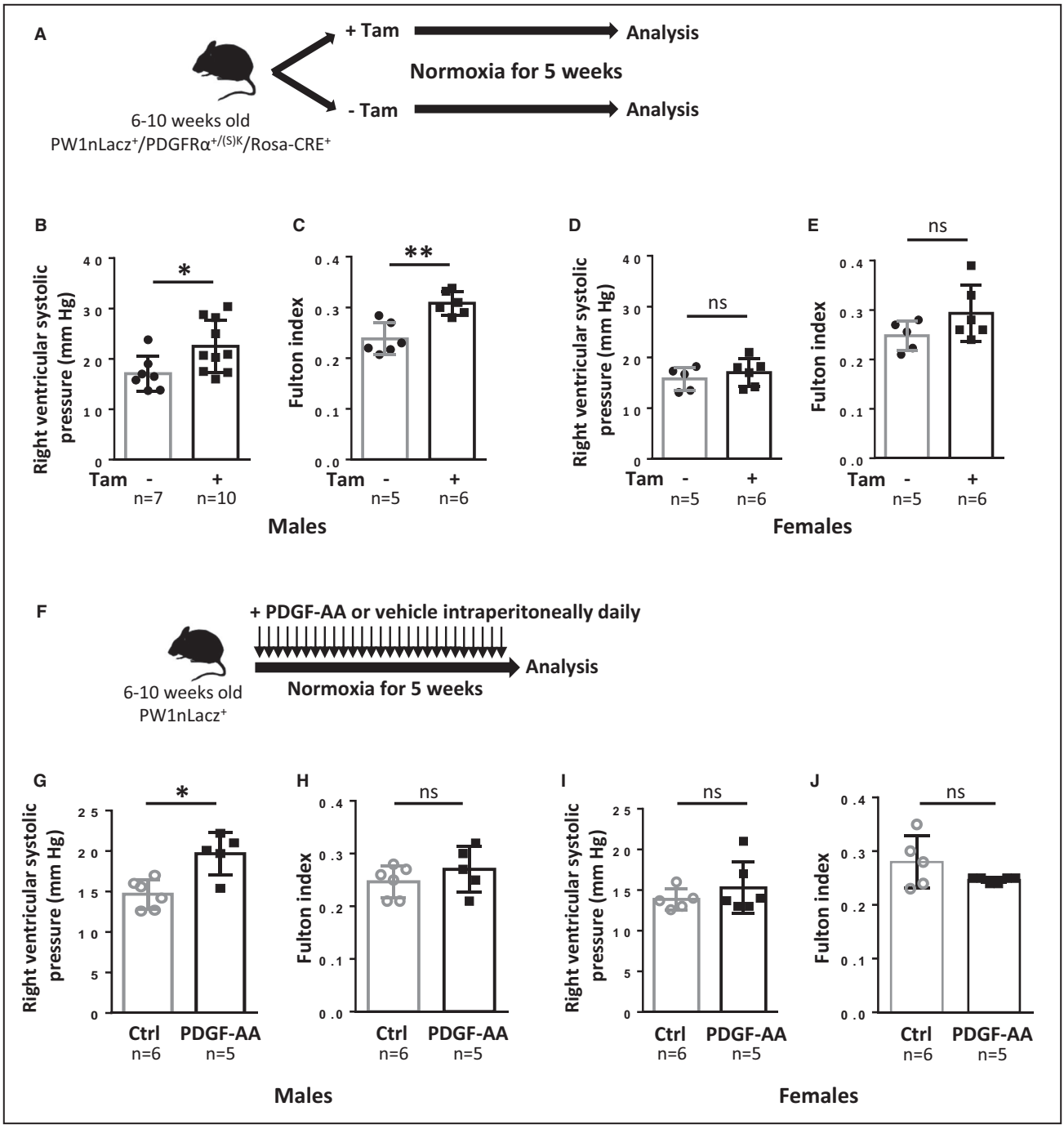



**Figure 7. Constitutive PDGFRα activation leads to pulmonary hypertension.**
**A**, Timeline of tamoxifen (Tam) induction and analysis of PW1$^{nLacz}$/PDGFRα$^{+/(S)K}$/Rosa-CRE$^{+}$ mice. **B to E,** Analysis of mice 5 weeks after tamoxifen induction. **B** and **D,** Right ventricular systolic pressure (RVSP) was measured in control or tamoxifen-treated (**B**), males (n=7–10) and (**D**), females (n=5–6). **C** and **E**, Right ventricular hypertrophy was determined using the Fulton index (right ventricular weight to left ventricular+septum weight ratio) in control or tamoxifen-treated (**C**), males (n=5–6) and (**E**), females (n=5–6). **F**, Timeline of PDGF-AA treatment and analysis of PW1nLacz$^{+}$ mice. **G** through **J,** Analysis of mice after 5 weeks of PDGF-AA treatment. **G** and **I,** Right RVSP was measured in control or PDGF-AA-treated (**G**), males (n=5–6) and (**I**), females (n=5–6). **H** and **J**, Right ventricular hypertrophy was determined using the Fulton index in control or PDGF-AA–treated (**H**), males (n=5–6) and (**J**), females (n=5–6). Bars represent means and whiskers represent SD. *$P$<0.05, **$P$<0.01; ns indicates not significant (2-tailed Mann-Whitney); PDGF, platelet-derived growth factor; PDGFRα, platelet-derived growth factor receptor type α; and PW1, protein widely 1.

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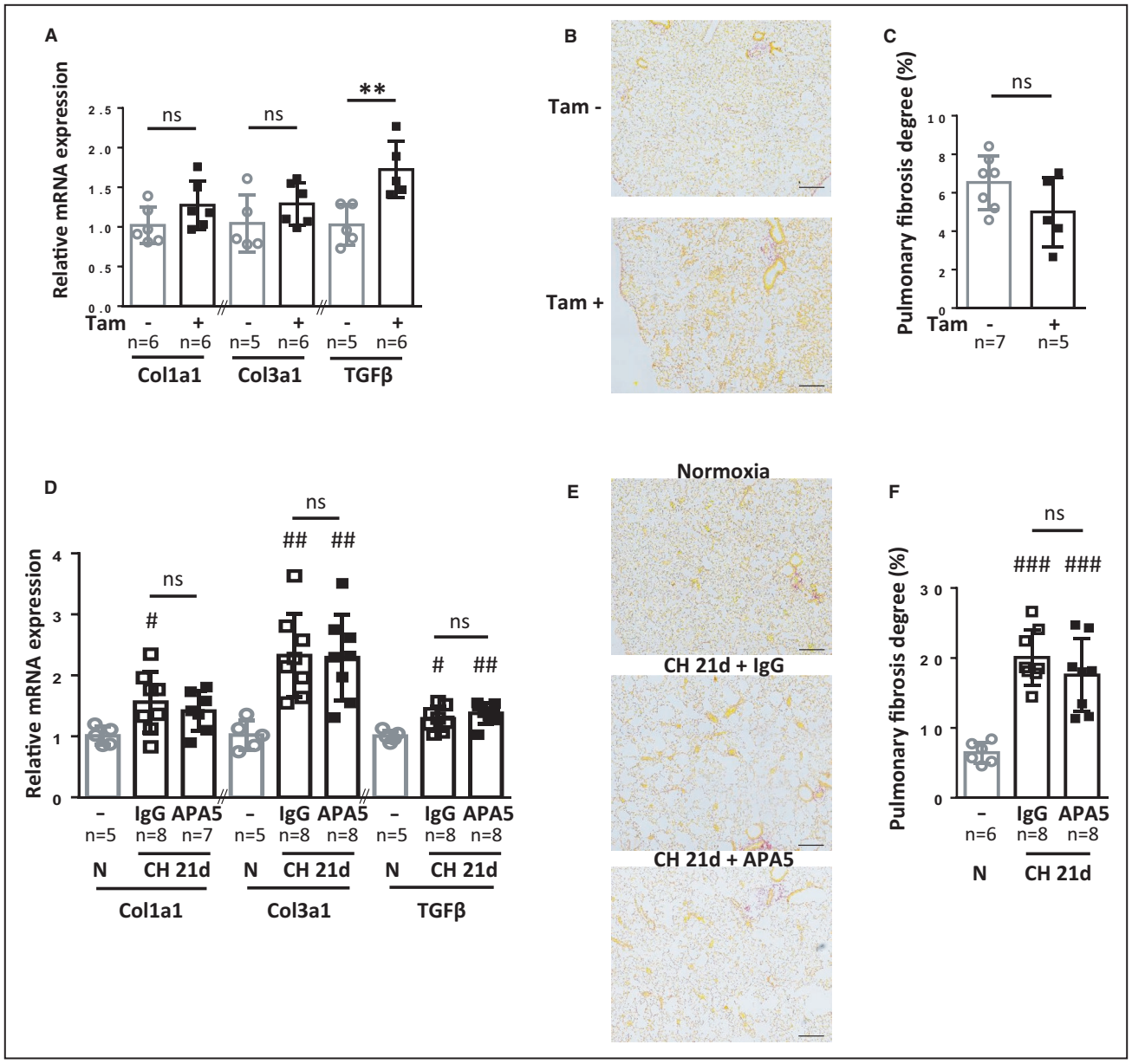



**Figure 8. Lung fibrosis is not induced by PDGFRα activation and is not reduced by PDGFRα inhibition.**
**A** through **C**, Fibrosis evaluation in noninduced (n=6–7) or tamoxifen-induced PW1$^{nLacz/+}$/PDGFRα$^{+/(S)K}$/Rosa-CRE$^{+}$ mice 5 weeks after tamoxifen induction (n=5–6). **D** to **F**, Fibrosis evaluation in untreated mice under normoxia (n=5) or IgG and APA5-treated mice after 21 days of CH (n=7–8). **A** and **D**, RT-qPCR measurements of mRNA expression for collagen 1a1, collagen 3a1, and transforming growth factor-β in lungs. **B** and **E**, Representative images of picrosirius red staining for collagen (red) in pulmonary parenchyma. Larger images are displayed in Figures S10 and S11. **C** and **F**, Quantification of the picrosirius red-stained area as a measure of fibrosis using Histolab analysis. Fibrosis area (% of total area) was determined as the mean fibrotic area of four large lung sections for each animal. Bars represent means and whiskers represent SD. *$P$<0.05, **$P$<0.01 Tam$^{+}$ vs Tam- group, 2-tailed Mann-Whitney; #$P$<0.05, ##$P$<0.01 vs normoxic, Kruskal-Wallis and Dunn. CH indicates chronic hypoxia; ns, not significant for APA5 vs IgG within CH 21d group in D and F (scale bar 200 μm); PDGFRα, platelet-derived growth factor receptor type α; PW1, protein widely 1; and RT-qPCR, reverse transcriptase quantitative polymerase chain reaction.

initially involved in increasing pulmonary blood pressure or pulmonary vascular neomuscularization. In addition, PDGFRα inhibition using APA5 blocking antibody did not prevent long-term CH-induced fibrosis, indicating that PDGFRα does not regulate CH-associated fibrosis. Fibrocytes have been shown to contribute to CH-induced fibrosis[27] and pericyte-like ABCG2$^{+}$ mesenchymal stem cells (PDGFRα$^{-}$) could also participate in fibrotic remodeling because of their myofibroblastic capacities.[28] Our in vitro results suggest that PDGFRα activation leads to proliferation of PDGFRα$^{+}$ progenitor cells, whose fate is then

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controlled by other factors depending on the pathophysiological context. During early CH, these factors orientate PDGFRα+ progenitor cells toward differentiation into vascular SMCs, and our data argue against a PDGFRα-dependent fibroblastic fate during late CH. Other factors may induce differentiation toward fibroblasts in different experimental conditions, such as aging.[19] The involvement of the PDGFRα pathway and of PDGFRα+ cells in the context of fibrosis is controversial. Several studies suggest that PDGFRα signaling is important for fibrosis development in various organs, including the lung.[13,16,19,23,29] However, Kishi et al recently showed that specific PDGFRα inhibition does not reduce bleomycin-induced fibrosis.[30] These results, together with our data, suggest that the relationship between PDGFRα+ cells, the PDGFRα pathway, and fibrosis is more complex than simple PDGFRα activation of PDGFRα+ fibroblast precursors, and may involve other factors such as transforming growth factor-β.[31] Interestingly, PDGF-BB intratracheal administration was previously shown to induce SMC proliferation, confirming our results, but also perivascular and peribronchial collagen deposition.[32] These observations combined with ours suggest a complex interplay between both PDGFR receptors to induce fibrosis. In line with these results, although PDGFRα is often considered as a fibroblast marker, growing evidence now shows that a proportion of PDGFRα+ cells are progenitor or stem cells.[12] Our results confirm that the pulmonary PDGFRα+ progenitor cell population contains cells with SMC differentiation potential together with cells able to differentiate into alveolar myofibroblasts and peribronchiolar SMC, as observed during developmental alveolar septation[33] or pneumectomy-induced realveolarization.[34] Indeed, the diversity of PDGFRα+ cells has been illustrated by single-cell transcriptome analysis[23]; therefore, PDGFRα activation may also target other progenitor cells because of the overlapping expression of different markers and pathways or via paracrine mechanisms.

We observed that PDGFRα activation by PDGF-AA increases vascular progenitor cell proliferation but does not induce their differentiation into SMCs, indicating that other pathways could be involved. This is in agreement with previous studies showing that PDGFRα or PDGF-AA regulate stem/progenitor cell renewal or proliferation[15,35] and can also maintain precursors in an immature state.[36,37] On the other hand, other groups have published that PDGFRα is important for stem/progenitor cell differentiation, in particular into α-SMA-expressing cells or fibroblasts.[13,38–40] These putative conflicting roles of PDGFRα might originate from the type of progenitor/stem cells used. We previously showed that CD34+/PDGFRα+/PW1+ cells differentiate toward terminally differentiated smooth muscle myosin heavy chain+ SMCs,[3] which might not be the case for the stem cells used in other studies. It is also possible that our in vitro experiments lack some factors that, in combination with PDGF-AA, could induce differentiation of these cells. For example, primitive endoderm cell proliferation seems to depend on both fibroblast growth factor receptor 1 and PDGFRα, whereas cell specification seems to be only fibroblast growth factor receptor 1 dependent.[41]

In keeping with our previous study,[3] PW1+ cells in control human lungs were found clustered and mostly perivascular with a large majority of these cells coexpressing PDGFRα. We observed increased numbers of perivascular PW1+/PDGFRα+ in CH mice and in patients with iPAH, suggesting similar proliferative mechanisms, in particular PDGFRα activation. PW1+/PDGFRα+/α-SMA+ cells were absent from control arteries but were observed in the remodeled arterial wall from patients with iPAH and could be differentiating PW1+/PDGFRα+ progenitor cells participating in vascular remodeling as suggested by our data in the CH mouse (herein and in Dierick et al[3]). However, we cannot rule out the hypothesis that a proportion of medial PW1+/PDGFRα+ cells may be derived from proliferating PW1+ SMCs re-expressing PDGFRα or from endothelial cells through endothelial-to-mesenchymal transition. The increased expression of PDGF and PDGFRα has been previously documented in pulmonary vessels from patients with iPAH and further strengthens our data.[5] Part of the beneficial effects observed in patients treated with imatinib could therefore be attributable to the inhibition of the PDGFRα pathway.[42] However, unlike imatinib,[11] blocking PDGFRα is not sufficient to reduce pulmonary pressure in CH mice. This suggests that the vasorelaxing effect of imatinib observed on pulmonary vessels could be attributable to PDGFRβ inhibition.[43,44] Therefore, a multitarget therapeutic strategy associating PDGFRα inhibition with current vasodilating therapies may provide beneficial effects by inhibiting progenitor cell recruitment and vessel neomuscularization.

In conclusion, our study highlights PDGFRα as an essential regulator of lung vessel neomuscularization via the recruitment of vascular progenitor cells and opens new avenues for pulmonary hypertension medication.

## ARTICLE INFORMATION

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### Affiliations

Faculté de Médecine Pitié-Salpêtrière, INSERM, UMR_S 1166 (J.S., J.R.-M., F.D., L.E.B., C.P., F.A., F.S., S.N.), Faculté de Médecine Pitié-Salpêtrière, INSERM, UMS28 (N.M.) and Faculté de Médecine Pitié-Salpêtrière, INSERM, Plateforme CyPS (B.H.-C.), Sorbonne Universités, Paris, France; ICAN Institute, Paris,

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France (J.S., J.R., L.E.B., C.P., F.A., F.S., S.N.); Lady Davis Institute for Medical Research, McGill University, Montréal, QC, Canada (F.D.); Université Paris-Saclay, School of Medicine, Le Kremlin-Bicêtre, France (L.T., R.T., M.H., C.G.); INSERM, Hôpital Marie Lannelongue, UMR_S 999 «Pulmonary Hypertension: Pathophysiology and Novel Therapies, Le Plessis-Robinson, France (L.T., R.T., M.H., C.G.); LRTOX, Fontenay aux Roses, France (V.M.); Université de Paris, INSERM, Paris Cardiovascular Research Center, Paris, France (D.S., G.M.); Victor Chang Cardiac Research Institute, Darlinghurst, Australia (R.P.H.); St. Vincent's Clinical School and School of Biotechnology and Biomolecular Science, UNSW Sydney, Sydney, Australia (R.P.H., P.S., D.C.); Immunology Division, Garvan Institute of Medical Research, Darlinghurst, Australia (P.S., D.C.); and Department of Respiratory and Intensive Care Medicine, Assistance Publique–Hôpitaux de Paris (AP-HP), Pulmonary Hypertension National Referral Center, Hôpital Bicêtre, Le Kremlin-Bicêtre, France (M.H.).

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## Supplemental Material

Data S1
Tables S1–S3
Figures S1–S11
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## REFERENCES

1. Ricard N, Tu LY, Le Hiress M, Huertas A, Phan C, Thuillet R, Sattler C, Fadel E, Seferian A, Montani D, et al. Increased pericyte coverage mediated by endothelial-derived fibroblast growth factor-2 and interleukin-6 is a source of smooth muscle-like cells in pulmonary hypertension. *Circulation*. 2014;129:1586–1597. doi: 10.1161/CIRCULATIONAHA.113.007469
2. Sheikh AQ, Misra A, Rosas IO, Adams RH, Greif DM. Smooth muscle cell progenitors are primed to muscularize in pulmonary hypertension. *Sci Transl Med*. 2015;7:308ra159. doi: 10.1126/scitranslmed.aaa9712
3. Dierick F, Héry T, Hoareau-Coudert B, Mougenot N, Monceau V, Claude C, Crisan M, Besson V, Dorfmüller P, Marodon G, et al. Resident PW1+ progenitor cells participate in vascular remodeling during pulmonary arterial hypertension. *Circ Res*. 2016;118:822–833. doi: 10.1161/circresaha.115.307035
4. Bordenave J, Tu LY, Berrebeh N, Thuillet R, Cumont A, Le Vely B, Fadel E, Nadaud S, Savale L, Humbert M, et al. Lineage tracing reveals the dynamic contribution of pericytes to the blood vessel remodeling in pulmonary hypertension. *Arterioscler Thromb Vasc Biol*. 2020;40:766–782. doi: 10.1161/ATVBAHA.119.313715
5. Perros F, Montani D, Dorfmüller P, Durand-Gasselin I, Tcherakian C, Le Pavec J, Mazmanian M, Fadel E, Mussot S, Mercier O, et al. Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2008;178:81–88. doi: 10.1164/rccm.200707-1037OC
6. Humbert M, Monti G, Fartoukh M, Magnan A, Brenot F, Rain B, Capron F, Galanaud P, Duroux P, Simonneau G, et al. Platelet-derived growth factor expression in primary pulmonary hypertension: comparison of HIV seropositive and HIV seronegative patients. *Eur Respir J*. 1998;11:554–559.
7. Humbert M, Guignabert C, Bonnet S, Dorfmüller P, Klinger JR, Nicolls MR, Olschewski A, Pullamsetti SS, Schermuly RT, Stenmark K, et al. Pathology and pathobiology of pulmonary hypertension: state of the art and research perspectives. *Eur Respir J*. 2019;53:1801887. doi: 10.1183/13993003.01887-2018
8. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev*. 2008;22:1276–1312. doi: 10.1101/gad.1653708
9. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, et al. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest*. 2005;115:2811–2821. doi: 10.1172/JCI24838
10. ten Freyhaus H, Berghausen EM, Janssen W, Leuchs M, Zierden M, Murmann K, Klinke A, Vantler M, Caglayan E, Kramer T, et al. Genetic ablation of PDGF-dependent signaling pathways abolishes vascular remodeling and experimental pulmonary hypertension. *Arterioscler Thromb Vasc Biol*. 2015;35:1236–1245. doi: 10.1161/ATVBAHA.114.304864
11. Dahal BK, Heuchel R, Pullamsetti SS, Wilhelm J, Ghofrani HA, Weissmann N, Seeger W, Grimminger F, Schermuly RT. Hypoxic pulmonary hypertension in mice with constitutively active platelet-derived growth factor receptor-β. *Pulm Circ*. 2011;1:259–268. doi: 10.4103/2045-8932.83448
12. Farahani RM, Xaymardan M. Platelet-derived growth factor receptor alpha as a marker of mesenchymal stem cells in development and stem cell biology. *Stem Cells Int*. 2015;2015:1. doi: 10.1155/2015/362753
13. Iwayama T, Steele C, Yao L, Dozmorov MG, Karamichos D, Wren JD, Olson LE. PDGFRα signaling drives adipose tissue fibrosis by targeting progenitor cell plasticity. *Genes Dev*. 2015;29:1106–1119. doi: 10.1101/gad.260554.115
14. Calver AR, Hall AC, Yu WP, Walsh FS, Heath JK, Betsholtz C, Richardson WD. Oligodendrocyte population dynamics and the role of PDGF in vivo. *Neuron*. 1998;20:869–882. doi: 10.1016/S0896-6273(00)80469-9
15. Rivera-Gonzalez GC, Shook BA, Andrae J, Holtrup B, Bollag K, Betsholtz C, Rodeheffer MS, Horsley V. Skin adipocyte stem cell self-renewal is regulated by a PDGFA/AKT-signaling axis. *Cell Stem Cell*. 2016;19:738–751. doi: 10.1016/j.stem.2016.09.002
16. Saito Y, Chikenji T, Ozasa Y, Fujimiya M, Yamashita T, Gingery A, Iba K. PDGFR signaling mediates hyperproliferation and fibrotic responses of subsynovial connective tissue cells in idiopathic carpal tunnel syndrome. *Sci Rep*. 2017;7:16192. doi: 10.1038/s41598-017-16443-w
17. Gouveia L, Betsholtz C, Andrae J. PDGF-A signaling is required for secondary alveolar septation and controls epithelial proliferation in the developing lung. *Development*. 2018;145. doi: 10.1242/dev.161976
18. Besson V, Smeriglio P, Wegener A, Relaix F, Nait Oumesmar B, Sassoon DA, Marazzi G. PW1 gene/paternally expressed gene 3 (PW1/Peg3) identifies multiple adult stem and progenitor cell populations. *Proc Natl Acad Sci USA*. 2011;108:11470–11475. doi: 10.1073/pnas.1103873108
19. Olson LE, Soriano P. Increased PDGFRalpha activation disrupts connective tissue development and drives systemic fibrosis. *Dev Cell*. 2009;16:303–313. doi: 10.1016/j.devcel.2008.12.003
20. Liao C-H, Akazawa H, Tamagawa M, Ito K, Yasuda N, Kudo Y, Yamamoto R, Ozasa Y, Fujimoto M, Wang P, et al. Cardiac mast cells cause atrial fibrillation through PDGF-A-mediated fibrosis in pressure-overloaded mouse hearts. *J Clin Invest*. 2010;120:242–253. doi: 10.1172/JCI39942

21. Ntokou A, Klein F, Dontireddy D, Becker S, Bellusci S, Richardson WD, Szibor M, Braun T, Morty RE, Seeger W, et al. Characterization of the platelet-derived growth factor receptor-α-positive cell lineage during murine late lung development. *Am J Physiol Lung Cell Mol Physiol*. 2015;309:L942–L958. doi: 10.1152/ajplung.00272.2014
22. Branchfield K, Li R, Lungova V, Verheyden JM, McCulley D, Sun X. A three-dimensional study of alveologenesis in mouse lung. *Dev Biol*. 2016;409:429–441. doi: 10.1016/j.ydbio.2015.11.017
23. Li R, Bernau K, Sandbo N, Gu J, Preissl S, Sun X. Pdgfra marks a cellular lineage with distinct contributions to myofibroblasts in lung maturation and injury response. *Elife*. 2018;7. doi: 10.7554/eLife.36865
24. Chow K, Fessel JP, Ihida-Stansbury K, Schmidt EP, Gaskill C, Alvarez D, Graham B, Harrison DG, Wagner DH, Nozik-Grayck E, et al. Dysfunctional resident lung mesenchymal stem cells contribute to pulmonary microvascular remodeling. *Pulm Circ*. 2013;3:31–49. doi: 10.4103/2045-8932.109912
25. Izikki M, Guignabert C, Fadel E, Humbert M, Tu LY, Zadigue P, Dartevelle P, Simonneau G, Adnot S, Maitre B, et al. Endothelial-derived FGF2 contributes to the progression of pulmonary hypertension in humans and rodents. *J Clin Invest*. 2009;119:512–523. doi: 10.1172/JCI35070
26. Tofovic SP, Jackson EK. Estradiol metabolism: crossroads in pulmonary arterial hypertension. *Int J Mol Sci*. 2020;21:116. doi: 10.3390/ijms21010116
27. Frid MG, Brunetti JA, Burke DL, Carpenter TC, Davie NJ, Reeves JT, Roedersheimer MT, van Rooijen N, Stenmark KR. Hypoxia-induced pulmonary vascular remodeling requires recruitment of circulating mesenchymal precursors of a monocyte/macrophage lineage. *Am J Pathol*. 2006;168:659–669. doi: 10.2353/ajpath.2006.050599
28. Marriott S, Baskir RS, Gaskill C, Menon S, Carrier EJ, Williams J, Talati M, Helm K, Alford CE, Kropski JA, et al. ABCG2pos lung mesenchymal stem cells are a novel pericyte subpopulation that contributes to fibrotic remodeling. *Am J Physiol–Cell Physiol*. 2014;307:C684–C698. doi: 10.1152/ajpcell.00114.2014
29. Mueller AA, van Velthoven CT, Fukumoto KD, Cheung TH, Rando TA. Intronic polyadenylation of PDGFRα in resident stem cells attenuates muscle fibrosis. *Nature*. 2016;540:276–279. doi: 10.1038/nature20160
30. Kishi M, Aono Y, Sato S, Koyama K, Azuma M, Abe S, Kawano H, Kishi J, Toyoda Y, Okazaki H, et al. Blockade of platelet-derived growth factor receptor-β, not receptor-α ameliorates bleomycin-induced pulmonary fibrosis in mice. *PLoS One*. 2018;13:e0209786. doi: 10.1371/journal.pone.0209786
31. Heinzelmann K, Noskovičová N, Merl-Pham J, Preissler G, Winter H, Lindner M, Hatz R, Hauck SM, Behr J, Eickelberg O. Surface proteome analysis identifies platelet derived growth factor receptor-alpha as a critical mediator of transforming growth factor-beta-induced collagen secretion. *Int J Biochem Cell Biol*. 2016;74:44–59. doi: 10.1016/j.biocel.2016.02.013
32. Yi ES, Lee H, Yin S, Piguet P, Sarosi I, Kaufmann S, Tarpley J, Wang NS, Ulich TR. Platelet-derived growth factor causes pulmonary cell proliferation and collagen deposition in vivo. *Am J Pathol*. 1996;149:539–548.
33. Green J, Endale M, Auer H, Perl A-KT. Diversity of interstitial lung fibroblasts is regulated by platelet-derived growth factor receptor α kinase activity. *Am J Respir Cell Mol Biol*. 2016;54:532–545. doi: 10.1165/rcmb.2015-0095OC
34. Chen L, Acciani T, Le Cras T, Lutzko C, Perl A-KT. Dynamic regulation of platelet-derived growth factor receptor α expression in alveolar fibroblasts during realveolarization. *Am J Respir Cell Mol Biol*. 2012;47:517–527. doi: 10.1165/rcmb.2012-0030OC
35. Kikuchi A, Pradhan-Sundd T, Singh S, Nagarajan S, Loizos N, Monga SP. Platelet-derived growth factor receptor α contributes to human hepatic stellate cell proliferation and migration. *Am J Pathol*. 2017;187:2273–2287. doi: 10.1016/j.ajpath.2017.06.009
36. Zhu Q, Zhao X, Zheng K, Li H, Huang H, Zhang Z, Mastracci T, Wegner M, Chen Y, Sussel L, et al. Genetic evidence that Nkx2.2 and PDGFRa are major determinants of the timing of oligodendrocyte differentiation in the developing CNS. *Development*. 2014;141:548–555. doi: 10.1242/dev.095323
37. Baroti T, Zimmermann Y, Schillinger A, Liu L, Lommes P, Wegner M, Stolt CC. Transcription factors Sox5 and Sox6 exert direct and indirect influences on oligodendroglial migration in spinal cord and forebrain. *Glia*. 2016;64:122–138. doi: 10.1002/glia.22919
38. Ball SG, Shuttleworth CA, Kielty CM. Platelet-derived growth factor receptor-alpha is a key determinant of smooth muscle alpha-actin filaments in bone marrow-derived mesenchymal stem cells. *Int J Biochem Cell Biol*. 2007;39:379–391. doi: 10.1016/j.biocel.2006.09.005
39. Kim B-J, Kim Y-H, Lee Y-A, Jung S-E, Hong YH, Lee E-J, Kim B-G, Hwang S, Do JT, Pang M-G, et al. Platelet-derived growth factor receptor-alpha positive cardiac progenitor cells derived from multipotent germline stem cells are capable of cardiomyogenesis in vitro and in vivo. *Oncotarget*. 2017;8:29643–29656. doi: 10.18632/oncotarget.16772
40. Xu T, Liu N, Shao Y, Huang Y, Zhu D. MiR-218 regulated cardiomyocyte differentiation and migration in mouse embryonic stem cells by targeting PDGFRα. *J Cell Biochem*. 2019;120:4355–4365. doi: 10.1002/jcb.27721
41. Molotkov A, Soriano P. Distinct mechanisms for PDGF and FGF signaling in primitive endoderm development. *Dev Biol*. 2018;442:155–161. doi: 10.1016/j.ydbio.2018.07.010
42. Frost AE, Barst RJ, Hoeper MM, Chang H-J, Frantz RP, Fukumoto Y, Galié N, Hassoun PM, Klose H, Matsubara H, et al. Long-term safety and efficacy of imatinib in pulmonary arterial hypertension. *J Heart Lung Transplant*. 2015;34:1366–1375. doi: 10.1016/j.healun.2015.05.025
43. Rieg AD, Bünting NA, Cranen C, Suleiman S, Spillner JW, Schnöring H, Schröder T, von Stillfried S, Braunschweig T, Manley PW, et al. Tyrosine kinase inhibitors relax pulmonary arteries in human and murine precision-cut lung slices. *Respir Res*. 2019;20:111. doi: 10.1186/s12931-019-1074-2
44. Abe K, Toba M, Alzoubi A, Koubsky K, Ito M, Ota H, Gairhe S, Gerthoffer WT, Fagan KA, McMurtry IF, et al. Tyrosine kinase inhibitors are potent acute pulmonary vasodilators in rats. *Am J Respir Cell Mol Biol*. 2011;45:804–808. doi: 10.1165/rcmb.2010-0371OC
45. Takakura N, Yoshida H, Kunisada T, Nishikawa S, Nishikawa SI. Involvement of platelet-derived growth factor receptor-alpha in hair canal formation. *J Invest Dermatol*. 1996;107:770–777. doi: 10.1111/1523-1747.ep12371802
46. Takakura N, Yoshida H, Ogura Y, Kataoka H, Nishikawa S, Nishikawa S. PDGFR alpha expression during mouse embryogenesis: immunolocalization analyzed by whole-mount immunohistostaining using the monoclonal anti-mouse PDGFR alpha antibody APA5. *J Histochem Cytochem*. 1997;45:883–893. doi: 10.1177/002215549704500613
47. Dudgeon K, Rouet R, Kokmeijer I, Schofield P, Stolp J, Langley D, Stock D, Christ D. General strategy for the generation of human antibody variable domains with increased aggregation resistance. *Proc Natl Acad Sci USA*. 2012;109:10879–10884. doi: 10.1073/pnas.1202866109
48. Asli NS, Xaymardan M, Patrick R, Farbehi N, Cornwell J, Forte E, Waardenberg AJ, Janbandhu V, Kesteven S, Chandrakanthan V, et al. PDGFRα signaling in cardiac fibroblasts modulates quiescence, metabolism and self-renewal, and promotes anatomical and functional repair. *bioRxiv*. 2019;225979. doi: 10.1101/225979
49. Relaix F, Weng X, Marazzi G, Yang E, Copeland N, Jenkins N, Spence SE, Sassoon D. Pw1, a novel zinc finger gene implicated in the myogenic and neuronal lineages. *Dev Biol*. 1996;177:383–396. doi: 10.1006/dbio.1996.0172


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# SUPPLEMENTAL MATERIAL

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**Data S1.**

## SUPPLEMENTAL METHODS

The detailed description of the reagents and resources can be found in the Major Resources Tables.

### Animals and experimental PH model

Care of the animals and surgical procedures were performed according to the Directive 2010/63/EU of the European Parliament. Animals were housed in an environmentally-controlled pathogen-free animal facility for the duration of the experiment. All animals had *ad libitum* access to food and water and were under a 12h-12h light-dark cycle. $Pw1^{IRESnLacZ}$ transgenic mice ($Pw1^{nlacZ/+}$), in which a nuclear operon lactose gene is expressed under the control of the Pw1 gene locus were bred in our animal facility and maintained in a C57BL6/J background [3,18]. C57BL6/J mice were from Janvier (Orléans, France). RosaCRE-ERT2 (B6.129-Gt(ROSA)$26^{Sortm1(}$cre/ERT2)Tyj/J) and $PDGFR\alpha^{+/(S)K}$ (B6.129S4-Pdgfratm12Sor/J) transgenic mice were bought from Jackson Labs and are in a C57BL6/J background. The $PDGFR\alpha^{+/(S)K}$ strain presents an inducible heterozygous mutation (D842V) located in the kinase domain leading to constitutive PDGFRα activation after Cre-dependent recombination [19]. The strains were crossed together to produce $Pw1^{nlacZ/+}$xRosaCre-ERT2x$PDGFR\alpha^{+/(S)K}$ mice. Mice (littermates of 6-10 weeks) were exposed to room air (normoxia) or chronic normobaric hypoxia (10% $O_2$) in a ventilated chamber for 4 or 21 days with the same light-dark cycle. Hypoxia was obtained by flushing nitrogen into the hypoxic chamber. Levels of $CO_2$ and $O_2$ in the chamber were constantly monitored and maintained by flushing oxygen in the chamber and by absorbing $CO_2$ with soda lime granules. The chamber temperature was maintained at 22–24°C and excess humidity was prevented by desiccant. To confirm chronic hypoxia (CH) after 21 days, hemoglobin concentration was measured in the peripheral blood using HemoCue (Hb 20+). Male and female mice show similar vascular remodeling to CH, so both females and males were used for studies after four days of CH. Females are known to

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display statistically lower RVSP than males, so we only studied males for long-term CH or after PDGF-AA treatment to obtain homogeneous groups. Cre recombination was induced by Tamoxifen injection (2 mg/day/mouse for five consecutive days). Tamoxifen (Sigma Aldrich, France) was prepared in 100% ethanol and diluted in corn oil for i.p. injection. Mice (males and females) were sacrificed two or five weeks after the last injection. Mice, in particular littermates, were randomly assigned to the different experimental groups.

**Hemodynamic measurements and tissue collection**

Hemodynamic parameters were measured blinded to the experimental treatment as already described [3]. Mice were anesthetized with a ketamine/Xylazine mix (100 and 10 mg/kg i.p.) and maintained at 37°C. After intubation, mice were connected to a Minivent Mouse ventilator (type 845, Harvard Apparatus, respiratory frequency 170 stroke/min and respiratory volume 200 µL). After incision of the abdomen and diaphragm, mouse right ventricular systolic pressure (RVSP) was measured by introducing a Millar pressure transducer (size 1.4 F, Millar Micro-tip catheter transducer, model SPR-671; Millar Instruments, Inc, Houston, TX, USA) into the right ventricle. After recording, the mice were killed by removal of the heart. Right ventricle (RV) was separated from the left ventricle+septum (LV+S) and both were weighed to calculate the Fulton index (RV/LV+S) measuring right ventricular hypertrophy.

**Mice treatment**

Pw1$^{nlacZ/+}$mice received a daily i.p. injection of imatinib (50 mg/kg/d, #SML1027, Sigma Aldrich, St. Quentin Fallavier, France, dissolved in DMSO and diluted in PBS prior to injection) or vehicle (2% DMSO in PBS) during the whole duration of CH. Specific PDGFRα blocking antibody was expressed and purified from hybridoma cell line APA5 [45,46] as previously described [47,48]. For the blocking antibody experiments, mice were injected i.p. with 500 µg of PDGFRα blocking antibody APA5 or isotype control (clone 2A3, InVivoMAb BE0089, BioXCell, USA) three days before starting CH, with additional 100 µg i.p. injections every three days during the course of CH. For proliferation measurements, mice were injected i.p. with

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ethynyl-deoxyuridine (EdU, 1 mg/mouse in PBS) 24 h prior to sacrifice. For PDGF-AA treatment, mouse PDGF-AA (PeproTech, Neuilly-Sur-Seine, France) or vehicle (PBS with 0.03% mouse serum albumin, Sigma Aldrich) was injected i.p. 6 days/week (150 ng/mouse) for five weeks.

**Flow cytometry isolation of PW1$^+$ progenitor cells**

Lung single-cell suspensions were obtained as previously described with minor changes. Tissue was digested in HBSS (Gibco, Thermo Fisher Scientific, Villebon, France) supplemented with 3% decomplemented fetal bovine serum (FBS; Thermo Fisher Scientific) and 0.5 mg/mL of collagenases IA-S, II-S, IV-S (Sigma Aldrich) for 35 min at 37°C. The remaining tissue fragments were next disrupted mechanically between two superfrost+ slides (Thermo Fisher Scientific), to separate the progenitor cells from the extracellular matrix. Mouse lung cells were stained for 20 min on ice in the dark. Antibodies used were CD45 PECy7, CD34 Brilliant Violet421, and c-kit PE [3]. DDAO-G (Life Technologies) was used to detect β-gal activity. Dead cells were labeled using Zombie Green Fixable Viability (Biolegend, San Diego). Cells were sorted using a FACSAria (Becton Dickinson) with appropriate isotype matching controls. Fluorescence Minus One controls (FMO) were used to identify and gate cells. The FACS gating strategy is described in Figure S7. We have previously shown that mouse pulmonary CD45$^-$/CD34$^+$/β-gal$^+$/c-kit$^-$ cells are vascular progenitor cells expressing PDGFRα$^+$ and PW1$^+$ [3].

**Cell culture**

FACS-isolated pulmonary CD45$^-$/CD34$^+$/β-gal$^+$/c-kit$^-$ progenitor cells were used to test the effect of PDGFRα activation or inhibition on their spontaneous differentiation into smooth muscle cells in *in vitro* culture. Following FACS, cells were plated at a density of 2000 cells/cm$^2$ and were maintained in DMEM medium (Thermo Fisher Scientific) supplemented with 20% FBS and 1% penicillin/streptomycin (Thermo Fisher Scientific) for 24 hours. For PDGFRα activation experiments, cells were incubated in DMEM medium supplemented with 1% FBS and 1% PS and were stimulated or not with 20 ng/ml of PDGF ligands (AA; AB and BB, R&D Systems). For PDGFRα inhibition experiments, cells were incubated with DMEM containing 5% FBS, 1% PS, and APA5 blocking antibody or control antibody (150 µg/mL).

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In all cases, medium was changed every other day and culture was stopped at day five for immunofluorescence analysis.

**Immunofluorescence analysis**

Mouse lungs were inflated by intratracheal injection of 1-2 ml Optimal Cutting Temperature (OCT) medium (Leica, 50% in PBS) and fixed by immersion in a 4% paraformaldehyde solution (PFA; Sigma) for 24-48 hours before paraffin embedding. Alternatively, lungs were immersed in isopentane, frozen in liquid nitrogen, embedded in OCT and stored at -80°C. Sections (3 µm) of paraffin-embedded tissue were deparaffinized, rehydrated, and citrate antigen retrieval (20 min; 95°C) was performed. Cryosections (12 µm) were fixed in 4% paraformaldehyde. After permeabilization with ice-cold acetone/methanol (1:1) (10min; -20°C), sections were blocked with 5% FBS (1h) prior to incubation with FBS+antibodies (Table S1). Immunolabeled sections were mounted with Dako fluorescent mounting medium (Dako) and examined under fluorescence (Nikon epifluorescence Ti microscope). Images were analyzed using NIH ImageJ. Control experiments were performed using secondary antibodies alone and showed no non-specific labeling.

Pulmonary vessel muscularization was analyzed by co-labeling with anti-α-SMA and anti-vWF antibodies. The percentages of fully-muscularized (SMC covering more than 90% of the vessel) and partially-muscularized (SMC covering more than 10% and less than 90% of the vessel) were determined on more than 100 $vWF^+$ vessels/animal. The number of $PW1^+$ progenitor-derived SMC was determined as the percentage of $\beta\text{-gal}^+/\alpha\text{-SMA}^+$ SMC relative to the number of $\alpha\text{-SMA}^+$ cells for each vessel (15 to 100 µm diameter) in the whole lung section (15-30 vessels/animal). Perivascular $\beta\text{-gal}^+/PDGFR\alpha^+$ cells were defined to be within 40µm from the $vWF^+$ vessels. Images were analyzed by an investigator blinded to the experimental status of all animals. Proliferating cells were detected using EdU (Thermo Fisher Scientific) incorporation (Click-iT EdU Alexa Fluor 555 Imaging Kits; Thermo Fisher Scientific) followed by PW1 immunodetection. The number of $EdU^+/PW1^+$ proliferative progenitor cells was measured relative to the total number of $PW1^+$ cells. Apoptotic cells were detected with the TUNEL

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method according to the manufacturer's protocol (Fluorescein In Situ Cell Death Kit; Roche). The number of TUNEL$^+$ apoptotic cells was assessed as a percentage of the total number of cells analyzed (2000 to 8000 cells/animal).

For human patients, 5 µm lung sections were deparaffinized and incubated with the antigen retrieval buffer. Then, sections were saturated with blocking buffer and incubated overnight with specific antibodies, followed by addition of the corresponding secondary fluorescent-labeled antibodies (Thermo Fisher Scientific). Nuclei were labeled using DAPI (Thermo Fisher Scientific). Mounting was performed using ProLong Gold antifade reagent (Thermo Fisher Scientific). All images were taken using an LSM700 confocal microscope (Zeiss, Marly-le-Roi, France).

Cultured cells were fixed with 4% PFA (6min) and permeabilized using acetone/methanol (1:1) (10 min; -20°C) prior to incubation with antibodies (Table S1). The number of proliferative cells (positive for Ki67$^+$) or differentiated (positive for α-SMA) was determined relative to the total number of cells in the well.

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**Table S1. Antibodies used for immunofluorescence experiments. ON, overnight. RT, room temperature.**

| Target antigen | Provider, reference | Dilution, conditions | Secondary antibody | Provider, reference | Dilution, conditions |
|---|---|---|---|---|---|
| PW1 (human) | Novus Biologicals NBP2-46379 | 1/100 ON; 4°C | Alexa Fluor Donkey anti-mouse 647 | Thermo Fisher Scientific A31571 | 1/200 1h ; RT |
| PDGFRα (human) | abcam ab61219 | 1/200 ON; 4°C | Alexa Fluor Donkey anti-rabbit 547 | Thermo Fisher Scientific A10040 | 1/200 1h ; RT |

| | | | | | |
|---|---|---|---|---|---|
| PW1 (mouse) | Relaix 1996<br>ref [49] | 1/5000<br>ON; 4°C | Goat anti-rabbit 488 | Thermo Fisher Scientific<br>A11034 | 1/500<br>1h; RT |
| β-galactosidase | Abcam<br>ab9361 | 1/6000<br>ON; 4°C | Goat anti-chicken 594 | Thermo Fisher Scientific<br>A11042 | 1/500<br>1h; RT |
| α-SMA-FITC | Sigma<br>F3777 | 1/500<br>1h30; RT | | | |
| α-SMA | ebioscience<br>14-9760-82 | 1/500<br>ON; 4°C | Goat anti-mouse 555 | Abcam<br>Ab150078 | 1/500<br>1h; RT |
| Von Willebrand Factor | DAKO<br>A0082 | 1/300<br>ON; 4°C | Goat anti-rabbit 488 | Thermo Fisher Scientific<br>A11034 | 1/500<br>1h; RT |
| Ki67 | Abcam<br>ab15580 | 1/300<br>ON; 4°C | Goat anti rabbit-488 | Thermo Fisher Scientific<br>A11034 | 1/500<br>1h; RT |
| DAPI | Santa Cruz<br>sc3598 | 1/1000<br>1h; RT | | | |

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**RT-qPCR**

Total lung RNA was extracted using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA (0.5 µg) was reverse transcribed (M-MuLV kit, Thermo Fisher Scientific) per manufacturer's instructions and real time PCR was performed and analyzed using a Light Cycler (LightCycler 480, Roche) with the Brilliant III kit (**Agilent Technologies, Les Ulis, France**). Gene expression was determined relative to RPL13a using the $2^{-\Delta\Delta CT}$ method. The primers sequences are:

**Table S2. Primer sequences for real-time PCR measurements of mRNA expression.**

| Gene | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|---|---|---|
| PDGF-A | CATCCGCTCCTTTGATGATCTT | GTGCTCGGGTCATGTTCAAGT |

| | | |
|---|---|---|
| PDGF-B | CATCCGCTCCTTTGATGATCTT | GTGCTCGGGTCATGTTCAAGT |
| PDGF-C | GCCAAAGAACGGGGACTCG | AGTGACAACTCTCTCATGCCG |
| PDGF-D | ATGGAACGGCTCGTTTTAGTC | CTTCCAGTTGACAGTTCCGCA |
| PDGFRα | GGACTTACCCTGGAGAAGTGAGAA | ACACCAGTTTGATGGATGGGA |
| PDGFRβ | GTGGTGAACTTCCAATGGACG | GTCTGTCACTGGCTCCACCAG |
| Col1a1 | CTCAGGGTGCTCGTGGAT | CTTAGGACCAGCAGGACCAG |
| Col3a1 | GATCTCCTGGTTCTCCTGGAT | TCGTCCAGGTCTTCCTGACT |
| TGFβ | CTGAACCAAGGAGACGGAAT | GGCTGATCCCGTTGATTTC |
| Rpl13a | GAGGAGGCGAAACAAGTCCA | GGGTGGCCAGCTTAAGTTCT |

**Picrosirius red staining**

Lung sections (3µm) of paraffin-embedded tissue were deparaffinized, rehydrated and collagen fibers were stained using a sirius red (Direct red 80, CI35780; Sigma) and picric acid solution (1:1) (45 min). Slides were immersed in two picric acid baths (2 min), two absolute ethanol baths (3 min), and two xylene baths (3 min). For each animal, the light microscopy image (Nikon epifluorescence Ti microscope) of the whole pulmonary parenchyma was analyzed to measure the degree of pulmonary fibrosis (percentage of collagen-stained area per total area) with Histolab software (Excilone, Elancourt, France).

**Image processing and quantification**

All images were composed, edited, and modifications applied to the whole image. Immunofluorescence images were analyzed using ZEN LSM (Carl Zeiss) and ImageJ (NIH) software for human samples and using NIS-Elements (Nikon) and ImageJ (NIH) for mouse samples. Images of picrosirius red staining were quantified using Histolab software (Excilone).

**Immunoblotting**

Lung samples were frozen in liquid nitrogen and conserved at -80°C. Tissue samples were homogenized with the IKA T10 Ultra Turrax® for 30 seconds on ice, in 20 volumes of RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% triton X-100, 0.5% sodium deoxycholate, 0.5% SDS , 1 mM EDTA, and 0.1% anti-protease Sigma P8340 added extra-temporally) per mg of tissue. Tissue lysis was performed by sonicating twice on ice for 10 seconds with the B15 Sonifier Cell disruptor (Proscience) then extracts

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were slowly agitated for 1 h at +4°C, aliquoted, and stocked for further use at -80°C. Protein concentration was quantified using the Bradford method. For Western blotting, protein extracts were denatured in Laemmli buffer (BioRad ref 1610747) with 10% β-mercaptoethanol at 65°C for 20 minutes. Forty µg of protein/sample were run on Criterion Tris-glycine 4% to 15% (BioRad ref 6571095) at 80v. Gel transfer was performed using the Biorad TransBlot Turbo system on 0.45 µm nitrocellulose membrane which was blocked in Tris-buffered Saline, 0.1% Tween, 5% non-fat milk for two hours, then hybridized overnight at 4°C with primary antibodies (PDGFRα from R&D ref AF-1062, PDGFRβ clone APB5 from, GAPDH 14C10 from Cell Signaling) followed by HRP-linked secondary antibodies (anti-goat Jackson Lab ref 305-035-003, anti-rat ref NA935 from Cytiva, anti-rabbit from Abcam ref AB6721). Western blot imaging was performed using Western Clarity ECL (ref 170-0560; BioRad) and a LAS 4000 camera (GE-Healthcare). Quantification was performed using ImageJ (NIH) analysis.

**Statistical analysis**

All measures were performed by an experimenter blinded to animal status or cell treatment. The statistical analysis of the data was performed using XLStat 2013 (Addinsoft, New York, USA) or GraphPad Prism 6 (GraphPad software). Kruskall-Wallis followed by Dunn post-hoc tests or, when appropriate, 2-way ANOVA followed by Tuckey post-hoc tests were used for multiple comparisons and Mann-Whitney or Wilcoxon signed-rank tests for single comparisons. Sample size is indicated in the figures and in the figure legends and was calculated based on our previous experience. P values less than 0.05 were considered to be statistically significant. Graphs display all experimental values with bars representing the means and whiskers representing SD.

**Study approval**

Animal experiments for the project were approved by our institutional review board (APAFIS#3566bis-2016022617318129 v8).

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Studies with patients complied with the Declaration of Helsinki and are part of the French Network on Pulmonary Hypertension, a program approved by our institutional Ethics Committee (2018-A01252-53). Written informed consent was received from participants prior to inclusion in the study.

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**Table S3. Patients' characteristics.**

| | Control patients | | | | | iPAH patients | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Case** | Control 1 | Control 2 | Control 3 | Control 4 | Control 5 | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 |
| **Age (y)** | 45 | 65 | 40 | 60 | 62 | 21 | 16 | 14 | 51 | 40 |
| **Sex** | M | F | F | M | F | M | M | F | F | F |
| **Pathology** | Invasive adeno-carcinoma | Invasive adeno-carcinoma | Invasive adeno-carcinoma | Carcinoïd | Invasive adeno-carcinoma | iPAH | iPAH | iPAH | iPAH | iPAH |
| **mPAP (mmHg)** | x | x | x | x | x | 86 | 110 | 75 | 64 | 49 |
| **PVR (Wood units)** | x | x | x | x | x | 17 | 12,89 | 10 | 9 | 7,5 |
| **capP (mmHg)** | x | x | x | x | x | 10 | 8 | 12 | 4 | 10 |
| **CI ($L.min^{-1}.m^{-2}$)** | x | x | x | x | x | 2.05 | 3.9 | 2.6 | 2.6 | 3,2 |
| **6MWD (m)** | x | x | x | x | x | 360 | 535 | 445 | 493 | 347 |
| **NYHA** | x | x | x | x | x | IV | II | II | III | III |

Five control patients and five iPAH patients were included. mPAP, mean pulmonary arterial pressure. PVR, pulmonary vascular resistance. CapP, capillary pressure. CI, cardiac index. 6MWD, 6 minutes walking distance test. NYHA, New York Heart Association Functional Classification.

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Figure S1

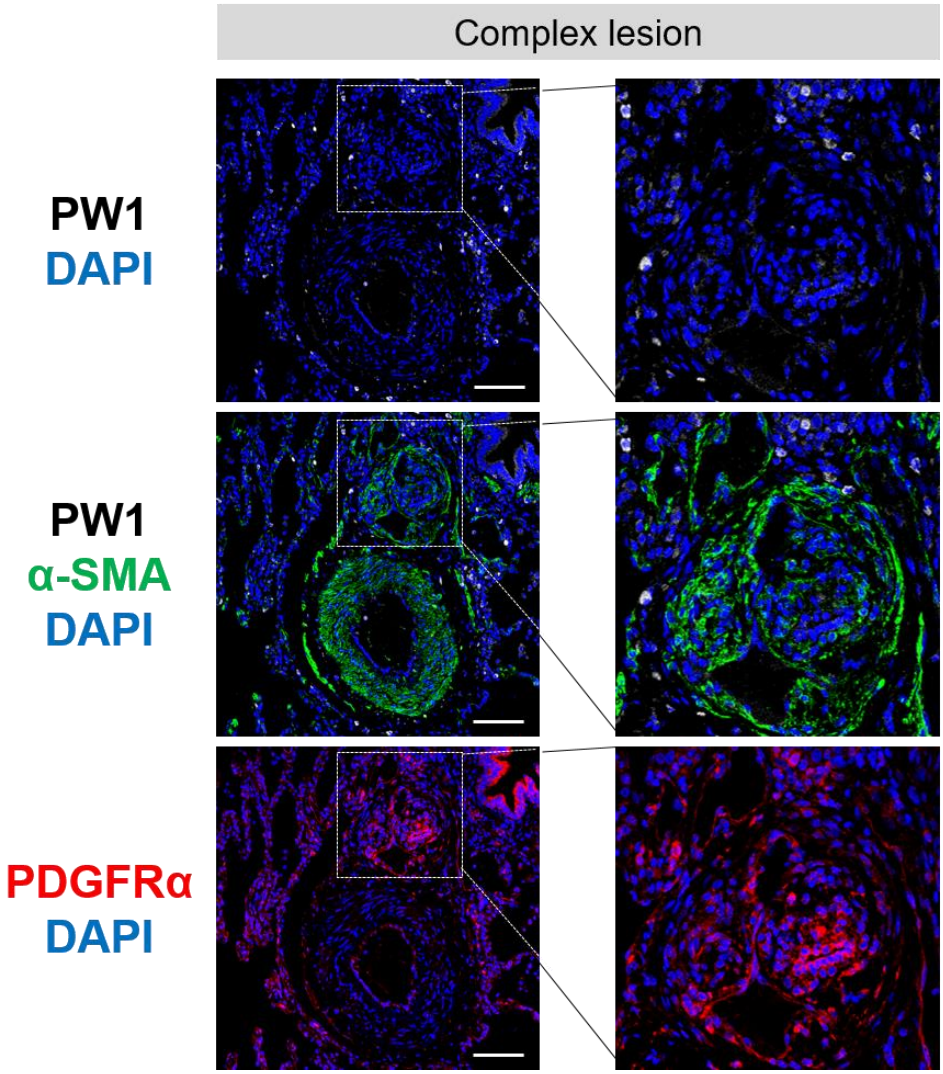



**Figure S1. PW1⁺/PDGFRα⁺ cells are not present within complex vascular lesions in iPAH patients.** Lung sections from or iPAH patients (iPAH) were labelled for PW1 (white), PDGFRα (red) and α-SMA (green). Representative confocal images of a complex vascular lesion in iPAH patient lung. Scale bar = 100µm.

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Figure S2

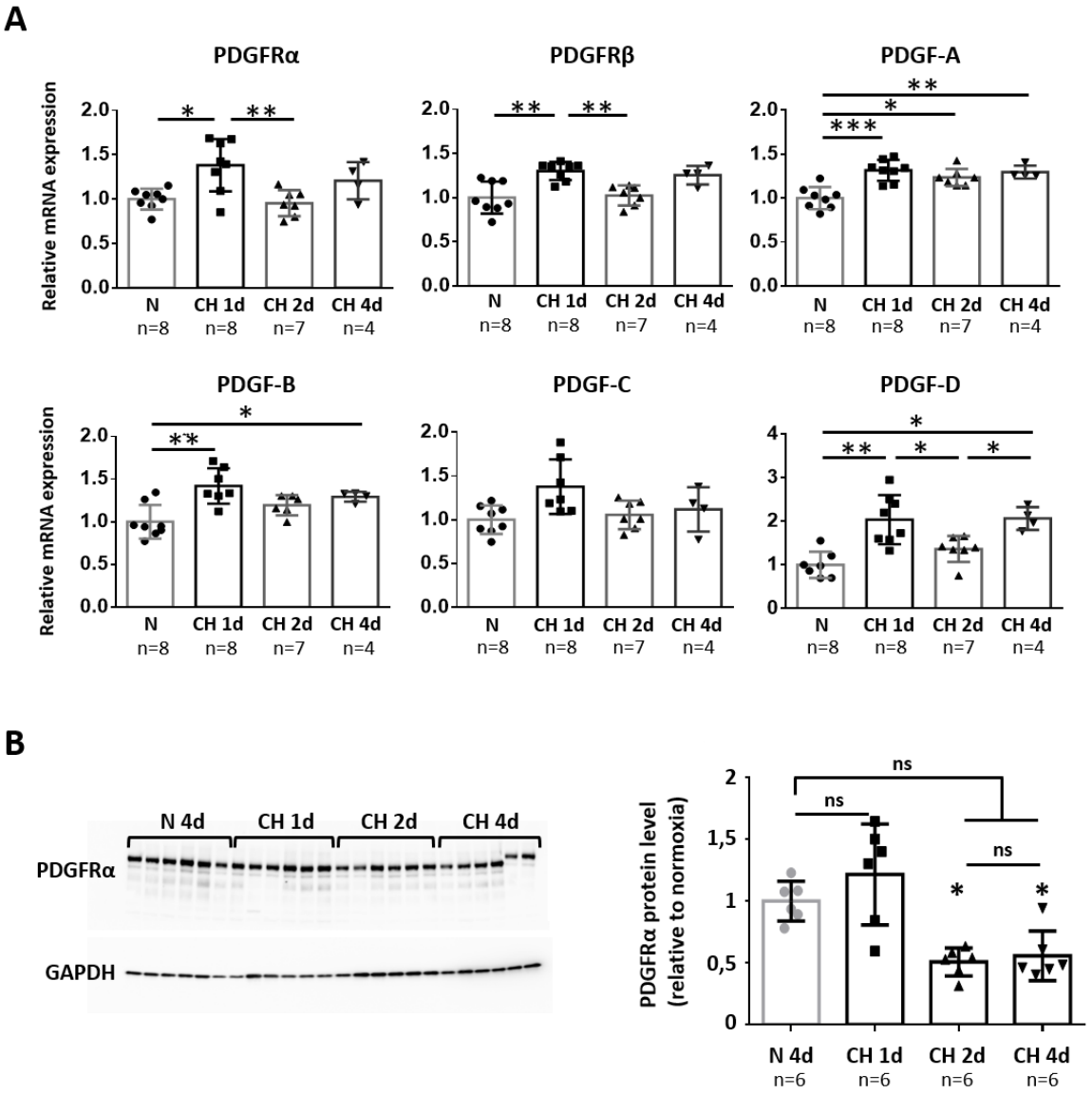



**Figure S2. Expression of PDGF ligands and receptors is increased within the first day of chronic hypoxia.** A, mRNAs for PDGFRα and β and PDGF-A, -B,- C, and -D were measured by real-time PCR on lung total RNA preparations from mice under normoxia (N) or after 1, 2, or 4 days of CH (CH 1d, CH 2d, and CH 4d respectively) (n=4-8). B, Western blot analysis of PDGFRα in lung extracts from control mice (N 4d) and from animals exposed to 1, 2, or 4 days of chronic hypoxia (CH 1-2-4d) (n=6/group). Quantification of PDGFRα normalized to GAPDH is shown in the bar graphs. Bars represent means and whiskers represent SD. *p<0.05, **p<0.01, ns=not significant (Kruskal-Wallis and Dunn).

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Figure S3

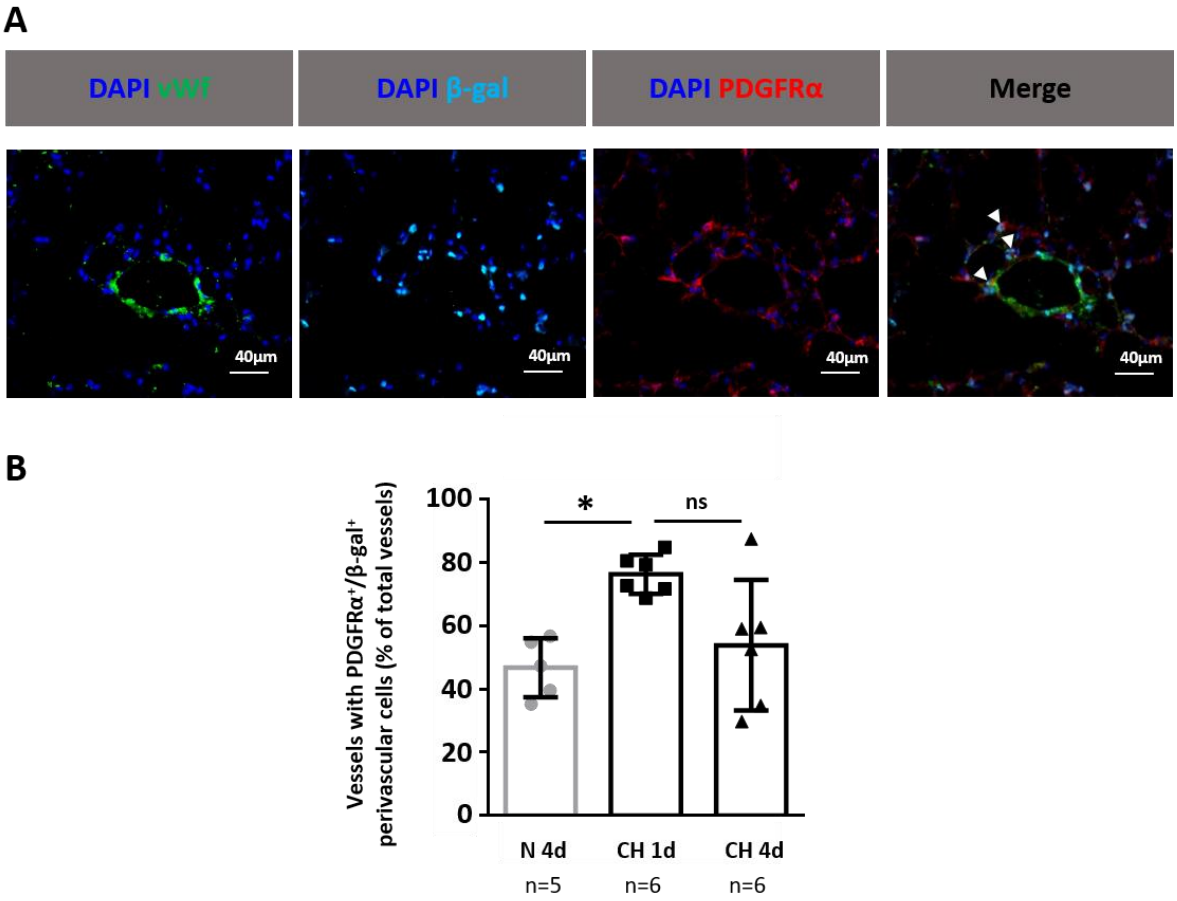



**Figure S3. Pulmonary β-gal+/PDGFRα+ perivascular cells are increased within the first day of chronic hypoxia.** PW1$^{nLacz}$ mice were maintained under normoxia (N 4d) or chronic hypoxia for 1 or 4 days (CH 1d or 4d). **A**, Representative immunofluorescence image of pulmonary vessels labeled for von Willebrand factor (vWF, green), β-gal (cyan), PDGFRα (red), and DAPI (nuclei, dark blue). Perivascular β-gal$^+$/PDGFRα$^+$ cells are marked by arrowheads. **B,** Quantification of vessels surrounded by one or more β-gal$^+$/PDGFRα$^+$ perivascular cells under normoxia (N 4d) and after 1 or 4 days of chronic hypoxia (CH 1d or 4d) (n=5-6 mice/group). Bars represent means and whiskers represent SD. *p<0.05 vs N 4d, ns=not significant (Kruskal-Wallis and Dunn).

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Figure S4

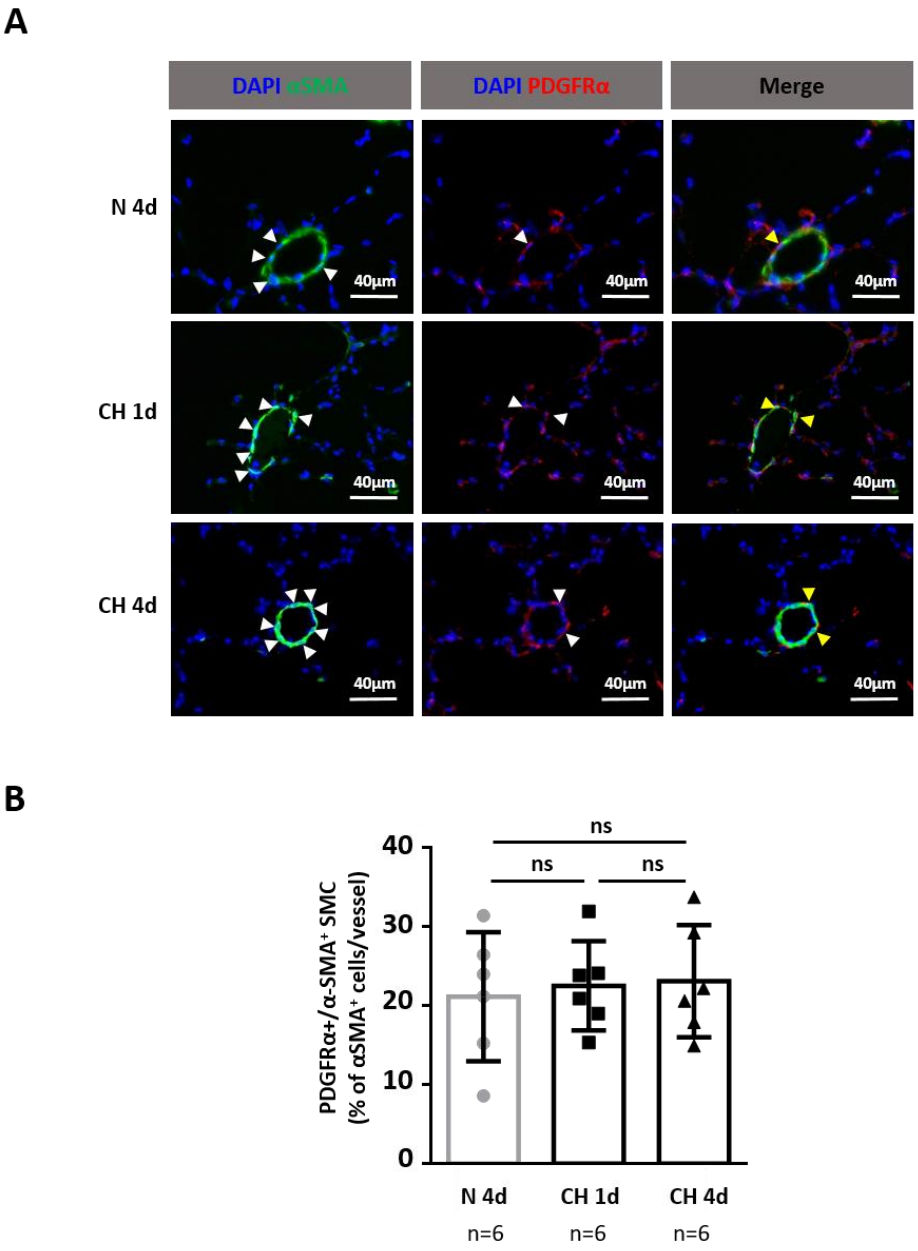



**Figure S4. Chronic hypoxia does not increase the number of PDGFRα$^+$/α-SMA$^+$ SMC in pulmonary muscularized vessels.** PW1$^{nLacz}$ mice were maintained under normoxia (N 4d) or chronic hypoxia for 1 or 4 days (CH 1d or 4d). **A,** Representative image of pulmonary muscularized vessel labeled for αSMA (green) and PDGFRα (red) (double-positives are marked by yellow arrowheads). **B,** Quantification of lung PDGFRα$^+$/α-SMA$^+$ SMC in muscularized vessels (n=6 mice/group). Bars represent means and whiskers represent SD. ns=not significant (Kruskal-Wallis and Dunn).

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Figure S5

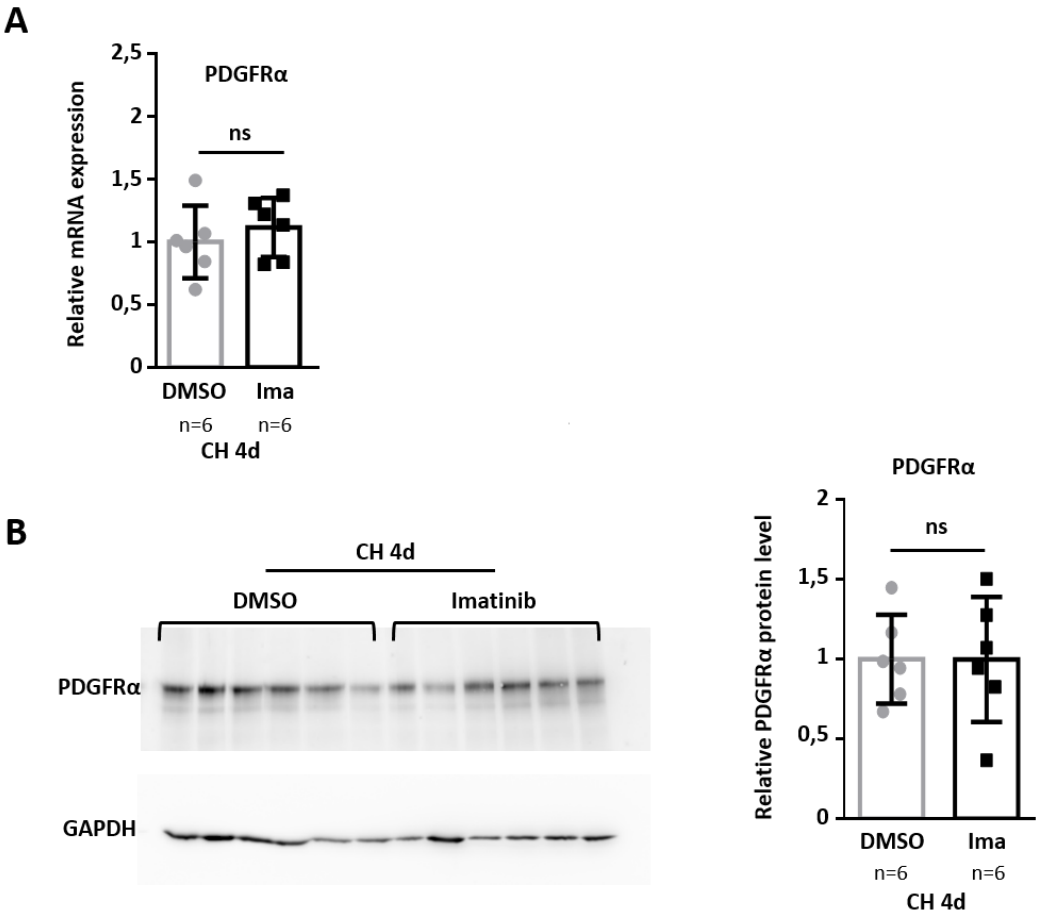



**Figure S5. Imatinib treatment does not modify PDGFRα mRNA and protein levels during chronic hypoxia.** Mice were maintained under chronic hypoxia for 4 days (CH 4d) and treated daily with DMSO or imatinib (Ima) **A,** PDGFRα mRNA was measured by real-time PCR on lung total RNA preparations from mice exposed to four days of chronic hypoxia (CH 4d) and treated with DMSO (vehicle) or imatinib (n=6 mice/group). **B,** Western blot analysis of PDGFRα in lungs from vehicle-treated mice (DMSO) or Imatinib-treated (Ima) (n=6 mice/group). Quantification of PDGFRα normalized to GAPDH is shown in the bar graphs. Bars represent means and whiskers represent SD. ns=not significant (Kruskal-Wallis and Dunn).

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Figure S6

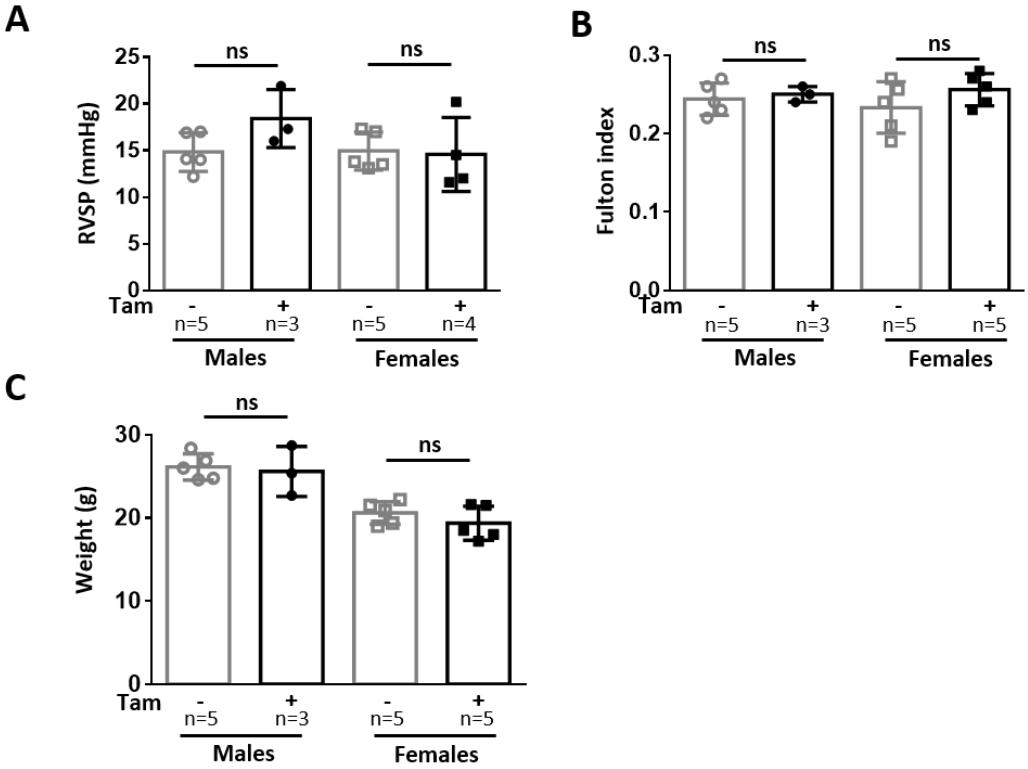



**Figure S6: Right systolic ventricular pressure (RSVP, A), Fulton index (B), and total heart weight (C), were not modified after two weeks of constitutive PDGFRα activation.** Constitutive PDGFRα activation was induced by Tamoxifen administration in PW1nLacz$^{+}$/PDGFRα$^{+/(S)K}$/Rosa-CRE$^{+}$ mice. Induced (Tam +) and non-induced (Tam -) mice were analyzed two weeks after the final Tamoxifen administration, n=3-5/group. Bars represent means and whiskers represent SD. ns, not significant, 2-tailed Mann-Whitney.

Figure S7

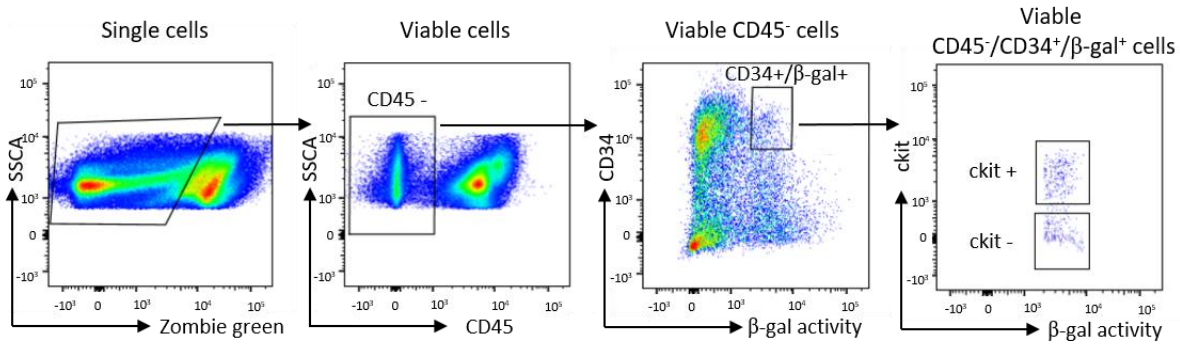



**Figure S7: Mouse pulmonary PW1$^{+}$ progenitor cells sorting strategy.** Representative FACS profile of total mouse lung cells indicating the gating strategy to sort the PDGFRα$^{+}$/PW1$^{+}$ populations based on Zombie green (viability marker), DDAO-G (β-galactosidase activity), and CD45, CD34, and c-kit cell surface expression. Viable CD45$^{-}$/CD34$^{+}$/c-kit$^{+}$ and c-kit$^{-}$ cells are PDGFRα$^{+}$ [3] and were sorted for cell culture.

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Figure S8

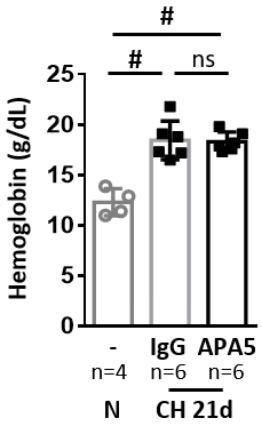



**Figure S8. Chronic hypoxia for 21 days increases blood hemoglobin concentration.** Blood hemoglobin concentration (g/dL) was measured in untreated normoxic (N, n=4) or in IgG- and APA5-treated PW1$^{nLacz}$ mice after 21 days of CH (CH 21d, n=6) . Bars represent means and whiskers represent SD. # p<0.05 vs normoxic, ns = not significant, Kruskal-Wallis and Dunn.

Figure S9

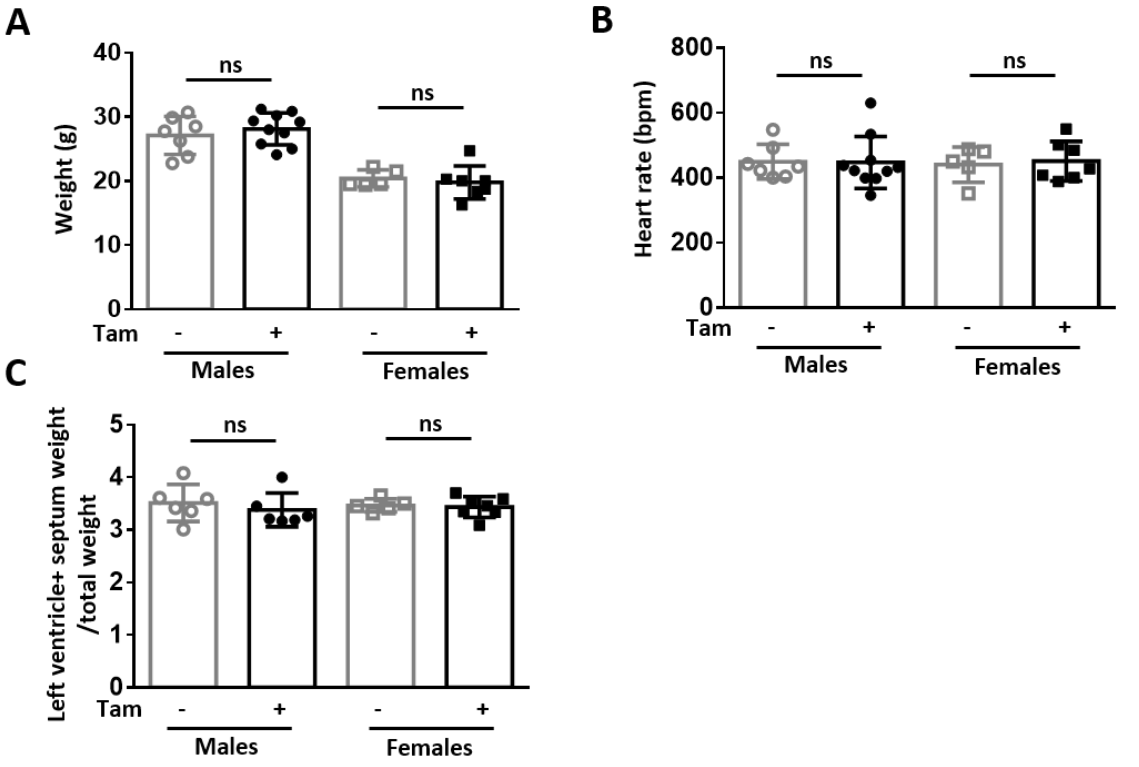



**Figure S9: Total heart weight (A), heart rate (B), and left ventricle+septum weight (C) were not modified five weeks after constitutive PDGFRα activation.** Constitutive PDGFRα activation was induced by Tamoxifen administration in PW1$^{nLacz/+}$/PDGFRα$^{+/(S)K}$/Rosa-CRE$^{+}$ mice. Induced (Tam +) and non-induced (Tam -) mice were analyzed 5-6 weeks after the final Tamoxifen administration, n=6-7/group. Bars represent means and whiskers represent SD. ns, not significant, 2-tailed Mann-Whitney.

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Figure S10

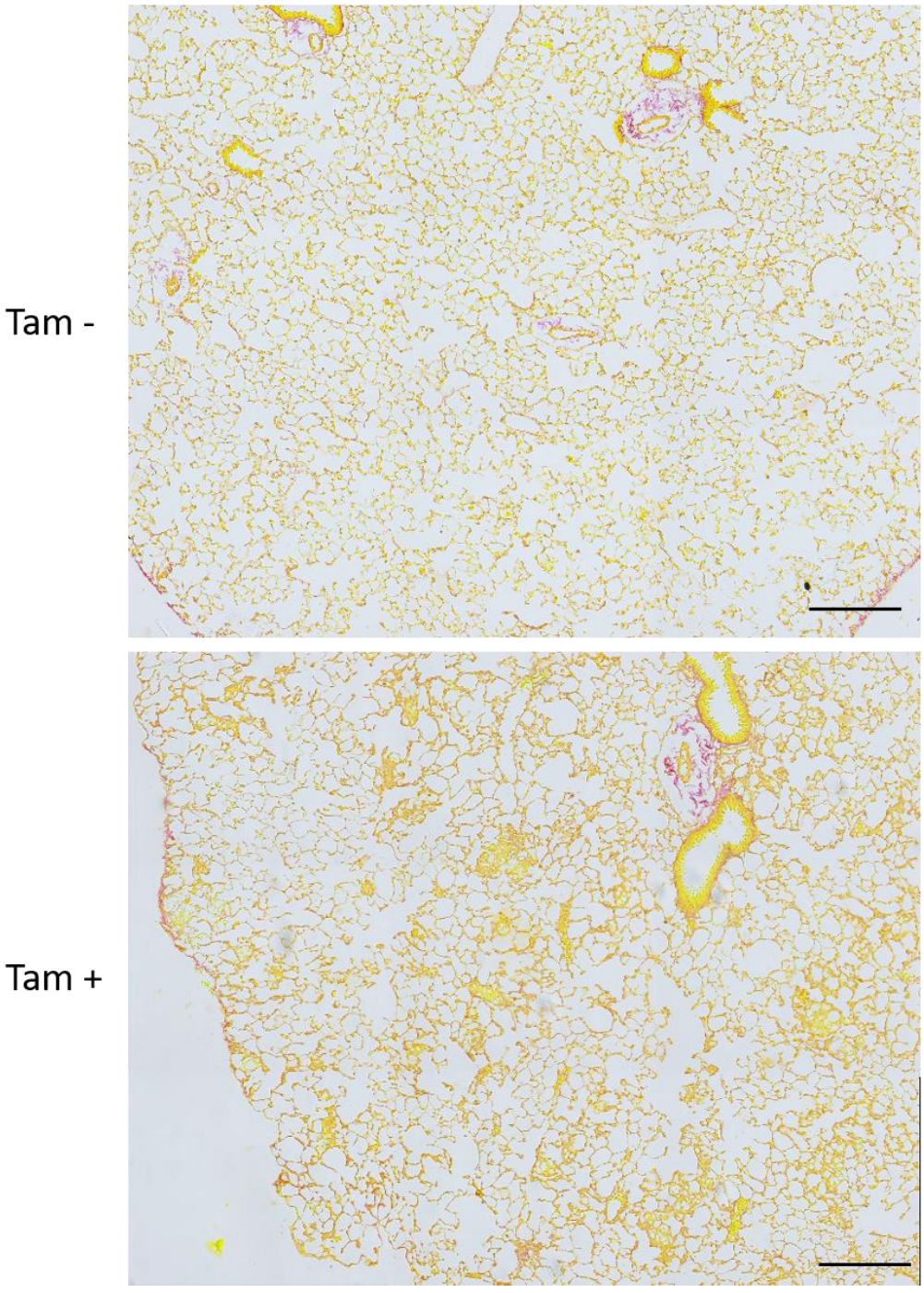



**Figure S10. Lung fibrosis is not induced by PDGFRα.** Representative images of picrosirius red staining for collagen (red) in pulmonary parenchyma of non-induced (Tam -) or tamoxifen-induced (Tam +) PW1$^{nLacz/+}$/PDGFRα$^{+/(S)K}$/Rosa-CRE$^{+}$ mice five weeks after Tamoxifen induction. Scale bar 200 µm.

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Figure S11

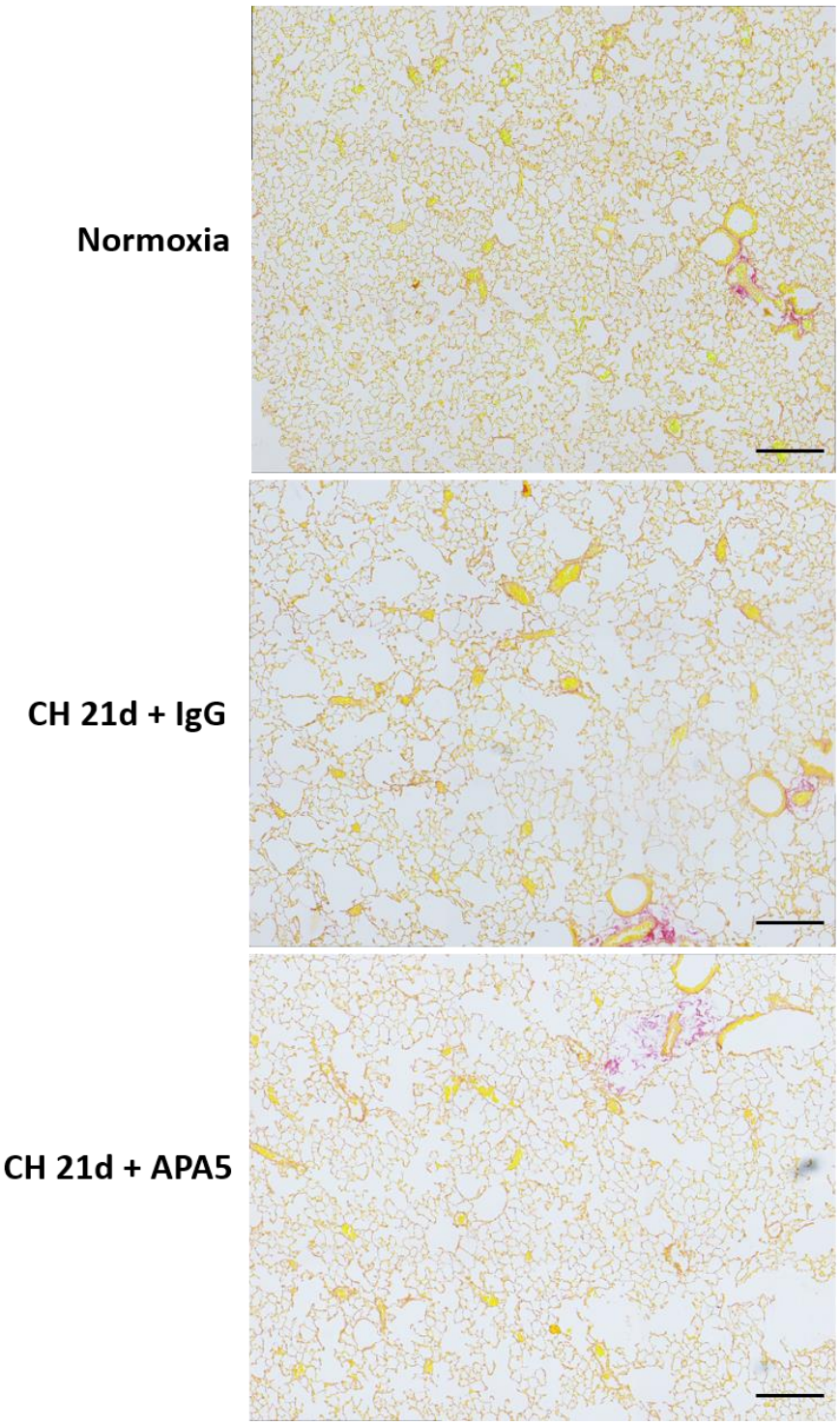



**Figure S11. Lung fibrosis is not reduced by PDGFRα inhibition during CH.** Representative images of picrosirius red staining for collagen (red) in pulmonary parenchyma of untreated mice under normoxia or IgG- and APA5-treated mice after 21 days of CH. Scale bar 200 µm.

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