Resident PW1\(^+\) Progenitor Cells Participate in Vascular Remodeling During Pulmonary Arterial Hypertension

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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 \(Pw^{fl}

\text{\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

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 state\(^6\) or from pericytes\(^7\) 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,
SMCs derive from resident PW1+ progenitor cells recruited into SMCs during CH. Our results strongly suggest that new progenitor cell populations located in the lung parenchyma and in perivascular zones of small pulmonary vessels, which are present in lung tissue are recruited to proliferate and differentiate into smooth and skeletal muscle as well as adipocytes in multiple adult somatic stem cells markers including Sca-1, c-kit, platelet-derived growth factor alpha polypeptide (PDGFR-α), CD34 or mesenchymal stem cells markers. In addition, perivascular cells identified by these same markers and expressing CD146, NG2, and PDGFR-β were also recognized as SMC progenitor cells.

Recently, a population of interstitial progenitor cells was identified in mouse skeletal muscle based on their expression of the PW1 protein, which are capable of giving rise to smooth and skeletal muscle as well as adipocytes in culture. These PW1-expressing cells were found in multiple adult tissues including skin, gut, BM, and the central nervous system. PW1 gene encodes a zinc-finger protein that has been shown to regulate cell cycle and cell stress responses caused by inflammation and p53. In addition, PW1 mediates β-catenin stability in the Wnt signaling pathway. 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. Moreover, PW1 expression is required for the myogenic and migratory capacities of both mouse and human mesangioblasts, reinforcing the relevance of PW1 to identify competent mesangioblasts before their use in stem cell-mediated therapeutic applications.

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, Pw1IRESnLacZtransgenic 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-GFPxPw1IRESnLacZ) 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 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 nonmascularized and muscularized vessels (Figure 1A and 1B). We characterized these progenitor cells from PW1 reporter mice after FACS purification based on β-galactosidase activity. 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±0.6% for PW1+/CD34+/c-kit− cells, 92±2.4% for PW1+/CD34+/c-kit−/PDGFR-α+ cells, and 86±6.1% for PW1+/CD34+/CD146+ cells; n=4; Figure 1D). The PW1+/CD34+/CD146+ cells were positive for pericyte markers NG2 (84±7.3%; n=2) and PDGFR-β (86±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±2.3%; n=3) and into terminally differentiated α-SMA+/SM-MHC+ SMCs (26±7%; n=3). In contrast, the PW1+/CD34+/CD146+ and PW1+/CD34+/c-kit− populations differentiated into α-SMA+ (66±4.2% and 90.5±0.7%, respectively) and α-SMA+/SM-MHC+ cells (4.5±6.3% and 14±4%, respectively) only on induction with transforming growth factor-β1 stimulated with BM cells of age- and sex-matched GFP+/β-gal+ (H2B-GFPxPw1IRESnLacZ) mice and exposed, after 9 months, to Normoxia (N) or CH for 4 days (CH 4 days).

Methods

Detailed Methods are provided in the Online Data Supplement. Pw1IRESnLacZ transgenic mice (Pw1IRESnLacZ) bear a nuclear operon lactose gene expressed under the control of the Pw1 gene locus. 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-GFPxPw1IRESnLacZ) mice and exposed, after 9 months, to Normoxia (N) or CH for 4 days (CH 4 days).
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 +/α-SMA+ progenitor cells. Indeed, in small pulmonary vessels under normoxic conditions, we observed many SMC coexpressing β-galactosidase and α-SMA (35±2.2% of β-gal+/α-SMA+ cells), calponin (44±3% of β-gal+/CNN1+ cells), or SM-MHC (54±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 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 cytospun 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 μm. 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 anti–skeletal myosin heavy chain (SM-MHC; red) show no staining. A, FACS isolated PW1+ cell populations immunostained directly after sorting for SMC markers α-SMA (green) and smooth muscle myosin heavy chain (SM-MHC; red). PW1+/CD34+/c-kit+ and PW1+/CD34+/c-kit−/PDGFR-α+ cells were left without induction. Representative images of multiple experiments (n=3). Confocal microscopy. B, 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 μm. L indicates Lumen of vessel.

**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)-β1 and PDGF-BB, whereas PW1+/CD34+/c-kit−/PDGFR-α+ cells were left without induction. Representative images of multiple experiments (n=3).

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±0.3% in N versus 2.5±1.3% in CH; P<0.001 and 0.4±0.2% in N versus 0.89±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±0.4% versus 3.16±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±3.1% versus 20±2%; P≤0.05),
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 μm), 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.
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. It has been suggested that the SMC cells that form in the lung in response to CH derive from circulating cells of BM origin. Therefore, we performed a total BM transplantation from PW1 reporter mouse crossed with H2B-GFP mice (expressing ubiquitous nuclear GFP fused to histone2B) onto lethally irradiated wild-type mice. After 8 months, chimera was evaluated at =82% (82.2±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 FACS 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 μm) 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...
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-α11 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 mesangioblasts 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)
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,20 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 CH12 (and our results). Yet, we showed that after 4 days of CH, resident proliferating PW1+ progenitor cells

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![Image](http://circres.ahajournals.org/)

**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 μm) 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 μm) were analyzed for muscularization (α-SMA−). Values are mean±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).
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\textsuperscript{5,7} suggest that hypoxia-induced pulmonary vessels muscularization is dependent on multiple resident SMC sources recruited successively: PW1+/CD34+ progenitor cells, PDGFR-\(\beta\)+ SMC, and NG2+ pericytes. Other investigations have also reported significant recruitment of resident perivascular progenitor cells during vascular remodeling/pathophysiology such as the restenosis\textsuperscript{33} or the atherosclerosis.\textsuperscript{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.\textsuperscript{8}

The SDF-1/CXCR4 pathway is one of the major regulators of progenitor and stem cells recruitment and homing.\textsuperscript{35} In addition, pulmonary concentrations of CXCR4 ligands SDF-1 and macrophage migration inhibitory factor were found to be elevated during CH,\textsuperscript{36,37} and CXCR4 inhibition by AMD3100 treatment was shown to partially prevent CH-induced increase in media thickness.\textsuperscript{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-alpha (HIF-1\(\alpha\)) pathways.\textsuperscript{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\textsuperscript{39,40} and inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-\(\alpha\),

![Figure 7. The pulmonary PW1+ cells recruited early in the chronic hypoxia (CH) mouse are not derived from bone marrow.](http://circres.ahajournals.org/)

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 \(\beta\)-galactosidase (\(\beta\)-gal) activity.\textsuperscript{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-\(\alpha\)-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 \(\mu\)m) were analyzed for muscularization (\(\alpha\)-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-\(\beta\)-gal and anti-\(\alpha\)-SMA antibodies. The percentage of \(\alpha\)-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 \(\mu\)m. Values are mean±SEM (2-tailed Mann–Whitney). ns: non significant. FM indicates fully muscularized; NM, nonmuscularized; and PM, partially muscularized.
or interleukin-10, play a crucial role in the proliferation and differentiation capacities of stem/progenitor cells. Another common mechanism between the 2 models could involve HIF-1α as it is well established that inflammation causes a hypoxia-independent HIF-1α activation in response to the proinflammatory interleukin-1β. HIF-1α regulates stem cells mobilization and could therefore be involved in the recruitment of PW1+ progenitor cells.

Finally, we demonstrate the presence of the PW1+ cells in the parenchyma and perivascular zones in the control human lung. Remarkably, these cells are found as small perivascular clusters similar to pulmonary c-kit+ cells as observed by Kajstura et al. These PW1+ progenitor cells were mainly absent from nonremodeled vessel walls, but we found numerous PW1+ cells within the vascular wall and perivascular zone of the remodeled vessels in patients with PAH. This result confirms previous observations that progenitor cells are present in remodeled pulmonary arteries in lung tissues from patients with PAH. Interestingly, numerous PW1+/α-SMA+ cells were present in the hypertrophic media and in the neointima. We did not observe these features in the mouse lung vessels after 28 days of CH (data not shown). As human PAH lung samples are obtained from transplanted patients with an end-stage disease, the severe inflammatory context and the proliferative and SMC-like/myofibroblast dedifferentiation conditions could be involved in maintaining the PW1 expression in these mature and dysfunctional cells. This is supported by the sustained increase in the number of pulmonary PW1+ cells observed in the monocrotaline-treated rat model after 21 days, whereas in the chronic hypoxic mouse model, this number is back to control levels after 28 days of CH. This suggests that the sustained mobilization of pulmonary PW1+ cells is associated with the severity of the PH disease.

In conclusion, we show here that the lung contains 3 myogenic PW1+ progenitor cell populations that are resident in parenchymal and perivascular zones in the adult mouse lung. On the basis of their myogenic capacities both in vivo and in

Figure 8. The PW1+ progenitor cells are also present in the human lung and are numerous around and within the remodeled vessels of patients with pulmonary arterial hypertension (PAH). Human lung sections were stained for α-smooth muscle actin (SMA; red) and PW1 (green). Images are representative of lung tissues from control lungs (n=3; A) and from patients with PAH (n=3) B. We noticed the presence of PW1+ cells in parenchyma from control lung tissues and in perivascular zones from both control and PAH lung tissues (A, B1, and B3; white stars). In pulmonary arterial lesions of patients with PAH, we showed the presence of PW1+ cells expressing α-SMA+ in the neointima (yellow stars; B2), and in the nonthickened (B3) and hypertrophic (B4) media (yellow arrowheads). White dotted line represents autofluorescent internal elastic lamina. Confocal microscopy. Scale bars, 20 μm. L indicates lumen of vessel; m, media; and n, neointima.
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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References


Disclosures

None.
In human, PW1-expressing cells were also present near-pulmonary vessels and recruited when mice are subjected to chronic hypoxia. These PW1-expressing resident progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. Inhibition of the SDF-1/CXCR4 axis attenuates neonatal hypoxia-induced pulmonary hypertension. 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. Patient PW1-positive progenitor cells 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 arteries and were found abundant in remodeled vascular structures of PAH patients.
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 Dorfmüller, 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. \textit{PwI\_IRE\_SnLacZ} transgenic mice (\textit{PwI\_nlacZ/+,} in which a nuclear operon lactose gene is expressed under the control of the \textit{PwI} gene locus were produced in our animal husbandry\textsuperscript{1}. 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\% \textit{O}_2) 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 CO\textsubscript{2} and O\textsubscript{2} in the chamber were constantly monitored and maintained by flushing oxygen in the chamber and by absorbing CO\textsubscript{2} with soda lime granules. The chamber temperature was maintained at 22–24°C and excess humidity was prevented by dessicant.

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 200\textmu L). 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 amplificator 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 super-frost+ 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 PECy7, 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 Pharmigen) 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 (Attachment 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-β1 (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 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 C57BL/6J 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 10.10^6 cells in PBS freshly isolated from bone marrow of age and sex-matched GFP^+/β-gal^+ (H2B-GFPxPw1^nlacZ/+) or β-gal^+ (Pw1^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 ± 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 Kruskall-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**


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: 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: 20μm.
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 β-galactosidase (red) and CNN1 (green). b) The percentage of lung β-gal+/CNN1+ cells (yellow arrowheads) in muscularized vessels (<100 μm) was determined by immunofluorescence from normoxic (n=5), and CH 4 days (n=6) mice. The number of β-gal-expressing SMC was significantly increased after 4 days of CH. L: Lumen. Confocal microscopy. Scale bars: 20μm. Values are means ± SEM, ** p<0.01 versus N (two-tailed MW).
Online Figure IV

**A**  
**Endothelial cell differentiation**

<table>
<thead>
<tr>
<th>PW1+/CD34+/c-kit+</th>
<th>PW1+/CD34+/c-kit+/PDGFR-α*</th>
<th>PW1+/CD34+/CD146+</th>
<th>HUVECs</th>
</tr>
</thead>
</table>

vWF DAPI

**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: 20μm.

**B**

β-gal vWF α-SMA DAPI

**Online Figure V**

**Skeletal muscle cell differentiation**

<table>
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<th>PW1+/CD34+/c-kit+</th>
<th>PW1+/CD34+/c-kit+/PDGFR-α*</th>
<th>PW1+/CD34+/CD146+</th>
<th>Differentiated satellite cells</th>
</tr>
</thead>
</table>

MF20 DAPI

**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: 20μm.
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 ± 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 ± SEM, * p<0.05, **p<0.01, ****p<0.0001 vs N (normoxia) (KW-Dunn).
Online Figure VIII: Flow cytometry analysis of peripheral blood cells from (A) GFP⁺/β-gal⁺ BM-engrafted 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.
Online Table I

<table>
<thead>
<tr>
<th>PAH patients</th>
<th>gender</th>
<th>age (yrs)</th>
<th>BMPR2 mutation</th>
<th>PAP (mmHg)</th>
<th>CI (L/min/m²)</th>
<th>PVR (Wood units)</th>
<th>NYHA</th>
<th>Therapy</th>
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<tr>
<td>1</td>
<td>Female</td>
<td>31</td>
<td>No</td>
<td>61</td>
<td>1.6</td>
<td>14</td>
<td>III</td>
<td>IV prostacyclin+ERA+ PDE5-i</td>
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<td>50</td>
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<table>
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<th>Control patients</th>
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<th>Disease</th>
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<td>36</td>
<td>pulmonary adenocarcinoma</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>51</td>
<td>pulmonary adenocarcinoma</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>47</td>
<td>pulmonary adenocarcinoma</td>
</tr>
</tbody>
</table>

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

<table>
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<tr>
<th>Abs</th>
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<tr>
<td>CD45 PECy7</td>
<td>25-0451-82</td>
<td>eBioscience</td>
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<td>CD34 BV421</td>
<td>119321</td>
<td>Biolegend</td>
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<td>PDGFR-α FITC</td>
<td>11-1401-82</td>
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<td>CD146 PE</td>
<td>134704</td>
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<td>c-kit APC</td>
<td>17-1172</td>
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<td>c-kit PE</td>
<td>135105</td>
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<tr>
<td>Lin Pacific blue</td>
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<tr>
<td>Sca-1 PE</td>
<td>12-5981-82</td>
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<td>CXCR4 PE</td>
<td>551966</td>
<td>BD</td>
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Online Table II: FACS antibodies, references and companies are indicated.

Online Table III

<table>
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<tr>
<th>Abs</th>
<th>Ref</th>
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<tbody>
<tr>
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<td>Relaix et al, 1996</td>
<td>-</td>
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<td>OVN, 4°C</td>
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<td>Abcam</td>
<td>1/300</td>
<td>1h30, RT (OCT sections)</td>
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<td>1/50</td>
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<td>Sigma</td>
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<td>Torres Pines Biolabs</td>
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</tr>
</tbody>
</table>

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