

Resident PW1⁺ Progenitor Cells Participate in Vascular Remodeling During Pulmonary Arterial Hypertension

France Dierick, Tiphaine Héry, Bénédicte Hoareau-Coudert, Nathalie Mougnot, Virginie Monceau, Caroline Claude, Mihaela Crisan, Vanessa Besson, Peter Dorfmueller, Gilles Marodon, Elie Fadel, Marc Humbert, Elisa Yaniz-Galende, Jean-Sébastien Hulot, Giovanna Marazzi, David Sassoon, Florent Soubrier, Sophie Nadaud

Rationale: Pulmonary arterial hypertension is characterized by vascular remodeling and neomuscularization.

PW1⁺ progenitor cells can differentiate into smooth muscle cells (SMCs) *in vitro*.

Objective: To determine the role of pulmonary PW1⁺ progenitor cells in vascular remodeling characteristic of pulmonary arterial hypertension.

Methods and Results: We investigated their contribution during chronic hypoxia-induced vascular remodeling in *Pw1^{lacZ/+}* mouse expressing β -galactosidase in PW1⁺ cells and in differentiated cells derived from PW1⁺ cells. PW1⁺ progenitor cells are present in the perivascular zone in rodent and human control lungs. Using progenitor markers, 3 distinct myogenic PW1⁺ cell populations were isolated from the mouse lung of which 2 were significantly increased after 4 days of chronic hypoxia. The number of proliferating pulmonary PW1⁺ cells and the proportion of β -gal⁺ vascular SMC were increased, indicating a recruitment of PW1⁺ cells and their differentiation into vascular SMC during early chronic hypoxia-induced neomuscularization. CXCR4 inhibition using AMD3100 prevented PW1⁺ cells differentiation into SMC but did not inhibit their proliferation. Bone marrow transplantation experiments showed that the newly formed β -gal⁺ SMC were not derived from circulating bone marrow-derived PW1⁺ progenitor cells, confirming a resident origin of the recruited PW1⁺ cells. The number of pulmonary PW1⁺ cells was also increased in rats after monocrotaline injection. In lung from pulmonary arterial hypertension patients, PW1-expressing cells were observed in large numbers in remodeled vascular structures.

Conclusions: These results demonstrate the existence of a novel population of resident SMC progenitor cells expressing PW1 and participating in pulmonary hypertension-associated vascular remodeling. (*Circ Res.* 2016;118:822-833. DOI: 10.1161/CIRCRESAHA.115.307035.)

Key Words: adult stem cells ■ hypertension, pulmonary ■ hypoxia ■ muscle, smooth, vascular ■ vascular remodeling

Pulmonary arterial hypertension (PAH), a rare and severe disease with no curative options, is characterized by a sustained increase in pulmonary vascular resistance leading to right heart failure and death.¹ Histologically, PAH is associated with neomuscularization of small pulmonary vessels, medial hypertrophy, neointima formation, endothelial proliferation, excessive extracellular matrix deposition, and recruitment of inflammatory cells in the vascular wall.²⁻⁴

To date, the cellular origin of neomuscularization and medial hypertrophy remains unknown. It was proposed that new smooth muscle cells (SMCs) are derived from resident SMC that re-enter a proliferative state⁵ or from pericytes^{6,7} and from endothelial cells that adopt a smooth muscle fate.⁸ Recent studies suggest that SMCs originate from the proliferation and differentiation of either resident or circulating bone marrow (BM)-derived progenitor cells.⁹ Indeed,

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From the INSERM, Institute of Cardiometabolism and Nutrition, UMR_S 1166-ICAN (F.D., T.H., V.M., C.C., V.B., E.Y.-G., J.-S.H., G.M., D.S., F.S., S.N.), UMS-030 CyPS, Paris, France (B.H.-C.), PECEMV UMS28 (N.M.), INSERM, CNRS, CR7, Centre d'Immunologie et des Maladies Infectieuses (CIMI), U1135, ERL 8255 (G.M.), Sorbonne Universités, UPMC Univ Paris 06, Paris, France; Erasmus MC Stem Cell Institute, Rotterdam, The Netherlands (M.C.); Univ Paris-Sud, Université Paris Saclay, INSERM UMR-S 999, Labex LERMIT, Le Plessis-Robinson, Paris, France (P.D., E.F., M.H.); Service d'Anatomie Pathologique, Centre Chirurgical Marie Lannelongue, Le Plessis-Robinson, Paris, France (P.D.); Service de Chirurgie Thoracique et Vasculaire, Centre Chirurgical Marie Lannelongue, Le Plessis-Robinson, France (E.F.); Univ Paris-Sud, Université Paris Saclay, Le Kremlin-Bicêtre, Paris, France (M.H.); and Assistance Publique Hôpitaux de Paris, Service de Pneumologie, Centre de Référence de l'Hypertension Pulmonaire Sévère, Hôpital Bicêtre, Le Kremlin Bicêtre, France (M.H.).

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

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Nonstandard Abbreviations and Acronyms

BM	bone marrow
CH	chronic hypoxia
GFP	green fluorescent protein
PAH	pulmonary arterial hypertension
PH	pulmonary hypertension
SMC	smooth muscle cell
SDF-1	stromal-derived factor-1

various types of resident SMC progenitor cells have been identified in numerous adult organs, including the lung. They are located in the vessel or in the perivascular zone and express several stem cells markers including Sca-1, c-kit, platelet-derived growth factor alpha polypeptide (PDGFR- α), CD34 or mesenchymal stem cells markers.^{10,11} In addition, perivascular cells identified by these same markers and expressing CD146, NG2, and PDGFR- β were also recognized as SMC progenitor cells.¹²

Recently, a population of interstitial progenitor cells was identified in mouse skeletal muscle based on their expression of the PW1 protein,¹³ which are capable of giving rise to smooth and skeletal muscle as well as adipocytes in culture.^{14,15} These PW1-expressing cells were found in multiple adult tissues including skin, gut, BM, and the central nervous system.¹⁴ *Pw1* gene encodes a zinc-finger protein that has been shown to regulate cell cycle and cell stress responses caused by inflammation¹⁶ and p53.¹⁷ In addition, PW1 mediates β -catenin stability in the Wnt signaling pathway.¹⁸ PW1 has also been shown to function as a transcription factor with a DNA-binding motif regulating a large array of genes involved in metabolic homeostasis.¹⁹ Moreover, PW1 expression is required for the myogenic and migratory capacities of both mouse and human mesoangioblasts, reinforcing the relevance of PW1 to identify competent mesoangioblasts before their use in stem cell-mediated therapeutic applications.²⁰

Given the distribution of PW1 expression in a wide array of adult somatic stem cells and the role of PW1 in cell stress responses, we investigated whether PW1⁺ progenitor cells present in lung tissue are recruited to proliferate and differentiate into SMCs during vascular remodeling in chronic hypoxia (CH)-induced pulmonary hypertension (PH). To address this hypothesis, we used a PW1 reporter transgenic mouse, PW1^{nLacZ/+}, that expresses the nuclear β -galactosidase within the *Pw1* gene context.¹⁴ These mice carry the β -galactosidase coding sequence (β -gal) driven under the control of *Pw1* and because of the high level and stability of the reporter gene product,²¹ we can identify cells recently derived from reporter-expressing progenitor cells. In this study, we used this reporter mouse to examine the role of the PW1⁺ progenitor cells in vascular remodeling during CH. We identified 2 resident PW1⁺ progenitor cell populations located in the lung parenchyma and in perivascular zones of small pulmonary vessels, which show an early recruitment and a potential to differentiate into SMCs during CH. Our results strongly suggest that new SMCs derive from resident PW1⁺ progenitor cells recruited during early CH.

Methods

Detailed Methods are provided in the Online Data Supplement.

Pw1IRESnLacZ transgenic mice (*Pw1*^{nLacZ/+}) bear a nuclear operon lactose gene expressed under the control of the *Pw1* gene locus.¹⁸ Mice (littermates of 6–8 weeks) were exposed to room air (normoxia) or chronic normobaric hypoxia (10% O₂) for 4 or 28 days. Fluorescence-activated cell sorting (FACS) was used to isolate the pulmonary PW1⁺ cell populations based on their β -gal activity and a combination of progenitor markers. Lethally irradiated C57BL/6J mice were reconstituted with BM cells of age- and sex-matched GFP⁺/ β -gal⁺ (*H2B-GFP* \times *Pw1*^{nLacZ/+}) mice and exposed, after 9 months, to Normoxia (N) or CH for 4 days (CH 4 days).

Results

Mouse Pulmonary PW1⁺ Cells Are Present in Perivascular and Parenchymal Zones, Express Progenitor and Pericyte Markers, and Differentiate Into SMC

PW1 expression was found to identify adult stem and progenitor cells in multiple organs^{14,15,20} although previous studies did not report on the expression in the lung. PW1 protein expression was detected by immunofluorescence in cells located in parenchymal and perivascular spaces, near small nonmuscularized and muscularized vessels (Figure 1A and 1B). We characterized these progenitor cells from PW1 reporter mice after FACS purification based on β -galactosidase activity.¹⁴ Among the CD45⁻ cells (Online Figure I), we observed the highest levels of reporter activity in 3 populations: the PW1⁺/CD34⁺/c-kit⁺ cells, the PW1⁺/CD34⁺/c-kit⁺/PDGFR- α ⁺ cells, and the PW1⁺/CD34⁺/CD146⁺ cells (Figure 1C). These results were confirmed by direct PW1 immunostaining for each purified population, which showed a high proportion of PW1-expressing cells (74 \pm 0.6% for PW1⁺/CD34⁺/c-kit⁺ cells, 92 \pm 2.4% for PW1⁺/CD34⁺/c-kit⁺/PDGFR- α ⁺ cells, and 86 \pm 6.1% for PW1⁺/CD34⁺/CD146⁺ cells; n=4; Figure 1D). The PW1⁺/CD34⁺/CD146⁺ cells were positive for pericyte markers NG2 (84 \pm 7.3%; n=2) and PDGFR- β (86 \pm 5.3%; n=2) and negative for CD31 (data not shown; Online Figure II). Immunofluorescence studies confirmed that, in the lung, PW1 expression partially colocalized with progenitor markers CD146, c-kit, and PDGFR- α (Figure 1E).

To investigate in vitro the potentials of these progenitor cells for differentiation in SMC, freshly sorted PW1⁺ cell populations were studied for SMC markers either on isolation or after cell culture and differentiation induction. Freshly sorted PW1⁺ cells were negative for the SMC markers α -smooth muscle actin (α -SMA) and smooth muscle myosin heavy chain (SM-MHC; Figure 2A). In culture, PW1⁺/CD34⁺/c-kit⁺/PDGFR- α ⁺ cells spontaneously differentiated into α -SMA⁺ cells (71 \pm 2.3%; n=3) and into terminally differentiated α -SMA⁺/SM-MHC⁺ SMCs (26 \pm 7%; n=3). In contrast, the PW1⁺/CD34⁺/CD146⁺ and PW1⁺/CD34⁺/c-kit⁺ populations differentiated into α -SMA⁺ (66 \pm 4.2% and 90.5 \pm 0.7%, respectively) and α -SMA⁺/SM-MHC⁺ cells (4.5 \pm 6.3% and 14 \pm 4%, respectively) only on induction with transforming growth factor- β ₁ and PDGF-BB (n=3; Figure 2B). After differentiation, 50% of the cells lost β -gal expression after \approx 5 days of culture confirming a downregulation of PW1 expression in differentiated cells (data not shown). Hence, all 3 PW1⁺ cell populations showed robust SMC differentiation capacity in vitro.

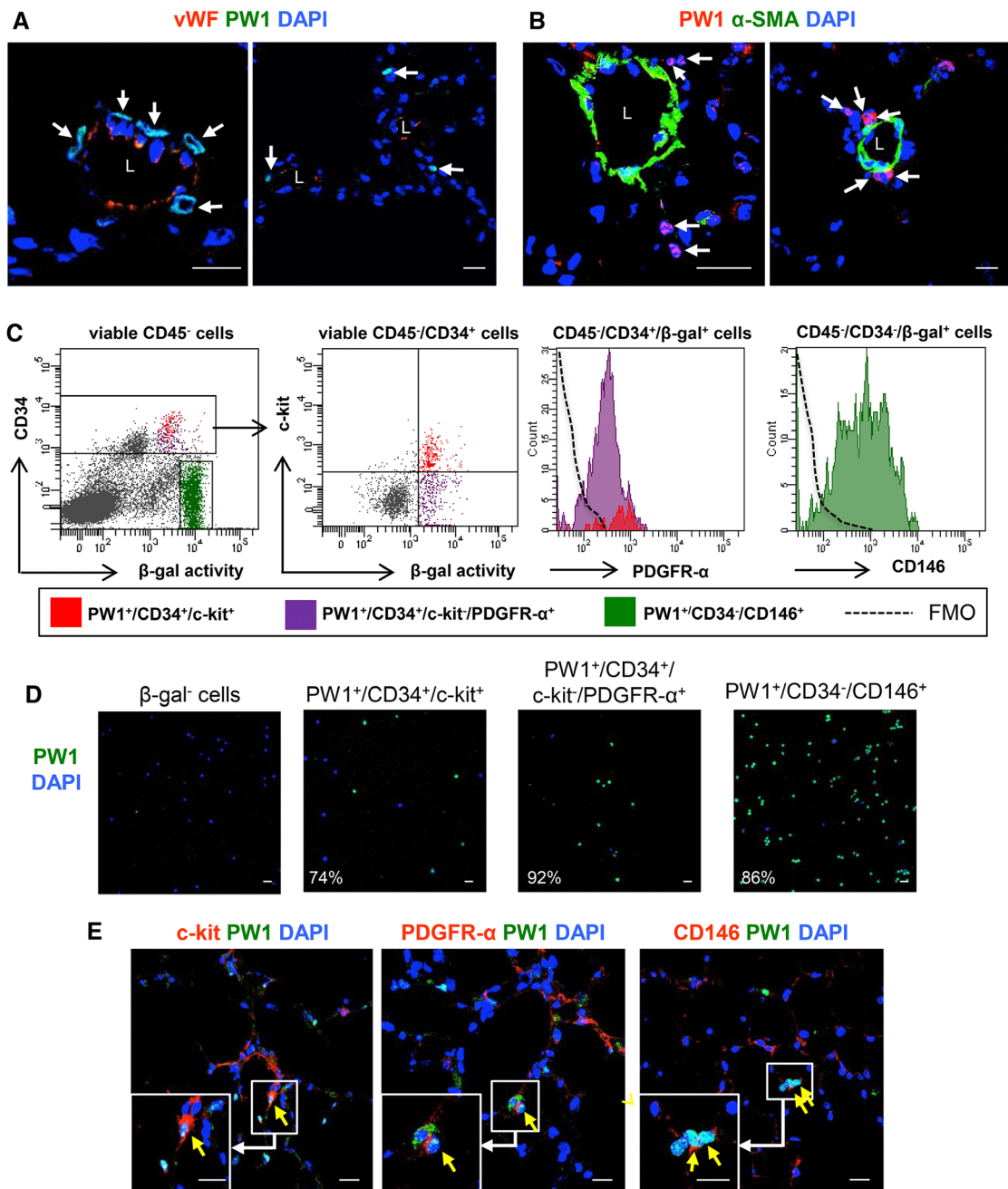


Figure 1. Mouse lung PW1⁺ cells are present in perivascular and parenchymal zones and express progenitor and pericyte markers. **A**, Immunofluorescent staining on mouse lung for PW1 (green) and von Willebrand factor (vWF; endothelial marker; red) and **(B)** for PW1 (red) and α -smooth muscle actin (SMA; smooth muscle cell marker; green) reveals PW1⁺ cells in parenchyma and perivascular zones (white arrowheads). **C**, Three PW1⁺ cell populations were sorted by fluorescence-activated cell sorting (FACS) based on cell surface markers expression profiles and β -galactosidase (β -gal) activity: PW1⁺/CD34⁺/c-kit⁺ cells, PW1⁺/CD34⁺/c-kit⁺/platelet-derived growth factor alpha polypeptide (PDGFR- α)⁺ cells and PW1⁺/CD34⁺/CD146⁺ cells. PDGFR- α and CD146 expression were also measured by FACS on these cell populations. **D**, PW1 expression (green) was confirmed in sorted cells, and the mean percentage of PW1⁺ cells is indicated. Representative images from mouse lung sorted cytopun cells (n=4). **E**, Colocalization of PW1 (green) and c-kit, PDGFR- α and CD146 (red) were confirmed on mouse lung by immunofluorescence. Yellow arrowheads indicate cells coexpressing PW1 and other progenitor markers. Confocal microscopy. Scale bars, 20 μ m. L indicates lumen of vessel.

The PW1 reporter mice were also used to follow the differentiation of PW1⁺ cells in vivo. Because of the long half-life of β -galactosidase protein,²¹ this transgenic strain allows for the detection of differentiated cells recently derived from β -gal⁺/PW1⁺ progenitor cells. Indeed, in small pulmonary vessels under normoxic conditions, we observed many SMC coexpressing β -galactosidase and α -SMA (35 \pm 2.2% of β -gal⁺/ α -SMA⁺

cells), calponin (44 \pm 3% of β -gal⁺/CNN1⁺ cells), or SM-MHC (54 \pm 7.3% β -gal⁺/SM-MHC⁺ cells), but we observed few SMC-expressing PW1 (<7% of SMC; Figures 2C and 5C; Online Figure III). Taken together, these results show that the lung contains 3 populations of PW1⁺ SMC progenitor cells and suggest that, in vivo, numerous SMCs are derived from PW1⁺ progenitor cells.

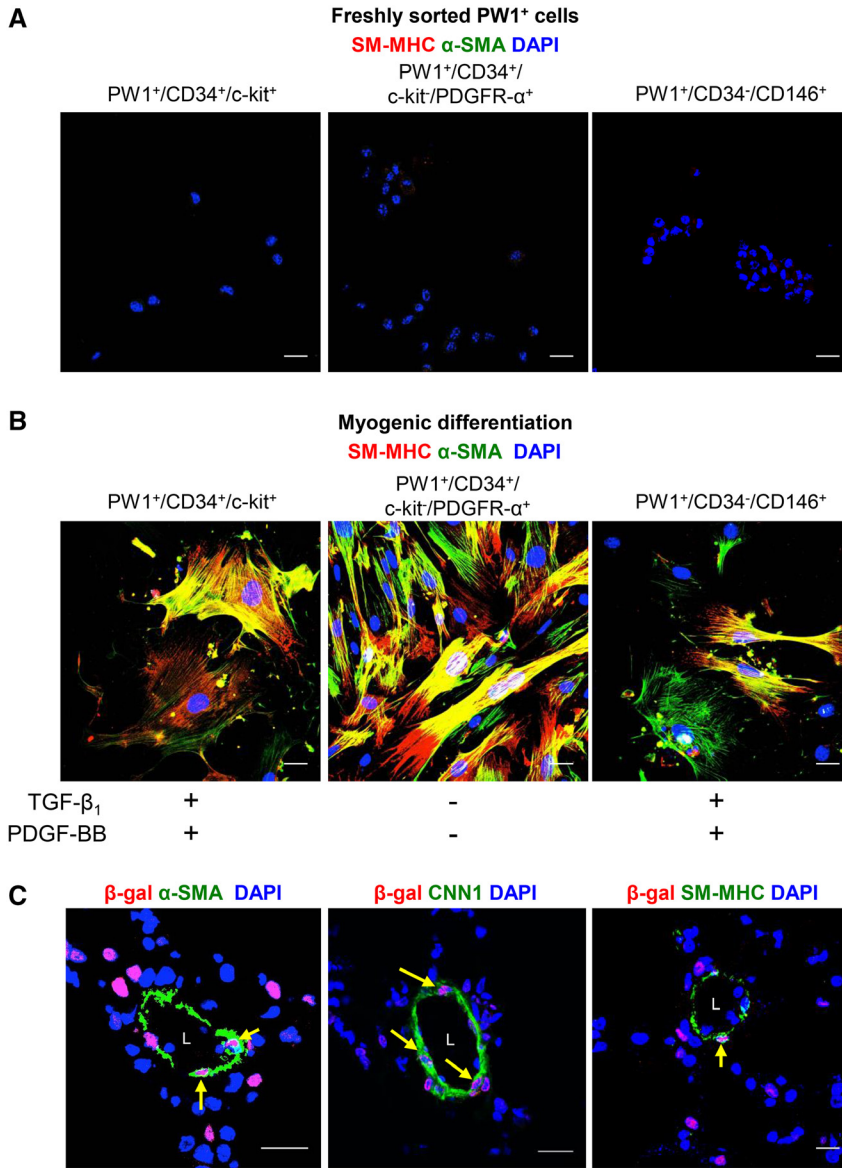


Figure 2. The mouse lung PW1⁺ cells differentiate into smooth muscle cells (SMC). **A**, fluorescence-activated cell sorting (FACS) isolated PW1⁺ populations immunostained directly after sorting for SMC markers α -smooth muscle actin (SMA; green) and smooth muscle myosin heavy chain (SM-MHC; red) show no staining. **B**, FACS isolated PW1⁺ cell populations immunostained after culture show staining for SMC markers α -SMA (green) and SM-MHC (red). PW1⁺/CD34⁺/c-kit⁺ and PW1⁺/CD34⁺/CD146⁺ cells differentiation was induced by addition of transforming growth factor (TGF)- β_1 and PDGF-BB, whereas PW1⁺/CD34⁺/c-kit⁺/PDGFR- α ⁺ cells were left without induction. Representative images of multiple experiments (n=3). Confocal microscopy. **C**, Mouse lungs immunostained for β -galactosidase (β -gal; red) and for SMC markers α -SMA, calponin (CNN1), or SM-MHC (green) show that multiple SMCs express β -gal in the media of pulmonary vessels (yellow arrowheads). Epifluorescence acquisition followed by deconvolution and z-axis projection of stacked images. Scale bars, 20 μ m. L indicates Lumen of vessel.

We also tested the capacity of lung PW1⁺ progenitor cells to differentiate into other vascular cells (endothelial cells) or other muscle cells (skeletal muscle cells). After in vitro differentiation induction, cells were labeled with anti-von Willebrand factor (endothelial marker) or antiskeletal myosin heavy chain (MF20, skeletal muscle marker) antibodies. For all 3 PW1⁺ cell populations, we did not observe any differentiation in neither endothelial cell nor skeletal muscle cells (Online Figures IVA and V). Moreover, whereas most of the vascular β -gal⁺ cells are SMC cells in *P_w^{Intac2}* mice lungs, we found only rarely vascular endothelial cells expressing β -gal (Online Figure IVB). These in vivo and in vitro data indicate that pulmonary PW1⁺ progenitor cells do not differentiate into endothelial or skeletal muscle cells.

Pulmonary PW1⁺ Progenitor Cells Are Recruited in 2 Rodent PH Experimental Models

We next aimed to determine whether PW1⁺ progenitor cells are recruited during CH and participate in pulmonary vascular remodeling by differentiating into SMC. Pulmonary

PW1⁺ cell populations were analyzed by FACS after 4 days of N or CH. We observed a significant increase in the number of PW1⁺/CD34⁺/c-kit⁺ and PW1⁺/CD34⁺/c-kit⁺/PDGFR- α ⁺ cells after 4 days of CH (0.4 \pm 0.3% in N versus 2.5 \pm 1.3% in CH; P <0.001 and 0.4 \pm 0.2% in N versus 0.89 \pm 0.27% in CH; P <0.001 respectively; Figure 3A). These results were confirmed by immunofluorescence experiments showing that the number of pulmonary PW1⁺ cells was also significantly increased after 4 days of CH (3.5 fold; P <0.05; Figure 3B). Double-labeled immunofluorescent staining of lung tissues from bromodeoxyuridine (BrdU)-injected mice revealed a significant increase in the number of proliferating BrdU⁺ PW1-expressing cells in the lung parenchyma (0.96 \pm 0.4% versus 3.16 \pm 0.66%; P <0.01; Figure 3C). We studied the recruitment of PW1⁺ progenitor cells in the monocrotaline-injected rat, a different, more severe, and inflammatory rodent PH model (Online Figure VI). The number of lung parenchymal PW1⁺ cells (Figure 4A) was found to be significantly increased in monocrotaline-treated rats when compared with control animals (11.7 \pm 3.1% versus 20 \pm 2%; P ≤0.05),

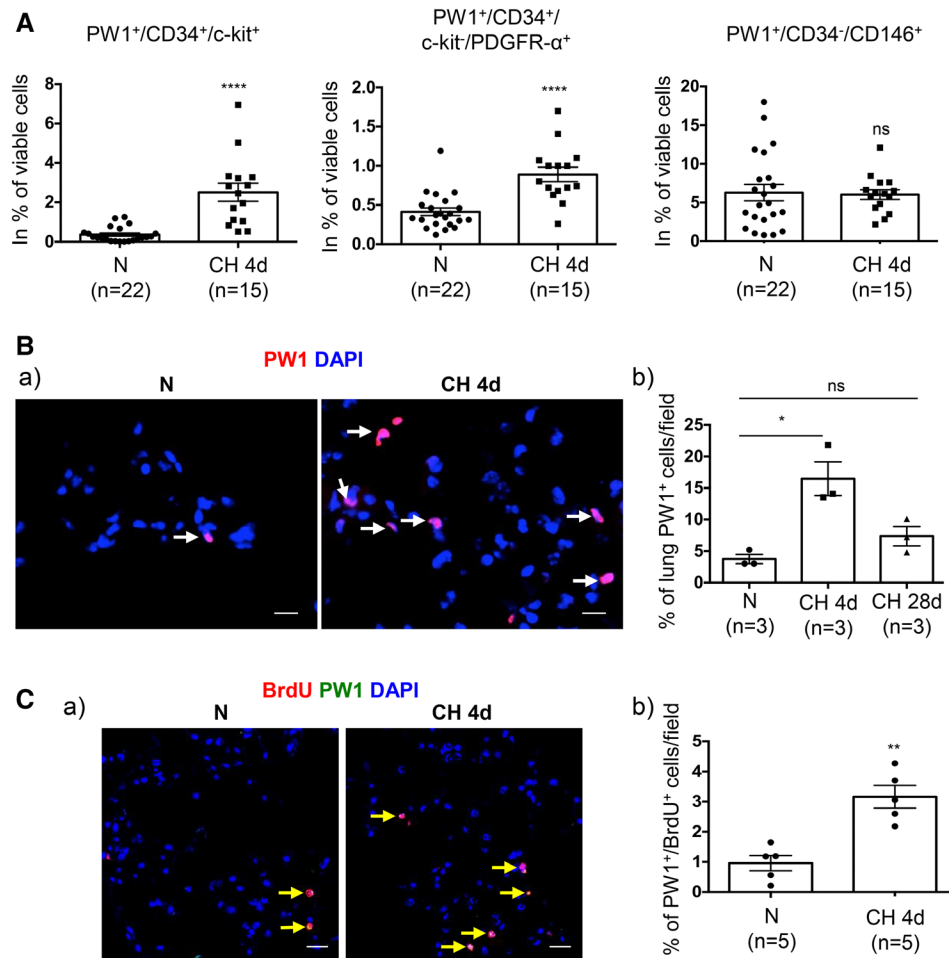


Figure 3. PW1⁺ progenitor cells are recruited and proliferate during early chronic hypoxia (CH) in mouse. **A**, Quantitative analysis of lung PW1⁺ cell populations from mice in normoxia (N; n=22) vs 4 d of CH (CH 4 d; n=15). The mean percentage of total viable cells for each PW1⁺ cell population was measured by fluorescence-activated cell sorting in normoxic and CH lungs. Values are mean \pm SEM, **** P <0.0001 vs N (2-tailed Mann–Whitney). **B**, Quantification of PW1⁺ cells in lung parenchyma from normoxic or CH mice. (a) Representative images of lung parenchyma from normoxic and CH mice stained for PW1 (PW1 in red, positive cells are marked by white arrowheads). (b) The percentage of lung PW1⁺ cells per field from mice submitted to normoxia and CH for 4 or 28 d was measured by immunofluorescence (n=3 for each condition). Values are mean \pm SEM, * P <0.05 vs N (Kruskal–Wallis, Dunn). **C**, Quantification of proliferating PW1⁺ cells in lung parenchyma from N or CH 4 d mice. (a) Representative images of lung parenchyma from normoxic and hypoxic mice stained for PW1 (green) and bromodeoxyuridine (BrdU) (red; double positive are marked by yellow arrowheads). (b) The percentage of lung BrdU⁺ proliferating PW1⁺ cells was determined by immunofluorescence (n=5 for each condition). Values are mean \pm SEM, ** P <0.01 vs N (2-tailed Mann–Whitney). Confocal microscopy. Scale bars, 20 μ m. ns indicates nonsignificant.

indicating that PW1⁺ cells are mobilized during PH independently of the experimental model used (Figure 4B).

CH-Induced Neomuscularization Is Associated With an Increased Number of PW1⁺ Cell-Derived SMC

We next studied the number of SMC differentiated from PW1⁺ cells during vascular remodeling in PW1 reporter mice after 4 or 28 days of CH. After 4 days of CH, we observed neomuscularization without any increase in vascular wall thickness, whereas after 28 days, both features were observed (Figure 5A and 5B). Right ventricular systolic pressure was already increased after 4 days of CH, whereas the cardiac right hypertrophy, estimated by the Fulton index, was not modified at 4 days but significantly increased after 28 days of CH (Online Figure VIIA–VIIC).

The number of β -gal⁺ SMC in small pulmonary vessels (<100 μ m), as determined by coimmunofluorescence, was significantly increased after 4 days of CH when compared

with normoxia, indicating an increased percentage of SMC recently derived from PW1⁺ cells at an early stage during the neomuscularization phase of CH-induced vascular remodeling (Figure 5C). This early increase was confirmed by using a more-specific SMC marker, calponin (CNN1; Online Figure III). These observations suggest that newly formed SMC during vessel neomuscularization are derived from PW1⁺ progenitor cells. This is supported by the observation that the number of PW1⁺ cells (Figure 3B) and of PW1⁺-derived β -gal⁺ SMC (Figure 5C) both return to basal levels at 28 days of CH, indicating that PW1⁺ cells and β -gal⁺ SMC follow similar kinetics. These observations do not exclude the possibility that SMC or myofibroblasts dedifferentiate into PW1-expressing cells during CH to proliferate and further differentiate into SMC. We therefore analyzed mouse lungs for coexpression of PW1 and α -SMA at 0, 1, 2, 3, and 4 days of CH. We found few cells coexpressing both α -SMA and PW1 at all time points

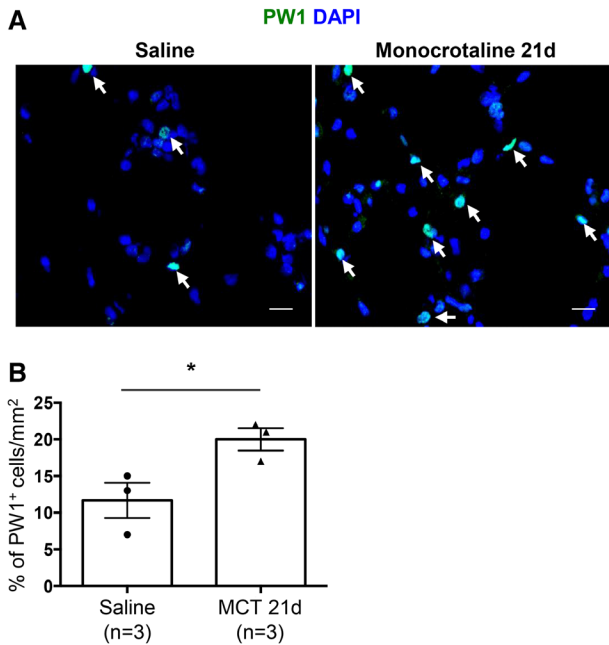


Figure 4. Lung PW1⁺ progenitor cells are also recruited in another pulmonary arterial hypertension model: the monocrotaline (MCT)-injected rat. **A**, Representative images of control and MCT-injected rat lungs stained with anti-PW1 antibody (green, white arrowheads). **B**, Quantification by immunofluorescence of the percentage of PW1⁺ cells from control (saline) and MCT-injected rat lungs (MCT, 21 d; n=3). Epifluorescence microscopy. Scale bars, 20 μ m. Values are mean \pm SEM, * P <0.05 vs saline (1-tailed Mann–Whitney).

(from 2.5% to 7% of all α -SMA⁺ cells). Overall, few vessels (10% to 20% of all vessels) contained PW1⁺/ α -SMA⁺ SMCs. Moreover, we did not observe any increase in the number of PW1⁺/ α -SMA⁺ cells during the first 4 days of CH (Figure 5D). These results are not in favor of SMC/myofibroblast dedifferentiation into PW1-expressing cells during CH but strongly suggest that newly formed SMC are differentiated from PW1⁺ progenitor cells recruited during early CH and lose their β -gal expression after 28 days of CH.

Pulmonary PW1⁺ Cells Differentiation Into Vascular SMCs During CH Is Dependent on the CXCR4 Pathway

The stromal-derived factor-1 (SDF-1)/CXCR4 pathway is an important regulator of stem and progenitor cells mobilization and homing. We hypothesized that the SDF-1/CXCR4 pathway plays an important role in the recruitment of the pulmonary PW1⁺ cells during CH. Therefore, we studied the effect of AMD3100, a CXCR4 antagonist, on PW1⁺ cells proliferation and differentiation and on vascular remodeling after 4 days of CH. AMD3100 treatment did not modify hematocrit values (data not shown). On the basis of flow cytometry analysis, we showed that the PW1⁺ progenitor cells are mainly positive for CXCR4 (Figure 6A). FACS analyses also showed that the number of cells of each PW1⁺ cell population was not modified by AMD3100 treatment neither in normoxia nor after CH (Figure 6B). We observed, however, by coimmunofluorescence that AMD3100 treatment completely prevented the increase in PW1⁺-derived β -gal⁺ vascular SMC obtained after CH but had no effect in normoxia (Figure 6C). These

results indicate that the CXCR4 pathway regulates the migration and differentiation of PW1⁺ progenitor cells into vascular SMC but does not regulate PW1⁺ progenitor cells' proliferation. In addition, AMD3100 treatment partially but significantly inhibited pulmonary vessels neomuscularization during CH without effect during normoxia (Figure 6D). These results indicate that CXCR4 pathway participates in the control of CH-induced neomuscularization.

Resident Pulmonary PW1⁺ Cells Are Recruited During Early CH

Previously, we have shown that the BM contains PW1⁺ cells that correspond to the BM progenitor populations.¹⁴ It has been suggested that the SMC cells that form in the lung in response to CH derive from circulating cells of BM origin.⁹ Therefore, we performed a total BM transplantation from PW1 reporter mice crossed with *H2B-GFP* mice (expressing ubiquitous nuclear GFP fused to histone2B) onto lethally irradiated wild-type mice. After 8 months, chimerism was evaluated at \approx 82% (82.2 \pm 4.2%; n=10) in the grafted mice blood by FACS analysis of GFP⁺/CD45⁺ blood mononuclear cells (Online Figure VIII). The reconstitution of the PW1⁺/ β -gal⁺ populations was verified by FACS¹⁴ by comparing the β -gal⁺ cells in control PW1 reporter mouse and in mouse transplanted with β -gal⁺ BM cells. We observed a similar amount and distribution of β -gal-expressing cells in the BM of control PW1 reporter mouse and in the transplanted mouse indicating that the BM PW1⁺ cell populations had been efficiently reconstituted in the transplanted mice (Figure 7A). Engrafted mice were then submitted to normoxia or CH for 4 days. CH induced pulmonary vessels neomuscularization as attested by a decreased number of nonmuscularized vessels quantified by immunofluorescence (Figure 7B). The percentage of GFP⁺/ α -SMA⁺ cells per vessel in small pulmonary vessels (<100 μ m) was not modified by CH, and we did not detect β -gal⁺ cells in the lungs of BM-engrafted normoxic or CH mice. These results indicate that circulating GFP⁺ BM-derived cells do not contribute to the early pulmonary vascular remodeling. We conclude that newly formed SMC during early CH are not derived from cells originating from the BM (Figure 7C).

PW1⁺ Progenitor Cells Are Present in the Human Lung and Accumulate in Remodeled Vessels

Our results reveal that a resident population of PW1⁺ cells is present in the lung that respond to CH and generate SMCs. Although the mouse can often serve to unravel cellular events in human disease progression, we set out to determine whether PW1⁺ cells are present in the human lung and to study them in the pathological context of PAH. We performed immunofluorescence on biopsies obtained from the healthy region of lung samples from cancer patients (control) and on PAH human lung samples. Patients' characteristics are indicated in Online Table I. Similar to our observations from mouse lungs, we found PW1⁺ cells in perivascular zones, adjacent to the media of small vessels and in the parenchyma. In contrast to the mouse lungs, PW1⁺ cells were not scattered but organized in clusters (from 2 to 5 cells; Figure 8A). In the human PAH lung, we observed PW1⁺ cells in the perivascular zone of the nonremodeled vessels, and only scarce PW1⁺/ α -SMA⁺ cells

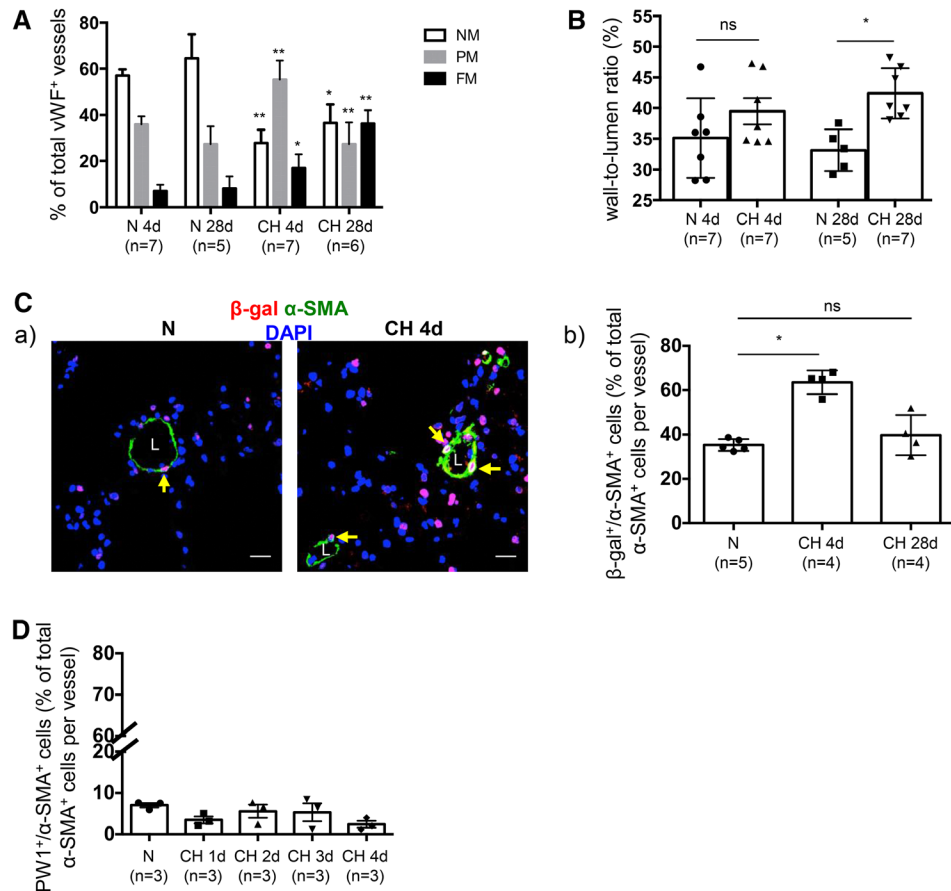


Figure 5. Chronic hypoxia (CH)-induced neomuscularization is associated with an increased number of PW1⁺-derived smooth muscle cell (SMC). **A**, Pulmonary vessel muscularization and **(B)** medial thickening of muscularized pulmonary vessels were determined by immunofluorescence in lungs from normoxic (N) or chronic hypoxic (CH 4 d) mice using anti- α -smooth muscle actin (SMA) and anti-von Willebrand factor (vWF) antibodies (n=5–7). For each animal, >100 vWF⁺ vessels (<100 μ m) were analyzed for muscularization (α -SMA⁺). **C**, Quantification of lung β -galactosidase (β -gal)-expressing SMC in normoxic and CH mice. (a) Representative images of lungs from N or CH (4 and 28 d) mice stained with β -gal (red) and α -SMA (green). (b) The percentage of lung β -gal⁺/ α -SMA⁺ cells (yellow arrowheads) in fully muscularized (FM) vessels (<100 μ m) was determined by immunofluorescence in normoxic (n=5), CH 4 d (n=4) and CH 28 d (n=4) mice. **D**, Quantification of vascular PW1⁺/ α -SMA⁺ cells in pulmonary vessels of N mice and of mice submitted to 1 to 4 d of CH (1–4 d), using anti-PW1, and anti- α -SMA antibodies (n=3 for each condition). Confocal microscopy. Scale bars, 20 μ m. Values are mean \pm SEM; * P <0.05, ** P <0.01 vs N. L indicates lumen of vessel; NM, nonmuscularized; ns, nonsignificant (Kruskal–Wallis, Dunn); and PM, partially muscularized.

were found similar to what we observed in the mouse. In the pulmonary remodeled vessels of patients with PAH, numerous PW1⁺ cells were observed in the perivascular area and within the vascular wall. We noticed the presence of numerous PW1⁺/ α -SMA⁺ cells in the broad neointima or in the thickened media of the remodeled vessels (Figure 8B).

Discussion

PAH is associated with vascular remodeling and in particular with the neomuscularization of small pulmonary vessels. In this study, we provide in vitro and in vivo evidence that resident pulmonary PW1⁺/CD34⁺ progenitor cells participate in the neomuscularization during CH, an experimental PH model. PW1⁺ progenitor cells have been isolated in several organs in mouse, human, dog, and pigs and share common characteristics with pulmonary PW1⁺ progenitor cells: (1) they are able to differentiate into α -SMA⁺ cells, and (2) they are characterized by the expression of CD34, c-kit, and PDGFR- α .^{14,15,20,22} These markers have also been used to

isolate vascular progenitor cells in other adult organs in rodents and human. In various tissues, resident progenitor cells expressing CD34,^{10,23,24} c-kit,^{11,24} or PDGFR- α ¹¹ differentiate into SMC. Resident pulmonary c-kit⁺ cells, in particular, have been recently described in human as multipotent stem cells able to generate pulmonary vessels.²⁵ We also report here the presence of a CD34-/PW1⁺ population expressing pericyte markers similar to the perivascular progenitor cells identified in multiple human organs.¹² Interestingly, PW1 expression has been recently demonstrated to be essential for progenitor competence in mesoangioblasts that are derived from vessels present in skeletal muscle.²⁰ Thus, the pulmonary PW1⁺ cell populations identified here are consistent with previously published vascular progenitor cells and confirm that PW1 identifies multiple progenitor cell populations.

Using PW1 reporter mice,¹⁴ we were able to follow the differentiation of these PW1⁺ progenitor cells into vascular SMC. Although this is not a bona fide lineage-tracing model, the long half-life of β -gal protein²¹ (and our own in vitro data)

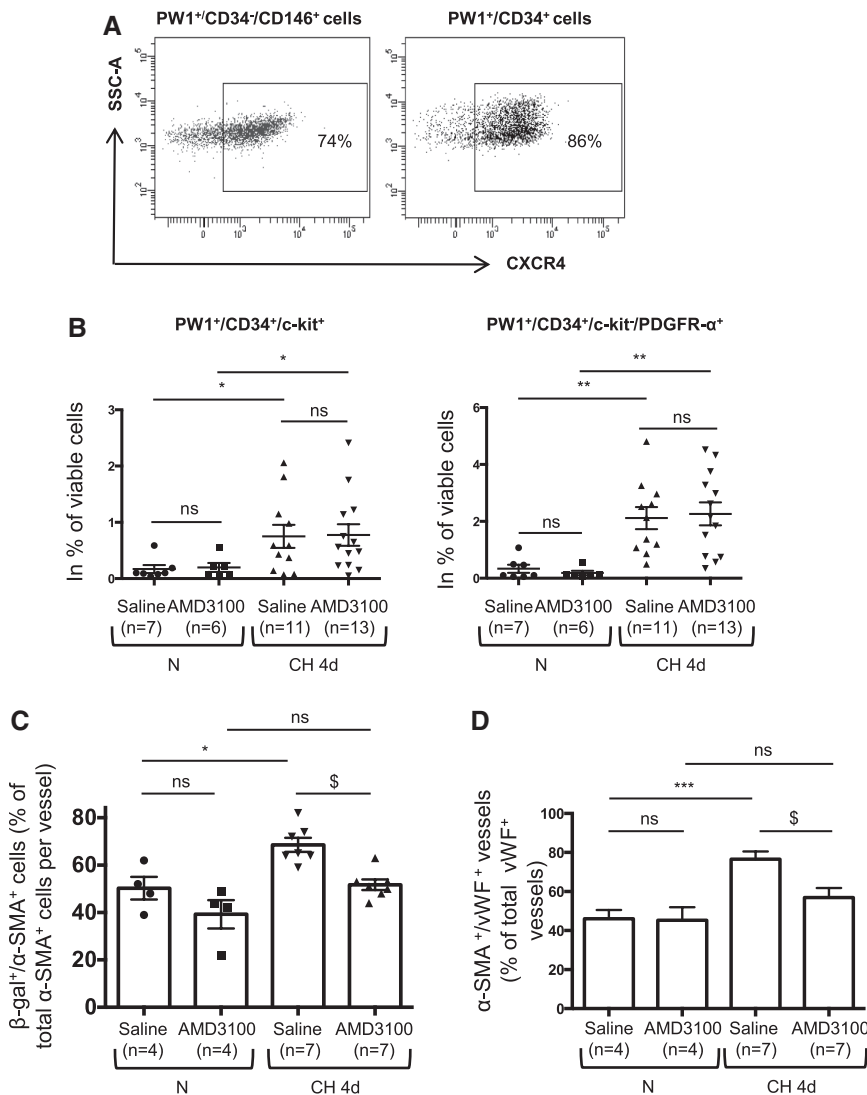


Figure 6. AMD3100 inhibits differentiation of PW1⁺ progenitor cells into vascular smooth muscle cells (SMCs) but not their proliferation.

A, CXCR4 expression was measured by fluorescence-activated cell sorting (FACS) on the PW1⁺/CD34⁺/CD146⁺ and the PW1⁺/CD34⁺ cell populations. The percentage of CXCR4⁺ cells for each population is indicated. SSC indicates side scatter area. **B**, Quantitative analysis of lung PW1⁺/CD34⁺ cell populations from normoxic (N) or chronic hypoxic (CH 4 d) mice treated with AMD3100 or saline. The mean percentage of total viable cells for each PW1⁺ cell population was measured by FACS. **C**, The percentage of lung β -galactosidase (β -gal)⁺/ α -smooth muscle actin (SMA)⁺ in fully muscularized vessels (<100 μ m) from mice treated with AMD3100 or saline and submitted to N (n=4) or CH for 4 d (n=7), was measured by immunofluorescence. **D**, Pulmonary vessel muscularization (percentage of fully and partially muscularized vessels) was determined by immunofluorescence in lungs from N or CH (4 d) mice treated with AMD3100 or saline, using anti- α -SMA and anti-von Willebrand factor (vWF; n=4–7 per group). For each animal, >50 vWF⁺ vessels (<100 μ m) were analyzed for muscularization (α -SMA⁺). Values are mean \pm SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 CH vs N and \$*P*<0.05 AMD3100 vs saline. ns indicates nonsignificant (Kruskal–Wallis, Dunn).

allowed us to detect differentiated PW1⁻ cells derived from PW1⁺ cells. Our results show that pulmonary resident PW1⁺ progenitor cells are a novel source of SMC during early CH-induced vascular remodeling. First, we observed an early increase in the number of these progenitor cells and in their proliferation. In addition, we show that 1 population mobilized during CH will spontaneously differentiate into terminally differentiated SM-MHC⁺ SMC. Second, we show an increased number of SMC derived from PW1⁺ cells during early CH-induced neomuscularization. Moreover, CH did not induce the expression of PW1 in pulmonary SMC, favoring a model in which direct differentiation of PW1⁺/ α -SMA⁻ progenitor cells into SMCs occurs as an early response to CH. Finally, we demonstrate that these new SMC are not differentiated from BM-derived progenitor cells. Other studies have demonstrated the existence of progenitor cells recruited in a pulmonary pathophysiological context. These studies were performed at late stages of the CH response when vascular remodeling has already concluded. The studies also showed that sustained CH stimulates the mobilization of BM-derived progenitor cells, such as mesenchymal stem cells,²⁶ c-kit⁺ cells,²⁷ and CD11b⁺ fibrocytes,²⁸ which contribute to pulmonary

vessels remodeling.^{27–29} Yet, few studies have been able to address the question of the mobilization of resident pulmonary progenitor cells. Long-term CH induces the proliferation of resident endothelial progenitor cells in the mouse lung.³⁰ Lineage-tracing experiments have shown that a perivascular lung mesenchymal stem cell population contributes to microvascular remodeling after 5 weeks of hypobaric hypoxia.³¹ Ricard et al⁷ showed that NG2⁺ pericytes, which contribute to forming SMC-like cells, are increased from day 7 to day 21 of CH demonstrating a later and more progressive recruitment of these cells in this model. This is consistent with our results, which indicate that the number of PW1⁺/CD34⁺/CD146⁺ cells (84% NG2⁺) is not significantly increased after 4 days of CH. Very recently, Sheikh et al⁵ have demonstrated that muscularization of specific distal arteries arise from the migration and clonal proliferation of vascular PDGFR- β ⁺/ α -SMA⁺ cells observed between 3 and 7 days of CH. However, after 3 days of CH, distal arterioles were barely muscularized and contained few proliferating SMCs. These results cannot explain the high level of neomuscularization already observed after 2 or 4 days of CH³² (and our results). Yet, we showed that after 4 days of CH, resident proliferating PW1⁺ progenitor cells

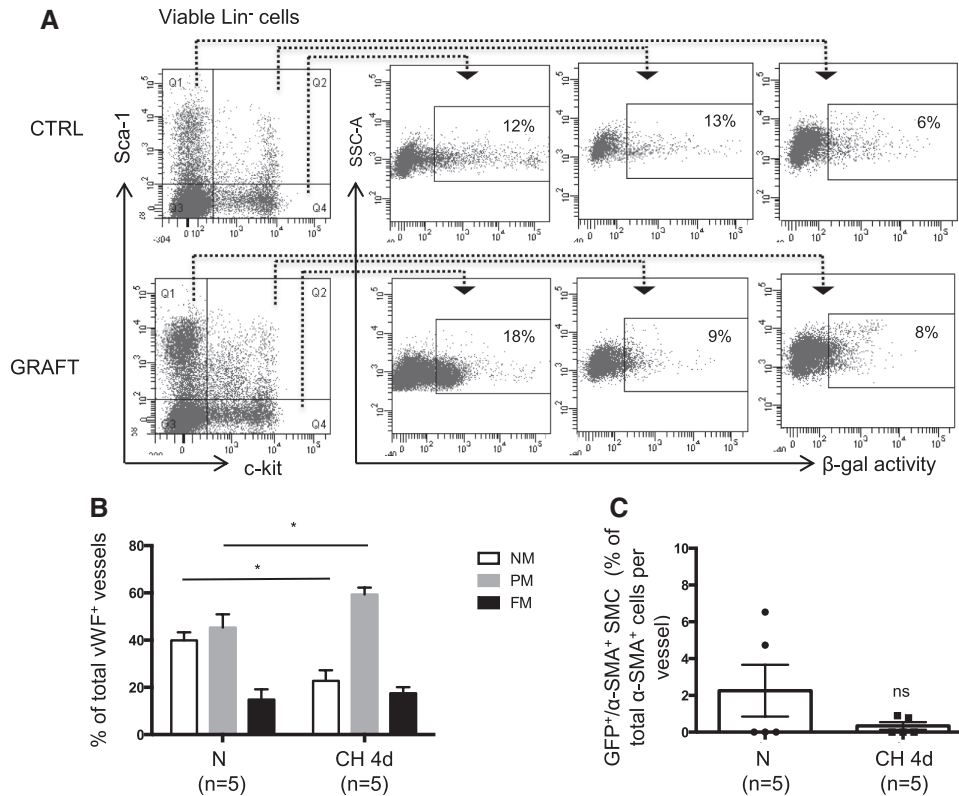


Figure 7. The pulmonary PW1⁺ cells recruited early in the chronic hypoxia (CH) mouse are not derived from bone marrow.

A, Flow cytometry analysis of the engrafted bone marrow (BM) cells 6 mo after transplantation compared with BM cells from a control PW1 reporter mouse (CTRL). Sca-1⁺/c-kit⁺ (Q1), Sca-1⁺/c-kit⁺ (Q2), and Sca-1⁻/c-kit⁺ (Q4) cells were analyzed for β-galactosidase (β-gal) activity.¹⁴ The percentage for each population is indicated in percentage of parent. SSC indicates side scatter area. **B**, Pulmonary vessels muscularization was determined by immunofluorescence using anti-α-smooth muscle actin (SMA) and anti-von Willebrand factor (vWF) on lungs of normoxic (N) and CH (4 d) transplanted mice (n=5). For each animal, >50 vWF⁺ vessels (<100 μm) were analyzed for muscularization (α-SMA⁺). Vessels showing a perimeter with >90% of muscularization were considered fully muscularized. Values are mean±SEM, **P*<0.05, ***P*<0.01 (2-tailed Mann-Whitney N vs CH 4 d). **C**, Quantification of BM-derived SMC in pulmonary vessels of N and CH (4 d or 21 d) mice, using anti-GFP, anti-β-gal and anti-α-SMA antibodies. The percentage of α-SMA⁺/GFP⁺ SMCs derived from BM was similar in the transplanted mice lungs under N and after 4 d of CH (4 d; n=5). Confocal microscopy. Scale bars, 20 μm. Values are mean±SEM (2-tailed Mann-Whitney). ns: non significant. FM indicates fully muscularized; NM, nonmuscularized; and PM, partially muscularized.

are important contributors of new SMC. Indeed, PW1⁺ cells represent 50% of all the proliferating cells found in the lung parenchyma after 4 days of CH (data not shown). Therefore, altogether our results with previous ones^{5,7} suggest that hypoxia-induced pulmonary vessels muscularization is dependent on multiple resident SMC sources recruited successively: PW1⁺/CD34⁺ progenitor cells, PDGFR-β⁺ SMC, and NG2⁺ pericytes. Other investigations have also reported significant recruitment of resident perivascular progenitor cells during vascular remodeling/pathophysiology such as the restenosis³³ or the atherosclerosis.³⁴ These resident progenitor cells contribute to maintain the tissue homeostasis and integrity and were proposed to be responsible for pathological features such as the SMC-like accumulation in remodeled vessels.⁹

The SDF-1/CXCR4 pathway is one of the major regulators of progenitor and stem cells recruitment and homing.³⁵ In addition, pulmonary concentrations of CXCR4 ligands SDF-1 and macrophage migration inhibitory factor were found to be elevated during CH,^{36,37} and CXCR4 inhibition by AMD3100 treatment was shown to partially prevent CH-induced increase in media thickness.²⁹ Consistent with these results, we here show that the increase in the number of PW1⁺-derived SMC and the neomuscularization are blocked by AMD3100,

indicating that they are dependent on the CXCR4 pathway. Although we cannot prove yet whether PW1⁺ progenitor cells are directly activated through the CXCR4 receptor, our observation that most PW1⁺ progenitor cells express CXCR4 suggests that the CXCR4 ligand may directly attract them to the vessels. Interestingly, our result shows that the CXCR4 pathway regulates PW1⁺ progenitor cells migration and differentiation but not their proliferation. This result is consistent with the major homing effect of the SDF-1/CXCR4 pathway, but this indicates that further studies are needed to understand which factors are responsible for inducing proliferation of PW1⁺ progenitor cells during early CH. It would be particularly interesting to study the effect of PAH-specific drugs on the proliferation, migration, and differentiation of PW1⁺ cells or to target pathways that regulate pulmonary vascular remodeling such as serotonin, PDGF, or hypoxia-inducible factor 1-α (HIF-1α) pathways.³⁸

We report here a similar increase in the number of lung parenchymal PW1⁺ cells in 2 well-recognized and widely used experimental models, the monocrotaline- and the CH-induced PH models. Both models induce inflammation as an initial process^{39,40} and inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α,

vitro, and on our immunofluorescence and FACS observations on normoxic and CH-submitted mouse lungs, we suggest that the 3 pulmonary PW1⁺ cell populations are a source of vascular smooth muscle progenitor cells in the adult mouse lung and that the PW1⁺/CD34⁺/c-kit⁺ and the PW1⁺/CD34⁺/c-kit⁻/PDGFR- α ⁺ cell populations are important players in neomuscularization during CH via activation of the CXCR4 pathway. However, our results do not allow to determine the respective roles of each PW1⁺ cell population. Strategies aiming to inhibit the mobilization, proliferation, or differentiation of these cells may provide important therapeutic avenues leading to an early treatment of PAH.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Pulmonary arterial hypertension (PAH) in humans and chronic hypoxia-induced pulmonary hypertension in mice are characterized by gain of a new muscular layer in previously nonmuscularized small pulmonary vessels (neomuscularization).
- The origin of the new vascular smooth muscle cells (SMC) is not clearly established.
- Resident smooth muscle progenitor cells have been detected in numerous tissues.

What New Information Does This Article Contribute?

- Resident SMC progenitor cells expressing the PW1 marker are present in the perivascular zone in the mouse and human lung and are recruited when mice are subjected to chronic hypoxia.
- New SMCs are derived from these PW1-expressing progenitor cells in a CXCR4-dependent manner.
- In human, PW1-expressing cells were also present near-pulmonary arterioles and were found abundant in remodeled vascular structures of PAH patients.

Neomuscularization of pulmonary arterioles and vascular SMCs proliferation are characteristic pathological features of PAH and contribute to reducing vascular compliance. The origin of the new vascular SMCs is not clearly established. In this study, we present evidence that these new vascular SMCs, which appear around previously not or partially muscularized small pulmonary vessels during PAH, are derived from PW1-expressing resident vascular progenitor cells. We have used an original transgenic mouse tool, which allows detection of PW1-expressing progenitor cells and of cells recently derived from these progenitors. We have shown that mouse pulmonary PW1⁺ progenitor cells are perivascular, and that they can differentiate into vascular SMCs in vitro and in vivo. Chronic hypoxia in mice induced the mobilization of these resident progenitor cells and their differentiation into new vascular SMCs in a CXCR4-dependent manner. PW1-expressing cells were also present in the human lung and were found abundant in remodeled vascular structures of patients with PAH, suggesting that these progenitor cells also contribute to pulmonary vascular remodeling in human. These results identify pulmonary PW1-expressing cells as a new resident progenitor cell population that participates in small vessel muscularization during PAH development and provide a new potential therapeutic target.

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Resident PW1⁺ Progenitor Cells Participate in Vascular Remodeling During Pulmonary Arterial Hypertension

France Dierick, Tiphaine Héry, Bénédicte Hoareau-Coudert, Nathalie Mougenot, Virginie Monceau, Caroline Claude, Mihaela Crisan, Vanessa Besson, Peter Dorfmueller, Gilles Marodon, Elie Fadel, Marc Humbert, Elisa Yaniz-Galende, Jean-Sébastien Hulot, Giovanna Marazzi, David Sassoon, Florent Soubrier and Sophie Nadaud

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SUPPLEMENTAL MATERIAL

Methods

Animals and experimental PAH models

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 animal facility for the duration of the experiment. All animals had access to food and water, ad libitum. *PwIIRESnLacZ* transgenic mice (*PwInlacZ/+*), in which a nuclear operon lactose gene is expressed under the control of the *PwI* gene locus were produced in our animal husbandry¹. They were backcrossed with C57BL6/J mice and maintained in a C57BL6/J background. C57BL6/J mice were from Janvier (Orléans, France). H2B-GFP mice were originally from Jackson Labs. Mice (littermates of 6-8 weeks) were exposed to room air (normoxia) or chronic normobaric hypoxia (10% O₂) in a ventilated chamber for 4 or 28 days with the same light-dark cycle. Hypoxia was obtained by flushing nitrogen into the hypoxic chamber. Levels of CO₂ and O₂ in the chamber were constantly monitored and maintained by flushing oxygen in the chamber and by absorbing CO₂ with soda lime granules. The chamber temperature was maintained at 22–24°C and excess humidity was prevented by desiccant. Adult male Wistar rats (300 g body weight obtained from Janvier Laboratories) were injected i.p. with monocrotaline (MCT, 60 mg/kg, Fluka ref 37024) or equal volume of isotonic saline. The animals were sacrificed 3 ± 0.5 weeks later.

BrdU (5'-bromo-2'-deoxyuridine) injections

Normoxic and chronic hypoxic mice were injected i.p. with BrdU solution (Sigma, sc-290815A) prepared in PBS (100 mg/kg) at 24, 16 and 4 hours before euthanasia.

AMD3100 injections

Normoxic and chronic hypoxic mice were injected daily i.p. with AMD3100 solution (ref. A5602, Sigma-Aldrich) prepared in PBS (10mg/kg) from day 1 to day 4.

Haemodynamic measurements and tissue collection

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 Minivent Mouse ventilator (type 845, Harvard Apparatus, respiratory frequency 170 stroke/min and respiratory volume 200µL). 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. Rats were anesthetized by

pentobarbital injection (50mg/kg, i.p.) and received a subcutaneous injection of METACAM (meloxicam, 1.5 mg/kg). Then, to perform RV pressure measurements, a pressure transducer catheter (size 2F, Millar model SPR-407) was advanced to the RV through the right jugular vein. The catheter was connected to a Gould amplifier to record the pressure (RS3200 Gould).

To confirm chronic hypoxia hemoglobin concentration was measured in the peripheral blood using HemoCue (Hb 20+). Right ventricular hypertrophy was assessed by calculating the weight ratio of the right ventricle (RV) to the left ventricle (LV) plus septum (S) (RV/(LV+S)).

Lungs were carefully removed and inflated by intratracheal infusion of 50% O.C.T. (Cryomatrix) in PBS, immersed in isopentane, frozen in liquid nitrogen, embedded in O.C.T and stored at -80°C. Alternatively, lungs were inflated and fixed by injecting Finefix (Milestone Srl) directly in lobes. After fixation for 24h à 4°C, lung tissues were paraffin-embedded.

FACS analysis

Lung single-cell suspensions were obtained by digestion the tissues in DMEM high glucose media (Gibco) supplemented with 20% FBS and 0.5 mg/mL of collagenases IA-S, II-S, IV-S (Sigma) for 45 min at 37°C. The remaining tissue fragments were next disrupted by mechanically between two superfrost+ slides (Thermo Scientific), to separate the progenitor cells from the extracellular matrix². Bone marrow was prepared by flushing bones with DMEM. Mice lung cells or bone marrow preparation were stained for 20 min on ice in the dark. Antibodies used were CD45 PE-Cy7, CD34 Brilliant Violet 421, PDGFR- α PE, CD146 PE, c-kit PE or APC, CXCR4 PE, Lin Pacific Blue cocktail (see Online Table II). To detect nuclear β -gal activity, two different substrates were optimized and used: FDG kit for bone marrow cells or DDAO-G kit for lung cells (both from Life Technologies). β -gal⁺ cells were defined as having a signal higher as compared to the cells isolated from non-transgenic mouse (β -gal⁻). 7-AAD (BD Pharmingen) for bone marrow cells and Live dead blue dye (Life Technologies) for lung cells were used as viability cell marker. Cells were sorted using FACSaria (Becton Dickinson) with appropriate isotype matching controls and the Fluorescence Minus One controls (FMO) were used to identify and gate cells.

Primary cell culture – Differentiation induction

Immediately after sorting, isolated cells were plated on matrigel-coated (ref. 354277, Life Sciences-Corning) dishes for smooth muscle and endothelial cell differentiation, or gelatin-coated (Attachement Factor protein, ref. S-006-100, ThermoFisher) dishes for skeletal muscle cell differentiation.

For smooth muscle cell differentiation, isolated cells were plated at a density of 2000 cells/cm² and were grown for three days in DMEM 20% FBS (Sigma), 2% (v/v) penicillin-streptomycin (Gibco) then cells were transferred in DMEM 2% FBS, 1% (v/v) penicillin-streptomycin, supplemented with TGF- β ₁ (2 ng/mL) (ref. 7666-MB-005, R&D systems) and PDGF-BB (20 ng/mL) (ref. 220-BB-010, R&D systems) for 10 days, this medium was changed every 2 days.

For skeletal muscle cell differentiation, isolated cells were plated at a density of 2000 cells/cm² and were grown for three days in DMEM supplemented with 2.5 ng/mL basic fibroblasts growth factor (bFGF, ref. 233-FB-025, R&D systems), 20% FBS (Sigma), 10% heat-inactivated horse serum (Gibco), 1% (v/v) penicillin-streptomycin (Gibco), 1% (v/v) L-glutamine (Gibco) and 1% (v/v) sodium pyruvate (Gibco). This growth medium was changed every 2 days. Then cells were transferred in DMEM 5% heat-inactivated horse serum and 1% (v/v) penicillin-streptomycin, for 2 days.

For endothelial cell differentiation, isolated cells were plated at a density of 5000 cells/cm² and were grown for 10 days in complete EGM-2 (Endothelial growth medium, Lonza) supplemented with 20 ng/mL VEGF₁₆₄ (ref. 493-MV-005, R&D systems) and 1% (v/v) penicillin-streptomycin. This medium was changed every 2 days.

Immunofluorescent labeling

Tissue 10µm thick cryosections were fixed in formalin (Sigma). Tissue paraffin-embedded 4µm thick sections were deparaffinised, rehydrated, and citrate antigen retrieval was performed. Sorted cells were centrifuged in 150 µL PBS onto a gelatin-coated slide using a Cytospin 3 cytocentrifuge (Shandon Instruments, PA) and were fixed in formalin (Sigma). Then cryosections and cytospun cells were permeabilized in methanol (6 min at -20°C) or with X-100 triton 0.01% in PBS (5 min at RT). Non specific binding was blocked with bovine serum albumin 10% in PBS (1h at RT) and sections were incubated overnight with PBS + primary Abs (see Online Table III) at 4°C. Secondary Abs and DAPI (DAPI) were diluted in PBS (respectively 1/500 and 1/1000) and incubated 45 min at RT. Immunolabeled sections were mounted with Dako fluorescent mounting medium (Dako) and examined under confocal microscope (Leica SPE confocal microscope). In some cases, as indicated, images were acquired with a cooled CoolSnap camera (Roper-Scientific) on an Olympus epifluorescent microscope (60x, UPlanSApo, 0.17). Images were processed and analyzed using Metamorph software (Molecular Devices) supplemented with the 3D-deconvolution module. For each sample, a series of consecutive planes (stack of images) were acquired (sectioning step, 0.2 µm) and deconvoluted using acquired point spread function. Control experiments were performed using secondary antibodies alone and showed no non-specific labeling.

Quantitative analysis by immunofluorescent microscopy

Images were analyzed by an investigator blinded to the experimental status of all animals. β-gal⁺ and β-gal⁻/α-SMA⁺ SMC were counted for all fully muscularized vessels (<100 µm) of one lung section per animal (15-30 vessels/animal) using stacks of 5-10 consecutive planes for one image to ensure co-localization of the nuclear labeling of β-gal and the cytoplasmic labeling of α-SMA. For other countings, single planes were analyzed for each image and a minimum of 30 to 60 fields were analyzed per animal.

Muscularization was measured by immunofluorescence in using using anti- α -SMA and anti-vWF antibodies. Vessels showing a perimeter with more than 90% of muscularization were considered fully muscularized. Medial thickness was measured as wall-to-lumen ratio.

Bone marrow transplantation

20 female 8 weeks-old C57BL6/J mice were subjected to 2x6 Gy (7.2 min; 0.83 Gy/min) lethal total body irradiation within 24h. Immediately after the second irradiation, isoflurane-anesthetized mice were reconstituted by direct intravenous injection (in retro-orbital plexus) with $10 \cdot 10^6$ cells in PBS freshly isolated from bone marrow of age and sex-matched GFP⁺/ β -gal⁺ (H2B-GFPxP_WI^{nLacZ/+}) or β -gal⁺ (P_WI^{nLacZ/+}) mice (both on C57BL6/J background). This latter was performed to confirm the good reconstitution of the PW1⁺/ β -gal⁺ populations in the grafted mice's bone marrow by FACS analysis using the FDG substrate. Reconstitution of the PW1⁺/ β -gal⁺ niche was found to be complete 6 months after transplantation. At this time, the percentage of GFP⁺/CD45⁺ cells in blood mononuclear cells of GFP⁺/ β -gal⁺ grafted mice was measured by FACS (LSRFortessa, Becton Dickinson), to confirm the total bone marrow reconstitution. These mice were then exposed to normoxia or chronic hypoxia for 4 days.

Statistical analysis

All values were expressed as the mean \pm SEM. Statistics were performed using the computer program GraphPad PRISM 6 (GraphPad software) or XLStat 2013 (Addinsoft, New York, USA) with a non-parametric Mann and Whitney test for single comparisons. Multiple comparisons were analyzed using Kruskal-Wallis test followed by Dunn post-hoc test. P values less than 0.05 were considered to be significant.

Study approval

Experiments for the project were approved by our institutional review board (authorizations Ce5/2012/023 and Ce5/2012/050).

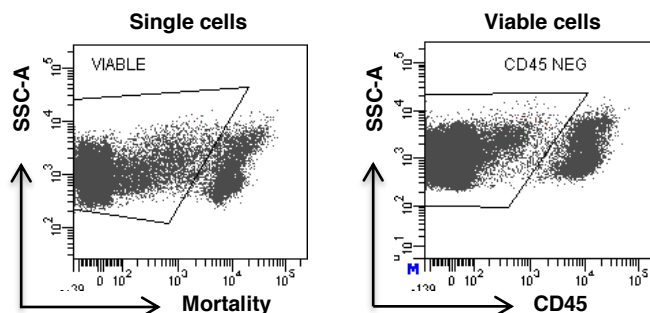
Study patients were part of the French Network on Pulmonary Hypertension, a program approved by our institutional Ethics Committee (Protocol N8CO-08-003, ID RCB: 2008-A00485-50, approved on June 18, 2008). Written informed consent was received from participants prior to inclusion in the study.

References

1. 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 U A* 2011;108:11470-5.
2. Crisan M, Huard J, Zheng B, Sun B, Yap S, Logar A, Giacobino J-P, Casteilla L, Péault B. Purification and culture of human blood vessel-associated progenitor cells. *Curr. Protoc. Stem Cell Biol.* 2008;Chapter 2:Unit 2B.2.1-2B.2.13.

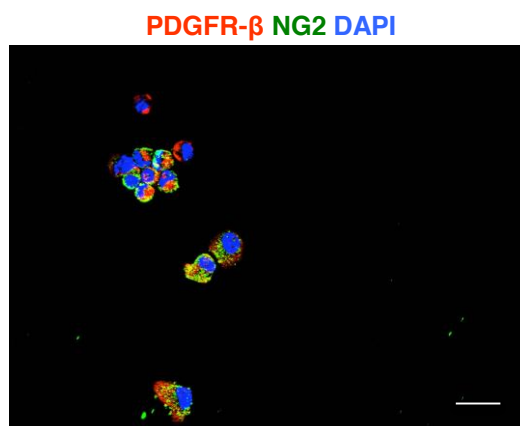
SUPPLEMENTAL FIGURES

Online Figure I



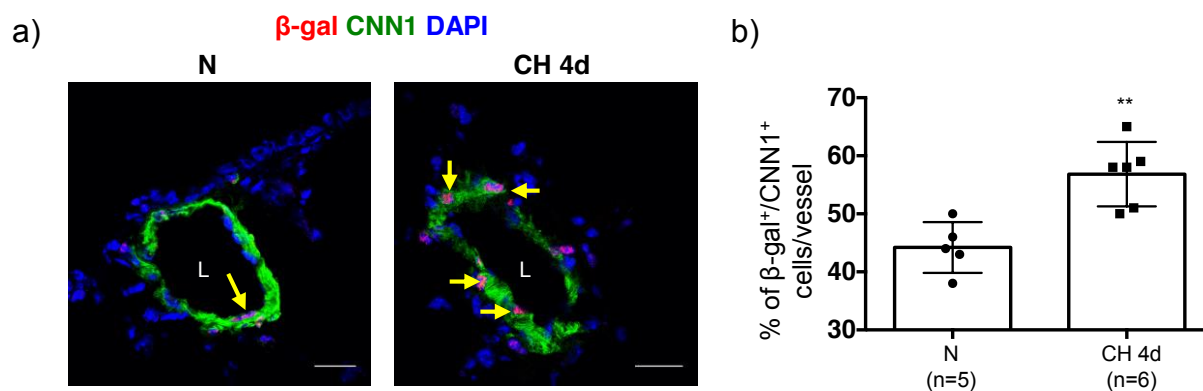
Online Figure I: Representative FACS profile of total mouse lung cells indicating the gating strategy to sort the three PW1⁺ populations with Live dead blue dye (viability marker) and CD45 antibody. Viable CD45⁻ cells were then separated by CD34, c-kit, and DDAO expression as shown in Figure 1B.

Online Figure II



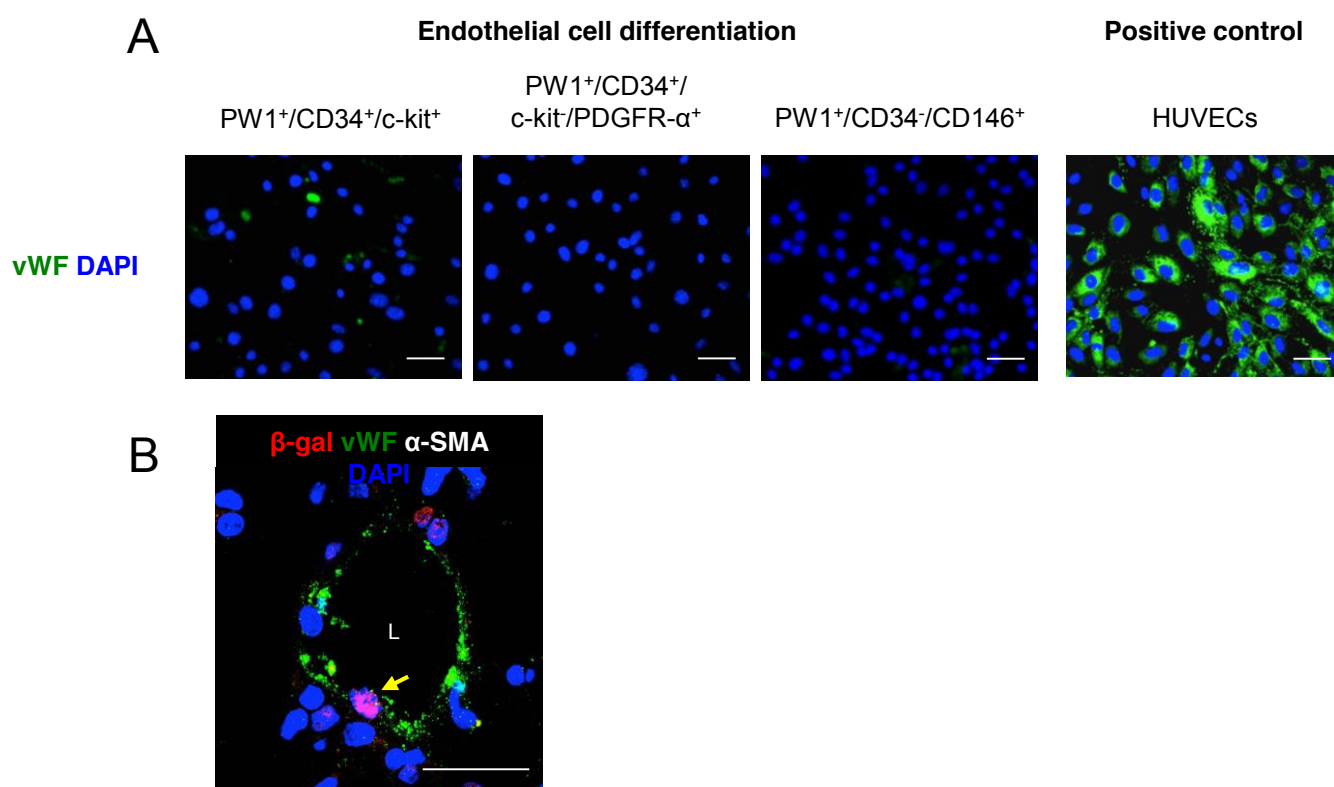
Online Figure II: Expression of other pericyte markers. PDGFR- β (red) and NG2 (green) were also analysed by immunofluorescence in the PW1⁺/CD34⁻/CD146⁺ population. Representative image (n=2). Epifluorescence microscopy. Scale bars: 20 μ m.

Online Figure III



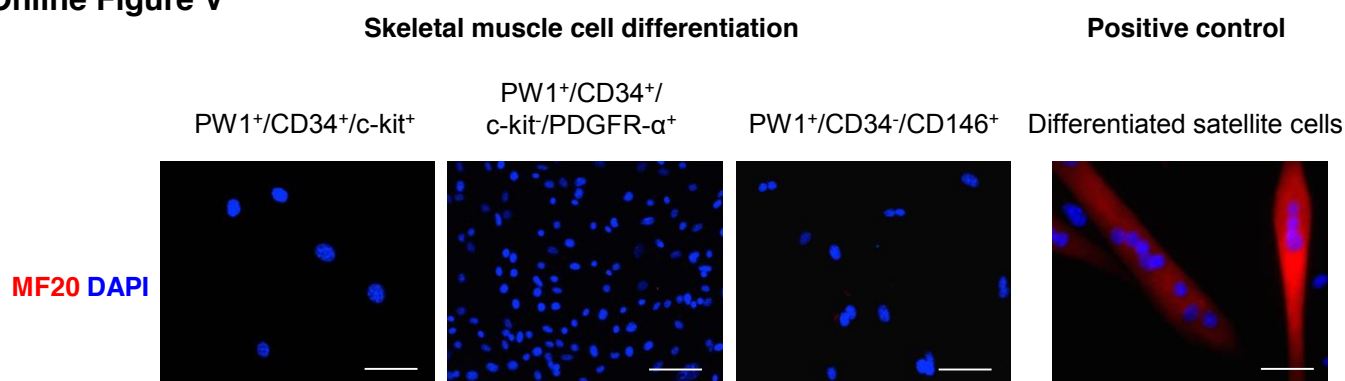
Online Figure III: Chronic hypoxia-induced neomuscularization is associated with an increased number of PW1⁺-derived β -gal⁺/CNN1⁺ SMC. Quantification of lung β -gal-expressing SMC in normoxic and chronic hypoxic 4 days mice (CH 4d). a) Representative images of lungs from N or CH 4d mice, stained with b-galactosidase (red) and CNN1 (green). b) The percentage of lung β -gal⁺/CNN1⁺ cells (yellow arrowheads) in muscularized vessels (<100 μ m) was determined by immunofluorescence from normoxic (n=5), and CH 4 days (n=6) mice. The number of b-gal-expressing SMC was significantly increased after 4 days of CH. L: Lumen. Confocal microscopy. Scale bars: 20 μ m. Values are means \pm SEM, ** p<0.01 versus N (two-tailed MW).

Online Figure IV



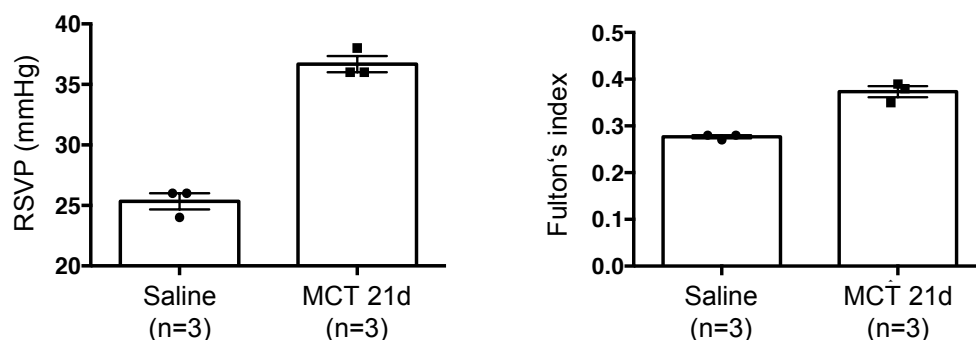
Online Figure IV : The mouse lung PW1⁺ cells do not differentiate into endothelial cell (EC). **(A)** FACS isolated PW1⁺ cell populations were grown in EGM-2 supplemented with VEGF and immunostained after culture for EC marker vWF (green) show no staining. HUVECs were used as positive control. Representative images of multiple experiments (n=2). **(C)** Mouse lungs immunostained for β -gal (red) and for EC marker vWF (green) show one of the rare EC expressing β -gal in the intima of pulmonary vessels (yellow arrowhead). Confocal microscopy. L: Lumen of vessel. Scale bars: 20 μ m.

Online Figure V



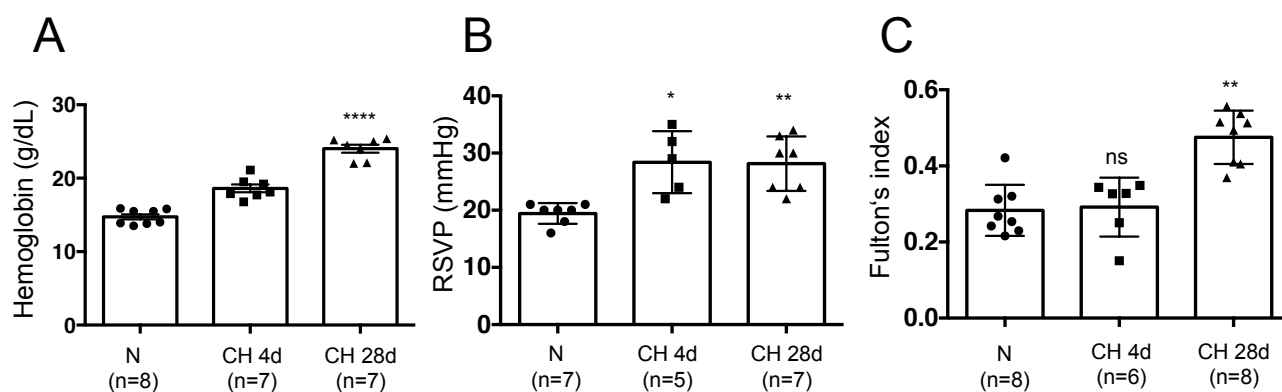
Online Figure V : The mouse lung PW1⁺ cells do not differentiate into skeletal muscle cells. FACS isolated PW1⁺ cell populations were grown in myogenic conditions and immunostained for skeletal muscle cell marker, skeletal myosin heavy chain, MF20 (red) show no staining. Differentiated satellite cells were used as positive control. Representative images of multiple experiments (n=2). Epifluorescence microscopy. Scale bars: 20 μ m.

Online Figure VI



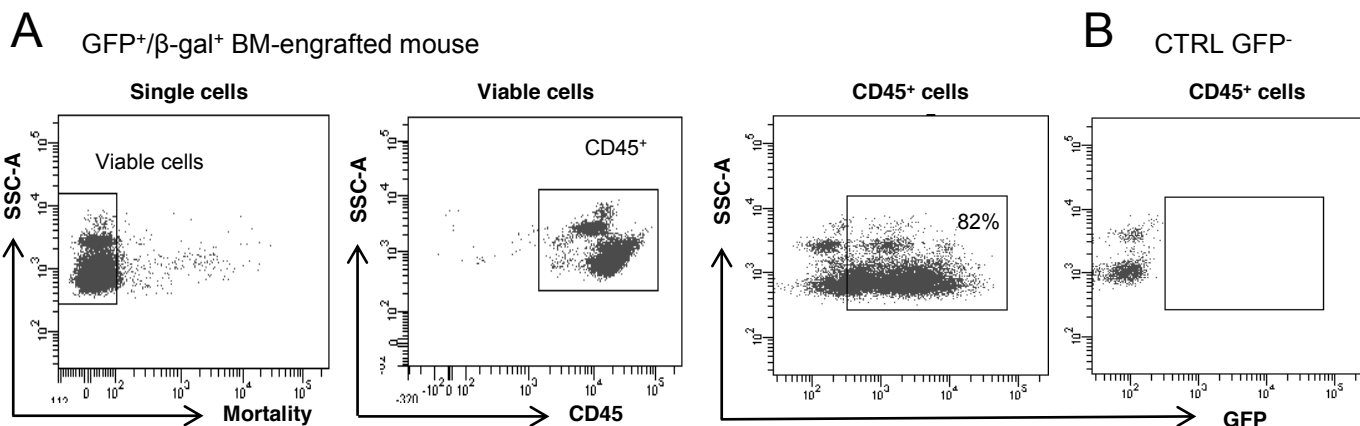
Online Figure VI: Monocrotaline (MCT) induces the increase of the right systolic ventricular pressure (RVSP, in mmHg) and the elevation of the fulton's index 21 days after the single MCT-injection (Ratio of RV to LV plus septum weight, RV/LV+S) in rats. Values are means \pm SEM.

Online Figure VII



Online Figure VII: Chronic hypoxia (CH) induces **(A)** the increase of the hemoglobin concentration (g/dL) in peripheral blood, **(B)** the elevation of the right systolic ventricular pressure (RVSP, in mmHg) and **(C)** the elevation of the fulton's index after 28 days of CH (Ratio of RV to LV plus septum weight, RV/LV+S) in mice. Values are means \pm SEM, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs N (normoxia) (KW-Dunn).

Online Figure VIII



Online Figure VIII: Flow cytometry analysis of peripheral blood cells from **(A)** GFP⁺ BM-transplanted mouse 6 months after transplantation and **(B)** control GFP⁻ mouse. Viable CD45⁺ cells were analyzed for GFP fluorescence. The transplanted mice showed a good chimerism (82,2±4,2%). Representative image of BM-engrafted mice (n=10). The percentage is indicated in percentage of viable CD45⁺ cells.

SUPPLEMENTAL TABLES

Online Table I

PAH patients	gender	age (yrs)	BMPR2 mutation	PAP (mmHg)	CI (L/min/m ²)	PVR (Wood units)	NYHA	Therapy
1	Female	31	No	61	1,6	14	III	IV prostacyclin+ERA+ PDE5-i
2	Male	50	No	43	3,23	5,61	III	IV prostacyclin+ERA
3	Female	38	No	43	2,2	8,16	III	IV prostacyclin+ERA+ PDE5-i

Control patients	gender	age (yrs)	Disease
1	Female	36	pulmonary adenocarcinoma
2	Female	51	pulmonary adenocarcinoma
3	Female	47	pulmonary adenocarcinoma

Online Table I: Clinical characteristics of patients in the study. PAP = pulmonary arterial pressure. CI = cardiac index. PVR = pulmonary vascular resistance. NYHA = New York Heart Association. IV prostacyclin = intravenous prostacyclin. ERA = endothelin receptor antagonist. PDE5-I = phosphodiesterase type 5 inhibitor.

Online Table II

Abs	Ref	Company
CD45 PECy7	25-0451-82	eBioscience
CD34 BV421	119321	Biolegend
PDGFR- α FITC	11-1401-82	eBioscience
CD146 PE	134704	Biolegend
c-kit APC	17-1172	eBioscience
c-kit PE	135105	Biolegend
Lin Pacific blue	88-7772-72	eBioscience
Sca-1 PE	12-5981-82	eBioscience
CXCR4 PE	551966	BD

Online Table II: FACS antibodies, references and companies are indicated.

Online Table III

Abs	Ref	Company	Dilution	Incubation time
PW1	Relaix et al, 1996	-	1/2000	OVN, 4°C
PEG3	ABIN 735653	Antibodies online	1/200	OVN, 4°C
β -galactosidase	ab9361	Abcam	1/6000	OVN, 4°C
vWF	ab11713	Abcam	1/300	1h30, RT (OCT sections)
vWF	ab9378	Abcam	1/100	OVN, 4°C (cell)
vWF	11778-1-AP	proteintech	1/50	OVN, 4°C (paraffin sections)
FITC α -SMA	F3777	Sigma	1/500	1h30, RT
α -SMA	M0851	Dakocytomation	1/300	1h30, RT
CNN1	ab466794	Abcam	1/300	1h30, RT
SM-MHC	ab53219	Abcam	1/100	OVN, 4°C
MF20	AB 2147781	Developmental Studies Hybridoma Bank	1/50	OVN, 4°C
c-kit	ab25022	Abcam	1/100	1h30, RT
PDGFR- α	BD558774	BD	1/500	1h30, RT
CD146	134701	Biolegend	1/2000	1h30, RT
NG2	AB5320	Millipore	1/500	OVN, 4°C
PDGFR- β	14-1402-82	eBioscience	1/2000	OVN, 4°C
BrdU	ab6326	Abcam	1/500	OVN, 4°C
GFP	TP401	Torres Pines Biolabs	1/300	OVN, 4°C

Online Table III: Antibodies used for immunofluorescence, references, companies, appropriate dilutions and incubation conditions are indicated. RT, room temperature; OVN, overnight.