Cell Autonomous and Non-cell Autonomous Regulation of SMC Progenitors in Pulmonary Hypertension

Graphical Abstract

Highlights

- KLF4 in primed SMCs is critical cell autonomously for initial distal migration in PH
- HIF1-α expression in primed cells is pivotal for subsequent steps of muscularization
- EC HIF1-α/PDGF-B regulates primed cell induction, proliferation, and differentiation
- Myeloid cell lineage marks SMCs and generates PDGF-B that induces muscularization

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In Brief
Sheikh et al. demonstrate that hypoxia-induced expression of KLF4 and HIF1-α in specialized lung arteriole SMC progenitors is required for distal migration and smooth muscle expansion, respectively. A HIF1-α/PDGF-B axis in endothelial cells non-cell autonomously regulates progenitor SMC induction, proliferation, and differentiation. The myeloid cell lineage marks SMCs.
Cell Autonomous and Non-cell Autonomous Regulation of SMC Progenitors in Pulmonary Hypertension

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SUMMARY

Pulmonary hypertension is a devastating disease characterized by excessive vascular muscularization. We previously demonstrated primed platelet-derived growth factor receptor β* (PDGF-R-β*)/smooth muscle cell (SMC) marker* progenitors at the muscular-unmuscular arteriole border in the normal lung, and in hypoxia-induced pulmonary hypertension, a single primed cell migrates distally and expands clonally, giving rise to most of the pathological smooth muscle coating of small arterioles. Little is known regarding the molecular mechanisms underlying this process. Herein, we show that primed cell expression of Kruppel-like factor 4 and hypoxia-inducible factor 1-α (HIF1-α) are required, respectively, for distal migration and smooth muscle expansion in a sequential manner. In addition, the HIF1-α/PDGF-B axis in endothelial cells non-cell autonomously regulates primed cell induction, proliferation, and differentiation. Finally, myeloid cells transdifferentiate into or fuse with distal arteriole SMCs during hypoxia, and Pdgfb deletion in myeloid cells attenuates pathological muscularization. Thus, primed cell autonomous and non-cell autonomous pathways are attractive therapeutic targets for pulmonary hypertension.

INTRODUCTION

Pulmonary hypertension (PH) is a grave disease marked by increased pulmonary arterial pressure and hypermuscularization of the lung vasculature. Treatment options are limited, and in severe cases, right heart failure and ultimately death ensue. Hypoxia and/or lung disease is a major cause of PH (World Health Organization [WHO] Group 3) and is characterized by smooth muscle cell (SMC) coating of the normally unmuscularized distal pulmonary arterioles (Arias-Stella and Saldana, 1963; Simonneau et al., 2013; Stenmark et al., 2006). While studies have shown extensive pathological changes in SMCs during the course of PH, there is limited understanding of the crosstalk between SMCs and other cell types that is undoubtedly integral to pathogenesis (Gao et al., 2016; Nogueira-Ferreira et al., 2014).

We have identified a specialized population of SMC progenitors that give rise to most hypoxia-induced distal arteriole SMCs in mice and initiated studies of the pathogenesis (Sheikh et al., 2014, 2015); however, critical aspects of the underlying mechanisms remain to be elucidated. We reasoned that these specialized cells are primed to muscularize the distal pulmonary arteriole because of their expression of the undifferentiated mesenchyme marker platelet-derived growth factor receptor β (PDGF-R-β) (in addition to SMC markers) and their position at the muscular-unmuscular border of each arteriole (Sheikh et al., 2015). With exposing mice to hypoxia, the ligand platelet-derived growth factor B (PDGF-B) is upregulated in the lung, which induces primed cell expression of the pluripotency factor Kruppel-like factor 4 (KLF4), and a single induced primed cell from each arteriole migrates distally and expands clonally, giving rise to pathological SMCs (Sheikh et al., 2015). The role of specific cellular sources of PDGF-B on primed cell biology and pathological muscularization have not been investigated. Similarly, hypoxia-inducible factors (HIFs) are implicated in pulmonary vascular remodeling (Brusselmans et al., 2003; Shimoda and Semenza, 2011; Yu et al., 1999), and the 5′ regulatory region of Klf4 includes a hypoxia response element, but the role of HIFs in hypoxic induction of primed cells is not known.

Furthermore, the effects of hypoxia on primed cell induction, migration, and proliferation are likely to depend on other cell types. Hypoxia induces endothelial cell (EC) expression of diverse agonists that have receptors on pulmonary artery (PA) SMCs and are implicated in PH and pulmonary vascular remodeling (Chen and Oparil, 2000; Dahal et al., 2011; Izikki et al., 2009; Luo et al., 2011; Nilsson et al., 2004; Savale et al., 2009; Wang et al., 2013; Yan et al., 1995). Yet EC-mediated regulation of primed cells has not been previously evaluated. In addition, macrophages are important players in PH pathogenesis, because they are found in the canonical plexiform lesions of vessels in pulmonary arterial hypertension (PAH) (WHO Group 1 classification of PH) (Rabinovitch et al., 2014; Tuder et al., 1994), and macrophage depletion attenuates PH and pulmonary arteriole media thickening in rat models (Rabinovitch et al., 2014; Thenappan et al., 2011; Tian et al., 2013; Zaloudikova et al., 2016). Understanding of macrophage-dependent effects on SMC biology is markedly limited in general and is essentially unknown in the context of PH.
**A**

**Normoxia**
- PDGFR-β
- SMA

**Hypoxia (day 1)**
- PDGFR-β
- SMA

**B**

- HIF1-α primed cells (%)
- Normoxia: HIF1-α
- Hypoxia (day 1): HIF1-α

**C**

**Normoxia**
- No tamoxifen
- Tamoxifen

**Hypoxia (day 21)**
- No tamoxifen
- Tamoxifen

**D**

- RVSP (mm Hg)
- Tamoxifen:
  - Normoxia
  - Hypoxia

**E**

- RV (LV+S)
- Tamoxifen:
  - Normoxia
  - Hypoxia

**F**

- PDGFR-β
- KLF4

**G**

- KLF4+ primed cells (%)
- Tamoxifen:
  - No tamoxifen
  - Tamoxifen

**H**

**Hypoxia (day 14):**
- Pdgfrb-CreER, Hif1α(flox/flox), ROSA26R(mTmG/+)

**I**

- Distal SMA* cells (%)
- Tamoxifen:
  - GFP CD68

**J**

- Days: 3, 5, 21

**K**

- No tamoxifen
- Tamoxifen

(legend on next page)
In the current study, we delineate cellular and molecular mechanisms underlying primed cell induction and expansion in the hypoxic model of PH and distinguish direct effects of hypoxia on primed cells and indirect effects via other cell types. Our findings indicate that primed cell expression of KLF4 and of HIF1-α is required in a cell autonomous manner for distal migration and distal arteriole SMC expansion, respectively. EC HIF1-α is critical for hypoxia-induced primed cell expression of KLF4, distal arteriole SMC proliferation and differentiation, and ultimately PH. Hypoxia induces EC PDGF-B expression, and PDGF-B is required for hypoxia-induced primed cell expression of HIF1-α. Similar to HIF1-α in ECs, EC-derived PDGF-B is critical for primed cell KLF4 expression, distal arteriole muscularization and SMC differentiation, and PH. Finally, ~10% of hypoxia-induced distal arteriole SMCs are marked by fate mapping of the monocyte or macrophage lineage, and deletion of Pdgfb in this lineage attenuates distal arteriole muscularization.

RESULTS

Primed Cell HIF1-α and KLF4 Play Distinct Roles in Distal Arteriole Muscularization

Similar to our prior studies, immunohistochemical analysis in this investigation focused on specific pulmonary arteriole beds adjacent to identified airway branches (left bronchus-first lateral secondary branch-first anterior branch [LL1.A1] or left bronchus-first medial branch [L.M1]) (Sheikh et al., 2014, 2015). Distal arterioles in these beds are unMuscularized under basal conditions and, with hypoxia exposure, undergo a stereotyped process of muscularization (Sheikh et al., 2014, 2015).

Myh11-CreERT2-mediated deletion of Hif1a attenuates hypoxia-induced pulmonary vascular remodeling (Bai et al., 2014), and we previously showed that SMMHC+ SMA PDGFR-β+ primed cells, located at muscular-unmuscular arteriole borders in the normal lung, give rise to hypoxia-induced distal pulmonary arteriole SMCs (Sheikh et al., 2015). Herein, we initially assessed the role of HIF1-α in PDGFR-β+ cells on hypoxia-induced distal muscularization and PH. Exposure of mice to hypoxia (10% FiO2) induces robust, widespread, and rapid upregulation of HIF1-α protein in the lung (Figure S1), and focusing specifically on primed cells, 82 ± 3% of these cells express HIF1-α at hypoxia day 1 (Figures 1A and 1B). Pdgfb-CreERT2, Hif1a(flox/flox) mice were induced with tamoxifen, rested, and then exposed to hypoxia for either 1 day to confirm HIF1-α deletion in PDGFR-β+ cells (Figure S2A) or 21 days to assess vascular remodeling and hemodynamics. In contrast to the heavily muscularized distal pulmonary arterioles of control-treated mice exposed to 21 days of hypoxia, the distal arterioles of tamoxifen-treated mice are only coated by sparse α-smooth muscle actin (SMA)+ cells (Figure 1C). In addition, Hif1a deletion in PDGFR-β+ cells attenuates PH and right ventricular hypertrophy (RVH) (Figures 1D and 1E) but does not alter KLF4 expression of primed cells in the early hypoxic lung (Figures 1F and 1G).

Through fate mapping of Pdgfrb-CreERT2, ROSA26R(mTmG+/+) mice, we previously demonstrated that most distal arteriole SMCs at hypoxia day 21 derive from PDGFR-β+ cells (Sheikh et al., 2015). Herein, Pdgfrb-CreERT2, Hif1a(flox/flox) mice were treated with tamoxifen, rested, and then exposed to hypoxia, and distal arteriole SMA+ cells were scored for expression of the GFP fate marker (Figures 1H, 1I, and S3). We also scored these cells for macrophage marker CD68 expression, because prior studies have found that bone marrow-transplanted cells in rodents contribute to lung vascular SMA+ cells in models of PH (Angelini et al., 2010; Hayashida et al., 2005; Spees et al., 2008; Yeager et al., 2011). Of the distal SMA+ cells at hypoxia day 14, 80% ± 2% are GFP+ and 11% ± 1% are CD68+, suggesting that, as explored more fully in Figure 6, some non-SMC-derived distal arteriole SMCs may originate from macrophages or upregulate a macrophage marker.

With hypoxia exposure, a primed SMC initially migrates beyond the muscular-unmuscular border at day 3 and then robustly proliferates, with distal arteriole SMC proliferation peaking at hypoxia day 7 (Sheikh et al., 2014, 2015). When tamoxifen is administered to Pdgfrb-CreERT2, Hif1a(flox/flox) mice on hypoxia days 3–5 (i.e., after the initial migration), the pattern of sparse SMC coating of the distal arteriole at hypoxia day 21 (Figures 1J and 1K) is indistinguishable from when tamoxifen is administered before hypoxia exposure (Figure 1C). This finding contrasts with the results for Klf4 deletion. KLF4 is expressed in primed cells at hypoxia day 2 (Sheikh et al., 2015), and hypoxia-induced distal muscularization, PH, and RVH are prevented by deleting Klf4 in SMA+ cells (Sheikh et al., 2015) or in PDGFR-β+ cells (Figures 2A–2C and S2B) before hypoxia treatment. Klf4 deletion in primed cells, however, does not alter HIF1-α expression in

Figure 1. HIF1-α Is Required for Distal Arteriole Muscularization and PH

(A) Wild-type mice were exposed to normoxia or hypoxia (10% FiO2) for 1 day, and then lung vibratome sections were stained for PDGFR-β, SMA, HIF1-α, and DAPI as indicated. The muscular-unmuscular borders of pulmonary arterioles near airway branch LL1.A1 (left bronchus-first lateral secondary branch-first anterior branch) are shown. The boxed regions in hypoxia are shown as close-ups on the right, with arrowheads indicating HIF1-α+ primed cells.

(B) Percentage of primed cells expressing HIF1-α at hypoxia day 1 is shown. n = 5 lungs, with 2–3 arterioles per lung. Total primed cells scored were 39 in normoxia and 37 in hypoxia.

(C–G) Pdgfrb-CreERT2, Hif1a(flox/flox) mice were injected with tamoxifen (1 mg/day for 5 days), rested, and then exposed to normoxia or hypoxia for 21 days (C–E) or 2 days (F and G). Arterioles were stained for SMA and panendothelial cell antigen (BECAM-32) in (C) and for PDGFR-β, KLF4, SMA, and DAPI in (F), as indicated. Right ventricle systolic pressure (RVSP) and the ratio of the weight of the right ventricle (RV) to that of the sum of the left ventricle (LV) and septum (S) are shown (D and E); n = 4 mice. The percentage of primed cells that are KLF4+ at hypoxia day 2 is quantified in (G); n = 4 mice. Total primed cells scored were 30 and 32 in no-tamoxifen and tamoxifen groups, respectively. NS, not significant.

(H and I) Arterioles of Pdgfrb-CreERT2, Hif1a(flox/flox), ROSA26R(mTmG+/+) mice were stained for GFP (lineage tag), SMA, and CD68 after 14 days of hypoxia in (H). The percentage of distal SMA+ cells expressing GFP or CD68 are quantified in (I); n = 3 mice and 3 arterioles per lung. 3S SMA+ cells were scored.

(J) Experimental strategy for (K), in which Pdgfrb-CreERT2, Hif1a(flox/flox) mice are induced with tamoxifen (1.5 mg/day) on days 3–5 of hypoxia.

(K) Arterioles were stained with SMA and MECA-32. n = 5 mice and 2–3 arterioles per lung.

All error bars indicate SD. Scale bars, 20 μm. See also Figures S1–S3.
primed cells of the early hypoxic lung (Figures 2D and 2E). In marked contrast to the effect of Klf4 deletion before hypoxia, tamoxifen treatment of Acta2-CreER\textsuperscript{T2}, Klf4\textsuperscript{(floxflox)} mice on hypoxia days 3–5 has no effect on distal muscularization at hypoxia days 7 or 21 (Figures 2F and 2G). Thus, KLF4 and HIF1-α are each critical in primed SMCs for distal muscularization but are so for distinct stages of the pathogenesis: KLF4 for initial breaching of the muscular-unmuscular border and HIF1-α for the subsequent robust clonal expansion of the primed cell lineage. In addition, deletion of Hif1a or Klf4 in PDGFR-β\textsuperscript{+} cells does not alter primed cell expression of the other transcription factor (Figures 1F, 1G, 2D, and 2E).

Figure 2. Primed Cell KLF4 Is Required for Distal Muscularization and PH

(A–E) Pdgfrb-CreERT2, Klf4\textsuperscript{(floxflox)} mice were induced with tamoxifen (1 mg/day for 5 days), rested for 5 days, and then exposed to normoxia or hypoxia for 1 day (D and E) or 21 days (A–C). Arterioles near L.L1.A1 were stained for SMA and MECA-32 in (A) and for HIF1-α, PDGFR-β, and DAPI in (D). RVSP and RV weight ratio are shown in (B and C), respectively, n = 5 mice. The percentage of HIF1-α\textsuperscript{+} primed cells at hypoxia day 1 is shown in (E); n = 4 mice, with 2–3 arterioles per lung. Total primed cells scored were 26 and 29 in no-tamoxifen and tamoxifen groups, respectively. ns, not significant.

(F) Experimental strategy for (G), in which Acta2-CreER\textsuperscript{T2}, Klf4\textsuperscript{(floxflox)} mice are induced with tamoxifen (1.5 mg/day) from hypoxia days 3–5.

(G) Arterioles of Acta2-CreER\textsuperscript{T2}, Klf4\textsuperscript{(floxflox)} mice were stained with SMA, MECA-32, and KLF4 after days 7 or 21 of hypoxia. n = 5 mice, with 2–3 arteriole per lung. All error bars indicate SD. Scale bars, 20 μm. See also Figure S2.
Figure 3. Endothelial Hif1a Deletion Attenuates Distal Muscularization and PH and Perturbs SMC Differentiation

Cdh5-CreER<sup>2</sup>, Hif1a<sup>flox/flox</sup> mice were injected with tamoxifen (1 mg/day for 5 days), rested for 5 days, and then exposed to normoxia or hypoxia for indicated duration. Arterioles near L.L1.A1 airway branches were analyzed in lung vibratome sections stained for SMA. Sections were also stained for PDGF-B and MECA-32 in (A); PDGFR-β, KLF4, and DAPI in (D); PDGFR-β and HIF1-α in (F); and SMMHC, PDGFR-β, and DAPI in (H). (B and C) RVSP and weight ratio are shown; n = 4 mice. The percentage of primed cells at hypoxia day 2 that are KLF4<sup>+</sup> is quantified in (E); n = 4 mice. Total primed cells scored were 33 and 30 in no-tamoxifen and tamoxifen groups, respectively. In (G), the percentage of SMA “PDGFR-β<sup>+</sup>” cells at hypoxia day 1 (primed cells) or hypoxia day 5 (middle or distal arteriole SMCs) that are also HIF1-α<sup>+</sup> is shown. n = 4 mice. 31 cells were scored at hypoxia day 1 for the no-tamoxifen group and 32 cells were scored for the
Endothelial HIF1-α Regulates Primed Cell Induction and Distal Muscularization

We next evaluated the effect of Hif1a deletion in ECs on hypoxia-induced distal muscularization and PH. Cdh5-CreERT2, Hif1aflox/flox mice were or were not induced with tamoxifen, and then mice were exposed to normoxia or hypoxia. With 21 days of hypoxia, similar to wild-type mice (Sheikh et al., 2014), Cdh5-CreERT2, Hif1aflox/flox mice that were not pre-treated with tamoxifen have a continuous single layer of SMA+ cells coating the distal arteriole (Figure 3A). However, following tamoxifen pre-treatment, hypoxic exposure results in sparse SMA+ cells that are loosely adherent to the underlying distal arteriole EC tube, often forming clumps of cells, and PH and RVH do not ensue (Figures 3A–3C). EC deletion of Hif1a decreases Pdgfb levels in lung ECs isolated from hypoxic mice (Figures S2C and S2D; Table S1) and markedly reduces the number of primed or distal arteriole SMCs expressing KLF4 at hypoxia day 2 or 7, respectively (Figures 3D, 3E, S4A, and S4B). In addition, the percentage of distal arteriole SMCs that are HIF1-α- at hypoxia day 5 is reduced by more than two-fold (Figures 3F and 3G). At hypoxia day 7, distal arteriole SMA+ cells normally express PDGFR-β, but not smooth muscle myosin heavy chain (SMMHC), and are highly proliferative (Sheikh et al., 2014). At this time in tamoxifen-pretreated Cdh5-CreERT2, Hif1a(flox/fox) mice, there is a 30% ± 2% reduction in distal SMA+ cells expressing PDGFR-β and a corresponding 31% ± 3% increase in SMA+SMMHC+ cells (Figures 3H, S4I, and S4J). In addition, proliferation is reduced by five-fold in these SMA+ cells (Figures S4D and S4E).

Under normoxic conditions, HIF-α protein levels are kept at a minimum by proline hydroxylation, which facilitates binding to the von Hippel-Lindau (VHL)-E3 ubiquitin ligase complex (Semenza, 2012). This complex induces protein ubiquitination and ultimately proteasomal-mediated degradation. With hypoxia, oxygen is not available as a substrate for proline hydroxylation, and hence, HIF-α accumulates. As a means of increasing HIF-α levels in ECs without hypoxia, we induced Cdh5-CreERT2, Vhl(flox/fox) mice with tamoxifen and then allowed them to rest for up to 47 days under normoxic conditions. HIF1-α and Pdgfb are markedly upregulated in lung ECs and in the lung parenchyma of these mice, and distal arterioles become muscularized (Figures 4A, 4B, and S2E). Similarly, KLF4 is expressed in distal arteriole SMCs (Figure 4B). Under normoxic conditions, primed cells are present at the muscular-unmuscular (i.e., middle-distal) arteriole border and are not induced (i.e., KLF4−) in wild-type mice (Sheikh et al., 2015). 12 days after EC Vhl deletion, primed cells remain present at the middle-distal arteriole border; however, 83.2% ± 2.5% of primed cells express KLF4 (Figures 4C and 4D). These results suggest that even under normoxic conditions, EC upregulation of HIF induces Pdgfb-B expression, as well as non-cell autonomous KLF4 expression in primed and distal arteriole SMCs, and distal arteriole muscularization ensues.

EC-Derived PDGF-B Regulates Primed Cell Induction and Distal Arteriole Muscularization

We next endeavored to investigate the role of EC-derived PDGF-B in hypoxia-induced PH. Hypoxia treatment of mice increases Pdgfb-B in lung ECs (Sheikh et al., 2013), and lung Pdgfb levels peak at hypoxia day 2 (Figure S5). In Pdgfb+/− mice, hypoxia-induced primed cell induction, distal muscularization, and PH are attenuated (Sheikh et al., 2015), and primed cell HIF1-α expression was almost abrogated at hypoxia day 1 (Figures 5A and 5B). To evaluate the effect of deleting Pdgfb from ECs (Figure S2F), Cdh5-CreERT2, Pdgfb(flox/fox) mice were induced with tamoxifen, rested, and then exposed to hypoxia. At hypoxia day 21, these mice have distal pulmonary arterioles covered by sparse clumps of SMA+ cells and have normal PA pressure and right ventricle (RV) weight ratio (Figures 5C–5E). EC deletion of Pdgfb results in a four-fold reduction in the percentage of primed cells expressing KLF4 at hypoxia day 2 (Figures 5F and 5G). Finally, tamoxifen pre-treatment of Cdh5-CreERT2, Pdgfb(flox/fox) mice induces a ~34% reduction of distal arteriole SMA+ cells expressing PDGFR-β and a corresponding ~33% increase in the SMA+SMMHC+ cells (Figures 5H, 5I, and 5J). Thus, EC deletion of Hif1a or Pdgfb before hypoxia exposure results in strikingly similar phenotypes (Figures 3 and 5).

Macrophages and Distal Pulmonary Arteriole Muscularization

Hypoxic exposure of Cdh5-CreERT2 mice carrying Hif1a(flox/fox) or Pdgfb(flox/fox) (after tamoxifen pre-treatment) induces the accumulation of distal arteriole SMA+ cells (Figures 3A and 5C), whereas Pdgfb+/− mice do not develop hypoxia-induced distal muscularization (Sheikh et al., 2015). These findings suggest that a non-EC source of PDGF-B is likely integral for pulmonary vascular remodeling. Macrophages produce PDGF-B (van Steensel et al., 2012), and hypoxia results in macrophage recruitment to the lung, which is implicated in hypoxia-induced PH (Amsellem et al., 2017; Frid et al., 2006; Vergadi et al., 2011). To evaluate the role of macrophages in distal muscularization, we initially conducted a timeline of anti-CD68 staining in lung vibratome sections from mice exposed to normoxia or hypoxia for up to 21 days (Figure S6A). Many CD68+ cells are present in the lung parenchyma and vasculature by day 2, and some CD68+ cells in the vasculature of the hypoxic lung at day 14 are SMA+. Deletion of Hif1a or Klf4 in primed cells or of Hif1a, Vhl, or Pdgfb in ECs does not affect the accumulation of CD68+ cells in the hypoxic lung (Figures S6B–S6F).

We next fate mapped monocytes and macrophages during hypoxia exposure, using mice carrying Csf1r-Mer-iCre-Mer (Qian et al., 2011) and ROSA26R(R1mTmG/+). Under normoxic conditions, tamoxifen treatment of Csf1r-Mer-iCre-Mer, ROSA26R(R1mTmG/+ mice results in marking of CD68+ cells, but not arterial SMCs (A. Misra, Z. Feng, R. Chandran, I. Kabir, N. Rottlan, B. Aryal, A.O.S., L. Ding, L. Qin, C. Fernández-
Hernando, G. Tellides, and D.M.G., unpublished data). Herein, these mice were induced with tamoxifen, rested, and then exposed to hypoxia for 19–21 days, at which time 12% of distal pulmonary arteriole SMCs (as indicated by SMA or SMMHC staining) express the GFP fate marker (Figures 6A, 6B, S6G, and S6H). Rare GFP+SMC marker+ cells in the distal pulmonary arteriole express CD68 at hypoxia day 19, but most are CD68− (Figures 6A and S6G), and at hypoxia day 21, GFP+SMC marker−CD68+ cells are not apparent (Figure 6B). Conversely, in Acta2-CreERT2, ROSA26R (mTmG+/+) mice induced with tamoxifen and exposed to hypoxia for 19 days, distal arteriole SMA+CD68+ cells are GFP− (Figure 6C). These results suggest that during hypoxia-induced PH, CSF1R+ cells are recruited into the pulmonary arteriole, and some transdifferentiate into SMC marker+ cells and/or fuse with SMCs, whereas pre-existing SMCs do not give rise to pulmonary arteriole SMA+CD68+ cells.

At hypoxia day 7, PDGF-B protein is located broadly in the lung parenchyma (Figures 6D and 6E) (Sheikh et al., 2015). Cdh5-CreER2 mice carrying Hif1a(flox/flox) or Pdgfb(flox/flox) treated with tamoxifen and then exposed to hypoxia for 7 days have a markedly reduced PDGF-B signal on lung immunohistochemical staining (Figures 6D and 6E). The residual PDGF-B expression is predominantly located near CD68+ cells, suggesting that macrophages are a substantial source of PDGF-B in the hypoxic lung. Thus, Csf1r-Mer-iCre-Mer was used to delete Pdgfb in monocytes and macrophages (Figure S2G), and to assess the role of PDGF-B derived from these cells in hypoxia-induced pulmonary arteriole remodeling, Csf1r-Mer-iCre-Mer, Pdgfb(flox/flox) mice were induced with tamoxifen and exposed to hypoxia for 21 days. There are many gaps in the SMA+ cell coating of the distal arterioles of these mice, evocative of a Swiss cheese pattern of muscularization (Figure 6F).
Figure 5. Endothelial-Specific Pdgfb Deletion Attenuates Distal Muscularization, KLF4 Expression, and PH and Perturbs SMC Differentiation

(A) Wild-type and Pdgfb\(^{+/+}\) mice were exposed to normoxia or hypoxia for 1 day, and then lung vibratome sections were stained for SMA, HIF1-\(\alpha\), PDGFR-\(\beta\), and DAPI.

(B) Quantification of primed cells that express HIF1-\(\alpha\) at day 1 of hypoxia. \(n = 5\) mice of each genotype, with 2–3 arterioles per mouse. 42 and 39 primed cells were scored in wild-type and Pdgfb\(^{+/+}\) lungs, respectively.

(C) Notoxifen

(D) Hypoxia (day 21)

(E) Hypoxia (day 2)

(F) No tamoxifen

(G) Tamoxifen

(H) Hypoxia (day 7)

(I) Distal SMA\(^+\) (%)

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DISCUSSION

Alveolar hypoxia resulting from chronic lung diseases, impaired breathing, or prolonged exposure to high altitude is a major cause of PH and is associated with pulmonary vascular remodeling. For instance, among humans who live at high altitudes, up to ~18% have PH and many have muscularized distal pulmonary arterioles that are not muscularized in control inhabitants of sea-level elevations (Arias-Stella and Saldana, 1963; Mirrakhimov and Strohl, 2016). Moreover, exposure of mice to chronic hypoxia induces PH and muscularization of distal arterioles (Rabinovitch, 2007; Stenmark et al., 2006, 2009). We have identified specialized primed PDGFR-β⁺ SMC progenitors at the muscular-unmuscular arteriolar border of the normal lung that give rise to most hypoxia-induced distal arteriolar SMCs (Sheikh et al., 2015). In the current study, we delineate mechanisms of primed cell induction and expansion through direct effects of hypoxia on primed cells and indirect effects via hypoxic ECs; we also analyze the role of macrophages in the distal muscularization process (Figure 7).

Our findings indicate that transcription factors KLF4 and HIF1-α play discrete and critical cell autonomous roles in primed cells during the pathogenesis of hypoxia-induced PH (Figures 1 and 2). In SMCs, KLF4 is integral in the pathogenesis of mouse models of diverse vascular diseases (Salmon et al., 2013; Shankman et al., 2015; Sheikh et al., 2015), but its role specifically in specialized SMC progenitors was not previously established. We observe that hypoxia-exposed, tamoxifen-induced Pdgfrb-CreER², Klf4(flox/flox) mice lack distal arteriole SMCs and do not develop PH. Thus, primed cell expression of KLF4 is essential cell autonomously for breaching the muscular-unmuscular border and initiating the muscularization process; however, KLF4 is not required for the subsequent steps of expansion of the early distal arteriole SMCs. In contrast to KLF4, primed cell HIF1-α is not required for SMCs to reach the distal pulmonary arteriolar under hypoxia but is critical for expansion of the number of SMCs in the distal compartment. Previously, Hif1a(+/-) mice have been shown to be protected against hypoxia-induced PH and pulmonary arteriolar muscularization (Brusselmans et al., 2003; Yu et al., 1999), as are mice with SMC deletion of Hif1a (Ball et al., 2014; Kim et al., 2013). These latter investigations used Hif1a(flox/flox) mice that carry Tagln-Cre or Myh11-CreER² (Ball et al., 2014; Kim et al., 2013), either of which would be expected to delete Hif1a in primed cells. Tamoxifen-induced Myh11-CreER², Hif1a(flox/flox) mice develop hypoxia-induced RVH (Ball et al., 2014), whereas Pdgfrb-CreER², Hif1a(flox/flox) do not (Figure 1), suggesting that hypoxia-induced HIF1-α in PDGFR-β⁺ pericytes of the heart may contribute to RVH. Based on our results, together with prior studies, we speculate that the protection against hypoxia-induced PH with SMC deletion of Hif1a results from Hif1a deletion in primed SMC progenitors.

Beyond the direct effects of hypoxia on SMCs, hypoxic ECs secrete factors that modulate the biology of SMCs (fzikki et al., 2009; Luo et al., 2011; Nilsson et al., 2004; Savale et al., 2009; Sheikh et al., 2015; Wang et al., 2013); however, such indirect effects of ECs on primed cells have not been previously investigated. Under normoxic conditions, enzymes containing the prolyl hydroxylase domain (PHD) use molecular oxygen as a substrate and catalyze the hydroxylation of prolines on HIF-α; in turn, VHL ubiquitinates hydroxylated HIF-α, targeting it for proteasome-mediated degradation (Semenza, 2012). In contrast, with hypoxia, PHD activity is impaired and HIF-α accumulates (Semenza, 2012). Prior studies have demonstrated that constitutive deletion of the gene encoding PHD2 with Cdh5-Cre or Tie2-Cre induces pulmonary vascular remodeling and PH (Dai et al., 2016; Kapitsinou et al., 2016; Wang et al., 2016), and humans or mice with a germline homozygous mutation in VHL develop polycythemia and PH (Hickey et al., 2010; Smith et al., 2006). In addition, EC-specific Hif1a deletion protects against hypoxia-induced pulmonary vascular remodeling, PH, and RVH (Cowburn et al., 2016; Kapitsinou et al., 2016). Herein, we find that EC deletion of Vhl results in enhanced lung expression of HIF1-α and PDGF-B, primed cell induction, and distal arteriolar muscularization (Figure 4). Specific and conditional deletion of Hif1a in ECs attenuates hypoxia-induced primed cell KLF4 expression and distal arteriolar accumulation and proliferation, and in contrast to the stereotyped pattern of distal muscularization in controls, distal arteriolar SMCs in mutants have perturbed expression of differentiation markers and form clumps, although not all SMCs are directly exposed to ECs (Figure 3). These mutants are protected against hypoxia-induced PH or RVH. EC deletion of Pdgfb phenocopies EC-specific Hif1a mutants (Figure 5), suggesting that in mice exposed to hypoxia, enhanced HIF1-α upregulates EC secretion of PDGF-B, which induces primed cells. In contrast to Pdgfb(+/-) mice, which lack hypoxia-induced distal arteriolar muscularization (Sheikh et al., 2015), Cdh5-CreER², Pdgfb(flox/flox) mice have some distal SMCs, suggesting that non-EC sources of PDGF-B (e.g., platelets and macrophages) are important in pathological SMC recruitment.

In further studies, we elected to evaluate macrophages in hypoxia-induced PH because macrophages secrete PDGF-B (van Steensel et al., 2012), and inflammation is an understudied area of high interest in PH (Erzurum et al., 2010; Nicolls and Voelkel, 2017; Rabinovitch et al., 2014). Only a few studies touch upon the interplay between macrophages and SMCs in PH. These investigations demonstrate that media conditioned from alveolar macrophages cultured in severe hypoxia (0.5% O₂ for...
Csf1r-Mer-iCre-Mer, ROSA26R\(^{(mTmG/+)}\)

A  

| Condition        | Hypoxia (day 19) | Hypoxia (day 21) |
|------------------|------------------|------------------|
| **GFP SMA**      |                  |                  |
| **SMA CD68**     |                  |                  |

B  

| Condition        | Hypoxia (day 21) |
|------------------|------------------|
| **SMA CD68**     |                  |

C  

| Condition        | Hypoxia (day 19) | Hypoxia (day 21) |
|------------------|------------------|------------------|
| **GFP SMA**      |                  |                  |
| **SMA CD68**     |                  |                  |

D  

| Condition        | Hypoxia (day 7) |
|------------------|-----------------|
| **Cdh5-CreER, Hif1a\(^{(flox/flox)}\)** |                  |

E  

| Condition        | Hypoxia (day 7) |
|------------------|-----------------|
| **Cdh5-CreER, Pdgfb\(^{(flox/flox)}\)** |                  |

F  

| Condition        | Hypoxia (day 21) |
|------------------|------------------|
| **Csf1r-Mer-iCre-Mer, Pdgfb\(^{(flox/flox)}\)** |                  |

(legend on next page)
48 hr) (Vergadi et al., 2011) or obtained from mice exposed to hypoxia (9% FiO2) (Amsellem et al., 2017) induce an increase of less than two-fold in proliferation of mouse PA SMCs. A similar effect is reported when leukotriene B4 or the chemokine CXCL1, which are both known to be expressed by macrophages, as well as other cell types, are added to cultured PA SMCs (Amsellem et al., 2017; Tian et al., 2013).

Our investigations demonstrate that ∼10% of hypoxia-induced distal pulmonary arteriolar SMCs (Amsellem et al., 2017; Tian et al., 2013) are labeled by fate-mapping cells expressing the monocyte or macrophage marker Csf1r (Figure 6). Prior studies with bone marrow-transplanted rodents in various models of PH indicate that bone marrow-derived cells are recruited into the lung and pulmonary vasculature and many of these cells express SMA (Angelini et al., 2010; Hayashida et al., 2005; Spees et al., 2008; Yeager et al., 2011); however, none of these investigations have demonstrated bone marrow-derived cells in the vasculature expressing the more specific markers of mature smooth muscle (i.e., SMMHC and smoothelin). Similarly, in humans or mice that have undergone bone marrow transplant and develop atherosclerosis, 5%–10% of SMA+ plaque cells derive from hematopoietic cells (Caplice et al., 2003; Iwata et al., 2010). The Myh11 transcriptional program is not active in bone marrow-derived plaque cells of Apoe<sup>−/−</sup> mice (Iwata et al., 2010); however, one image from a study of bone marrow-transplanted humans indicates that some SMMHC+ coronary atherosclerotic plaque cells derive from the bone marrow (Caplice et al., 2003). Finally, our results demonstrate that Pdgfb deletion in CSF1R<sup>+</sup> cells substantially abrogates hypoxia-induced distal pulmonary arteriolar muscularization.

Altogether, our findings emphasize the importance of primed cell autonomous and non-cell autonomous pathways in the context of hypoxia-induced distal arteriolar muscularization and PH. Primed cell expression of KLF4 is requisite for initial breach-ing of the muscular-unmuscular border, and subsequently, primed cell HIF1-α is integral for the expansion of early distal arteriolar SMCs. In addition to these cell autonomous effects, an EC HIF1-α/PDGF-B axis regulates primed cell induction, proliferation, and stereotyped differentiation. Finally, macrophages contribute to distal arteriolar muscularization through both transdifferentiation to SMMHC<sup>+</sup> cells and macrophage-derived PDGF-B. The interplay among distinct cell types is critical for the pathogenesis of diverse vasculoproliferative diseases but generally is understudied and poorly understood. Thus, pathological studies delineating intercellular mechanisms—such as this investigation of specialized SMC...
progenitors in PH—are needed, because the resulting insights promise to have major ramifications for the development of novel therapeutic strategies.

**EXPERIMENTAL PROCEDURES**

**Animals and Tamoxifen Treatment**

All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University. Mouse strains are described in Supplemental Information. Studies used male and female mice of 1.5–4 months of age. For CreER-catalyzed recombination, mice were injected intraperitoneally with tamoxifen (1 mg/day for 5 days), rested for 5 days, and then exposed to normoxia or hypoxia, unless otherwise noted. For experiments with injections during hypoxia days 3–5, tamoxifen (1.5 mg/day) was used. Because of low recombination efficiency, experiments with Csf1r-Mer-iCre-Mer mice used daily tamoxifen injections (1 mg/day) for 15–20 days.

**Hypoxia Treatment and Hemodynamic Measurements**

Adult mice were exposed to hypoxia (10% FiO2) for up to 21 days in a rodent hypoxia chamber equipped with a calibrated controller and an oxygen sensor (BioSpherix). Right ventricle systolic pressure (RVSP; equivalent to PA systolic pressure) and the weight ratio of the RV to the sum of the left ventricle (LV) and septum (S) were measured (Sheikh et al., 2014, 2015).

**Lung Preparation**

After appropriate hypoxia or normoxia exposure, mice were euthanized by isoflurane inhalation, the pulmonary vasculature was flushed with PBS, and the lungs were inflated with 2% low-met agarose. Solidified agarose-filled lobes were immersed in Dent’s fixative (4:1 methanol:DMSO) at 4°C overnight and then washed and stored in 100% methanol (Sheikh et al., 2014, 2015). Before immunohistochemical analysis, lungs were bleached in 5% H2O2, rehydrated into PBS, and vibratome sectioned at a thickness of 150 μm.

**Immunohistochemical Analysis**

Vibratome lung sections were blocked with 5% normal goat serum in 0.5% Triton X-100/PBS (PBS-T) at 4°C overnight. Sections were then incubated in primary antibodies for 1–3 days at 4°C, washed in PBS-T, incubated in secondary antibodies overnight at 4°C, washed again in PBS-T, and placed on slides in mounting media (Dako). Primary antibodies used were rat anti-MECA-32 (1:15, Developmental Studies Hybridoma Bank [DSHB]), rabbit anti-GFP (1:250, Invitrogen), rabbit anti-SM-MHC (1:250, Biomedical Technologies), rabbit anti-PDGFR-B (1:100, Abcam), goat anti-KLF4 (1:100, R&D Systems), rabbit anti-HIF1α (1:100, Novus Biologicals), rabbit anti-pH3 (1:200, Millipore), rat anti-CD68 (1:200, Bio-Rad), directly conjugated Cy3 or fluorescein isothiocyanate (FITC) mouse anti-SMA clone 1A4 (1:250, Sigma), and goat biotinylated anti-PDGFR-β (1:10; R&D Systems). ABC Elite reagents (Vector Laboratories) and fluorescein tyramide system (PerkinElmer) were used to amplify the biotinylated PDGFR-β staining as described previously (Gref et al., 2012; Metzger et al., 2008). Secondary antibodies were conjugated to Alexa 488, Alexa 564, or Alexa 647 (Invitrogen) or to DyLight 649 (Jackson Laboratory) fluorophores (1:500). Nuclei were stained with DAPI (1:500).

**In Vivo Quantification**

For quantification of immunohistochemical data, the number of cells was quantified on confocal sections by counting the DAPI-marked nuclei co-localizing with stains of specific markers. The total number of primed cells (PDGFR-β*SMAMDAPI* cells) at the muscular-unmuscular border of specific pulmonary arterioles or distal arteriole SMCs (DAPI* cells expressing SMA or SM-MHC) were used in calculations. The results for up to three specific arterioles (Sheikh et al., 2014) were determined, and the values for each arteriole were averaged to yield the results for each lung. These individual lung results were then averaged, and the SD was determined to yield the overall results that were plotted in graphs and reported.

**Imaging**

Lung sections were imaged on confocal microscopes (PerkinElmer UltraView VOX spinning disc or Leica SP8 point scanning). Velocity software (PerkinElmer) and Adobe Photoshop was used to process images.

**Statistical Analysis**

All data are presented as mean ± SD. Student’s t test, multi-factor ANOVA, and post hoc test with Bonferroni corrections were used to analyze the data (GraphPad Prism software). The statistical significance threshold was set at p ≤ 0.05. All tests assumed normal distribution and were two sided.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.043.

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**AUTHOR CONTRIBUTIONS**

A.Q.S., F.Z.S., A.N., and D.M.G. conceived and designed experiments and analyzed results. A.Q.S., F.Z.S., A.N., and R.M. performed experiments. A.Q.S., R.M., and D.M.G. wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**

Amsellem, V., Abid, S., Poupel, L., Parpaleix, A., Rodero, M., Gary-Bobo, G., Latiri, M., Dubois-Rande, J.L., Lipskia, L., Combediere, C., and Adnot, S. (2017). Roles for the CX3CL1/CX3CR1 and CCL2/CCR2 chemokine systems in hypoxic pulmonary hypertension. Am. J. Respir. Cell Mol. Biol. 56, 597–608.

Angelini, D.J., Su, Q., Kolosova, I.A., Fan, C., Skinner, J.T., Yamaji-Kegan, K., Collector, M., Sharkis, S.J., and Johns, R.A. (2010). Hypoxia-induced mitogenic factor (HIMF/FIZZ1/RELM alpha) recruits bone marrow-derived cells to the murine pulmonary vasculature. PLoS ONE 5, e11251.

Arias-Stella, J., and Saldana, M. (1963). The terminal portion of the pulmonary arteriole tree in people native to high altitudes. Circulation 28, 915–925.

Ball, M.K., Waypa, G.B., Mungai, P.T., Nielsen, J.M., Czech, L., Dudley, V.J., Beussink, L., Dettrman, R.W., Berkelhamer, S.K., Steinhom, R.H., et al. (2014). Regulation of hypoxia-induced pulmonary hypertension by vascular smooth muscle hypoxia-inducible factor-1α. Am. J. Respir. Crit. Care Med. 189, 314–324.

Brusselmans, K., Compernolle, V., Tjwa, M., Wessener, M.S., Maxwell, P.H., Collen, D., and Carmeliet, P. (2003). Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and...
right ventricular dysfunction during prolonged hypoxia. J. Clin. Invest. 111, 1519–1527.

Caplice, N.M., Bunch, T.J., Stalboerger, P.G., Wang, S., Simper, D., Miller, D.V., Russell, S.J., Litzow, M.R., and Edwards, W.D. (2003). Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. Proc. Natl. Acad. Sci. USA 100, 4754–4759.

Chen, Y.F., and Oparil, S. (2000). Endothelin and pulmonary hypertension. J. Cardiovasc. Pharmacol. 35, Suppl. 2, S49–S53.

Cowburn, A.S., Crosby, A., Macias, D., Branco, C., Colago, R.D., Southwood, M., Toshner, M., Crotty Alexander, L.E., Morrell, N.W., Chivers, E.R., and Johnson, R.S. (2016). HIF2α-arginase axis is essential for the development of pulmonary hypertension. Proc. Natl. Acad. Sci. USA 113, 8801–8806.

Dahal, B.K., Heuchel, R., Pullamsetti, S.S., Wilhelm, J., Ghofrani, H.A., Weissman, N., Seeger, W., Grimminger, F., and Schermuly, R.T. (2011). Hypoxic growth factor receptor-

Erzurum, S., Rounds, S.I., Stevens, T., Aldred, M., Aliotta, J., Archer, S.L., Aso, K., Balaban, R., Bauer, N., Bhattacharya, J., et al. (2010). Strategic plan for lung vascular research: an NHLBI-ORDR workshop report. Am. J. Respir. Crit. Care Med. 182, 1554–1562.

Frid, M.G., Brunetti, J.A., Burke, D.L., Carpenter, T.C., Davie, N.J., Reeves, J.T., Roedersheimer, M.T., van Rooijen, N., and Stenmark, K.R. (2006). Hypoxia-induced pulmonary vascular remodeling requires recruitment of circulating mesenchymal precursors of a monocyte/macrophage lineage. Am. J. Pathol. 168, 659–669.

Gao, Y., Chen, T., and Raj, J.U. (2016). Endothelial and smooth muscle cell interactions in the pathobiology of pulmonary hypertension. Am. J. Respir. Crit. Care Med. 193, 451–460.

Greif, D.M., Kumar, M., Lighthouse, J.K., Hum, J., An, A., Ding, L., Red-Horse, A., Kuro-o, M., Sata, M., and Nagai, R. (2010). Bone marrow-derived cells contribute to pulmonary vascular remodeling in hypoxia-inducible pulmonary hypertension. Chest 137, 1793–1798.

Hayashida, K., Fujita, J., Miyake, Y., Kawada, H., Ando, K., Ogawa, S., and Fukuda, K. (2005). Bone marrow-derived cells contribute to pulmonary vascular remodeling in hypoxia-inducible pulmonary hypertension. Chest 127, 168–172.

Izikki, M., Guignabert, C., Fadel, E., Humbert, M., Li, J., Zhang, Y., Kato, T., et al. (2014). Effect of IL-1β on hypoxia-inducible factor-1α- and MMP-9-positive circulating cells. J. Immunol. 193, 497–505.

Kapitsinou, P.P., Rajendran, G., Asteford, L., Michael, M., Schonfeld, M.P., Fields, T., Shay, S., French, J.L., West, J., and Haase, V.H. (2016). The endothelial prolyl-4-hydroxylase domain 2/hypoxia-inducible factor 2 axis regulates pulmonary artery pressure in mice. Mol. Cell. Biol. 36, 1584–1594.

Kim, Y.M., Barnes, E.A., Alivia, C.M., Ying, L., Reddy, S., and Cornfield, D.N. (2013). Hypoxia-inducible factor-1α in pulmonary artery smooth muscle cells lowers vascular tone by decreasing myosin light chain phosphorylation. Circ. Res. 112, 1230–1233.

Luo, J., Qiao, F., and Yin, X. (2011). Hypoxia induces FGF2 production by vascular endothelial cells and alters MMP9 and TIMP1 expression in extravilous trophoblasts and their invasiveness in a cocultured model. J. Reprod. Dev. 57, 84–91.

Metzger, R.J., Klein, O.D., Martin, G.R., and Krasnow, M.A. (2008). The branching programme of mouse lung development. Nature 453, 745–750.

Mirkakimov, A.E., and Strohl, K.P. (2016). High-altitude pulmonary hypertension: an update on disease pathogenesis and management. Open Cardiovasc. Med. J. 10, 19–27.

Nicolis, M.R., and Voelkel, N.F. (2017). The roles of immunity in the prevention and evolution of pulmonary arterial hypertension. Am. J. Respir. Crit. Care Med. 195, 1292–1299.

Nilsson, L., Shibuya, M., and Wennström, S. (2004). Differential activation of vascular genes by hypoxia in primary endothelial cells. Exp. Cell Res. 299, 478–485.

Nogueira-Ferreira, R., Ferreira, R., and Henriques-Coelho, T. (2014). Cellular interplay in pulmonary arterial hypertension: implications for new therapies. Biochim. Biophys. Acta 1843, 885–893.

Qian, B.Z., Li, J., Zhang, H., Kitamura, T., Zhang, J., Campion, L.R., Kaiser, E.A., Snyder, L.A., and Pollard, J.W. (2011). CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 475, 222–225.

Rabinovitch, M. (2007). Pathobiology of pulmonary hypertension. Annu. Rev. Pathol. 2, 369–399.

Rabinovitch, M., Guignabert, C., Humbert, M., and Nicoll, M.R. (2014). Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. Circ. Res. 115, 165–175.

Salmón, M., Johnston, W.F., Woo, A., Pope, N.H., Su, G., Upchurch, G.R., Jr., Owens, G.K., and Alliawadi, G. (2013). KLF4 regulates abdominal aortic aneurysm morphology and deletion attenuates aneurysm formation. Circulation 128 (Suppl 1), S163–S174.

Savale, L., Tu, L., Rideau, D., Izziki, M., Maitre, B., Adnot, S., and Edddahihi, S. (2009). Impact of interleukin-6 on hypoxia-induced pulmonary hypertension and lung inflammation in mice. Respir. Res. 10, 6.

Semenza, G.L. (2012). Hypoxia-inducible factors in physiology and medicine. Cell 148, 399–408.

Shankman, L.S., Gomez, D., Cherepanova, O.A., Salmón, M., Alencar, G.F., Haskins, R.M., Swiatloska, P., Newman, A.A., Greene, E.S., Straub, A.C., et al. (2015). KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. Nat. Med. 21, 628–637.

Sheik, A.Q., Lighthouse, J.K., and Greif, D.M. (2014). Recapitulation of developing artery muscularization in pulmonary hypertension. Cell Rep. 6, 809–817.

Sheik, A.Q., Misra, A., Rosas, I.O., Adams, R.H., and Greif, D.M. (2015). Smooth muscle cell progenitors are primed to muscularize in pulmonary hypertension. Sci. Transl. Med. 7, 308ra159.

Shimoda, L.A., and Semenza, G.L. (2011). HIF and the lung: role of hypoxia-inducible factors in pulmonary development and disease. Am. J. Respir. Crit. Care Med. 183, 152–156.

Simeone, G., Gatzoulis, M.A., Atadia, I., Celermajer, D., Denton, C., Ghofrani, A., Gomez Sanchez, M.A., Krishna Kumar, R., Landzberg, M., Machado, R.F., et al. (2013). Updated clinical classification of pulmonary hypertension. J. Am. Coll. Cardiol. 62 (Suppl P), D34–D41.

Smith, T.G., Brooks, J.T., Balanos, G.M., Lappin, T.R., Layton, D.M., Leedham, D.L., Liu, C., Maxwell, P.H., McMullin, M.F., McNamara, C.J., et al. (2006). Mutation of von Hippel-Lindau tumour suppressor and human pulmonary physiological. PLoS Med. 3, e290.

Spies, J.L., Whitney, M.J., Sullivan, D.E., Lasky, J.A., Laboy, M., Ylostalo, J., and Prockop, D.J. (2008). Bone marrow progenitor cells contribute to repair and remodeling of the lung and heart in a rat model of progressive pulmonary hypertension. FASEB J. 22, 1226–1236.

Stemrak, K.R., Fagan, K.A., and Frid, M.G. (2006). Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. Circ. Res. 99, 675–691.

Stemrak, K.R., Meyrick, B., Galie, N., Mool, W.J., and McMurtry, I.F. (2009). Animal models of pulmonary arterial hypertension: the hope for etiological
discovery and pharmacological cure. Am. J. Physiol. Lung Cell. Mol. Physiol. 297, L1013–L1032.

Thenappan, T., Goel, A., Marsboom, G., Fang, Y.H., Toth, P.T., Zhang, H.J., Kajimoto, H., Hong, Z., Paul, J., Wietholt, C., et al. (2011). A central role for CD68(+) macrophages in hepatopulmonary syndrome. Reversal by macrophage depletion. Am. J. Respir. Crit. Care Med. 183, 1080–1091.

Tian, W., Jiang, X., Tamosiuniene, R., Sung, Y.K., Qian, J., Dhillon, G., Gera, L., Farkas, L., Rabinovitch, M., Zamanian, R.T., et al. (2013). Blocking macrophage leukotriene b4 prevents endothelial injury and reverses pulmonary hypertension. Sci. Transl. Med. 5, 200ra117.

Tuder, R.M., Groves, B., Badesch, D.B., and Voelkel, N.F. (1994). Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. Am. J. Pathol. 144, 275–285.

van Steensel, L., Paridaens, D., van Meurs, M., van Hagen, P.M., van den Bosch, W.A., Kuijpers, R.W., Drexhage, H.A., Hooijkaas, H., and Dik, W.A. (2012). Orbit-infiltrating mast cells, monocytes, and macrophages produce PDGF isoforms that orchestrate orbital fibroblast activation in Graves’ ophthalmopathy. J. Clin. Endocrinol. Metab. 97, E400–E408.

Vergadi, E., Chang, M.S., Lee, C., Liang, O.D., Liu, X., Fernandez-Gonzalez, A., Mitsialis, S.A., and Kourembanas, S. (2011). Early macrophage recruitment and alternative activation are critical for the later development of hypoxia-induced pulmonary hypertension. Circulation 123, 1986–1995.

Wang, Z., Li, A.Y., Guo, Q.H., Zhang, J.P., An, Q., Guo, Y.J., Chu, L., Weiss, J.W., and Ji, E.S. (2013). Effects of cyclic intermittent hypoxia on ET-1 responsiveness and endothelial dysfunction of pulmonary arteries in rats. PLoS ONE 8, e58078.

Wang, S., Zeng, H., Xie, X.J., Tao, Y.K., He, X., Roman, R.J., Aschner, J.L., and Chen, J.X. (2016). Loss of prolyl hydroxylase domain protein 2 in vascular endothelium increases pericyte coverage and promotes pulmonary arterial remodeling. Oncotarget 7, 58848–58861.

Yan, S.F., Tritto, I., Pinsky, D., Liao, H., Huang, J., Fuller, G., Brett, J., May, L., and Stern, D. (1995). Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. Central role of the binding site for nuclear factor-IL-6. J. Biol. Chem. 270, 11463–11471.

Yeager, M.E., Frid, M.G., and Stenmark, K.R. (2011). Progenitor cells in pulmonary vascular remodeling. Pulm. Circ. 1, 3–18.

Yu, A.Y., Shimoda, L.A., Iyer, N.V., Huso, D.L., Sun, X., McWilliams, R., Beaty, T., Sham, J.S., Wiener, C.M., Sylvester, J.T., and Semenza, G.L. (1999). Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. J. Clin. Invest. 103, 691–696.

Zaloudíkova, M., Vytašek, R., Vajnerová, O., Hnilíčková, O., Vízek, M., Hampí, V., and Herget, J. (2016). Depletion of alveolar macrophages attenuates hypoxic pulmonary hypertension but not hypoxia-induced increase in serum concentration of MCP-1. Physiol. Res. 65, 763–768.
Supplemental Information

Cell Autonomous and Non-cell Autonomous Regulation of SMC Progenitors in Pulmonary Hypertension

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**Figure S1: Hypoxia induces HIF1-α expression in a time dependent manner.** (Related to Figure 1). Adult wild type mice were exposed to normoxia or to 1, 5, or 7 days of hypoxia (FiO₂ 10%). Coronal left lung sections were stained for HIF1-α, SMA and MECA-32 (EC marker). Boxed regions with arterioles in proximity to the left bronchus–first lateral secondary branch–first anterior-first medial airway branch (L.L1.A1.M1) and are displayed as close-ups on the right for each time point without or with staining for MECA-32 (n=3 lungs). Scale bar, 20 mm.
Figure S2: Gene deletion efficiency after tamoxifen induced recombination in mice carrying CreERs driven by diverse promoters (Related to Figures 1-6). A, B, Pdgfrb-CreER$^{T2}$ mice also carrying Hif1a$^{(floxed/floxed)}$ (A) or Klf4$^{(floxed/floxed)}$ (B) were or were not injected with tamoxifen (1 mg/day for 5 days), rested for 5 days and exposed to normoxia or hypoxia for 1 or 2 days, respectively. Arterioles were analyzed in lung vibratome sections stained for PDGFR-β, SMA and DAPI as well as for HIF1-α in (A) or for KLF4 in (B). The boxed regions are shown as close-ups on the right with arrowheads indicating primed cells; n=4 lungs, 2-3 arterioles per lung. Scale bars, 20 µm. C-F, ECs were isolated from lungs of mice using anti-CD31-coated beads, and levels of indicated transcripts relative to 18S levels were measured by qRT-PCR. At least n=3 mice for each genotype and tamoxifen treatment condition were assessed. In (C), levels of Pecam1, Cd68, Myh11 and Acta2 were measured in ECs and non-endothelial cells (control) isolated from the lungs of wild type mice. In (D, F), Cdh5-CreER$^{T2}$ mice also carrying Hif1a$^{(floxed/floxed)}$ (D) or Pdgfb$^{(floxed/floxed)}$ (F) were or were not injected with tamoxifen (1 mg/day for 5 days), rested for 5 days and then exposed to normoxia or hypoxia for indicated days. mRNA levels of Hif1a and Pdgfb in isolated lung ECs were assayed. In (E), Cdh5-CreER$^{T2}$, Vhl$^{(floxed/floxed)}$ mice were injected with tamoxifen (1 mg/day for 5 days) and then rested for 12 days in normoxia. Transcript levels of Vhl, Hif1a and Pdgfb in isolated lung ECs were measured. G, Csf1r-Mer-iCre-Mer, Pdgfb$^{(floxed/+)}$ mice were or were not injected with tamoxifen (1 mg/day for 15 days), rested for 5 days and then exposed to hypoxia for 3 days. CD64$^+$ cells were isolated via FACS, and Pdgfb mRNA levels relative to Gapdh were measured via qRT-PCR. n=3 mice. ^ vs. EC-depleted lung cells, p<0.05; * vs. no tamoxifen, p<0.05; ns, not significant.
Figure S3: Primed cell HIF1-α is required for distal pulmonary arteriole muscularization (Related to Figure 1). 

*Pdgfrb-CreER* 

*Pdgfrb-CreER*T2, ROSA26R(mTmG/+) mice that were also Hif1α(flox/flox) or wild type for Hif1α were injected with tamoxifen (1 mg/day for 5 days), rested and then exposed to normoxia or hypoxia for 7 days. Arterioles in the proximity of L.L1.A1.M1 airway were stained for SMA, GFP (lineage tag) and MECA-32. Boxed regions are shown as close-ups below; n=3 mice, 2-3 arterioles per mouse. Scale bar, 20 μm.
Figure S4: Hif1a deletion in ECs attenuates hypoxia-induced distal arteriole SMC expression of KLF4, proliferation and dedifferentiation and similarly, EC Pdgfb deletion attenuates SMC dedifferentiation (Related to Figures 3, 5). Cdh5-CreER\textsuperscript{Tg}, mice also carrying Hif1a\textsuperscript{floxflox} (A-E) or Pdgfb\textsuperscript{floxflox} (F) were injected with tamoxifen (1 mg/day for 5 days), rested for 5 days and exposed to normoxia or hypoxia for 7 days. Arterioles in proximity to L.L1.A1 airway branches were analyzed in lung vibratome sections by immunostaining. A, D, Sections were stained for SMA, MECA and DAPI and either for KLF4 in (A) or for pH3 in (D). Scale bars, 20 \mu m. B, E, The percent of distal arteriole SMA\textsuperscript{+} cells at hypoxia day 7 that are KLF4\textsuperscript{+} (B) or pH3\textsuperscript{+} (E) is quantified; n=3 mice. For no tamoxifen and tamoxifen groups, the total distal arteriole SMCs scored were 186 and 68 in (B) and were 197 and 76 in (E). C, F, Individual channels and merge of sections shown in Figures 3H and 5H, respectively, stained for PDGFR-\beta, SMA, SMMHC and DAPI.
Figure S5: Lung Pdgfb mRNA levels peak at hypoxia day 2 (Related to Figure 5). Pdgfb transcript levels were quantified by qRT-PCR from the lung lysates of mice exposed to normoxia or hypoxia for the indicated days. n=3 mice; * vs. normoxia; p<0.05; ^ vs. hypoxia day 2, p<0.05.
Figure S6: Accumulation of CD68^+ cells and cells-derived from CSF1R^+ cells in the hypoxic lung. (Related to Figure 6.) A, Wild type mice were exposed to normoxia or hypoxia (FiO2 10%) for up to 21 days, and then lung vibratome sections were stained for CD68, SMA and MECA. Pulmonary arterioles in proximity to airway branches L.L1.A1 are shown. The boxed region at hypoxia day 14 is shown as an inset close-up with arrowhead indicating CD68^+ SMA^- cell, n=3 mice, 2-3 arterioles per mouse. Scale bar, 20 µm. B-F, Pdgfrb-CreERT2, Hif1α^floxflox^, Pdgfrb-CreERT2, Klf4^floxflox^, Cdh5-CreERT2, Hif1α^floxflox^, Cdh5-CreERT2, Vhl^floxflox^, or Cdh5-CreERT2, Pdgfb^floxflox^, mice were or were not treated with tamoxifen and then were exposed to hypoxia for indicated days. Lung sections were stained for CD68, SMA, DAPI as well as for either HIF1-α, KLF4 or PDGF-B accordingly. At least 10 random lung regions per mouse were selected, and CD68^+ cells were scored per lung area (mm^2). n=3 mice per genotype and tamoxifen treatment condition; ns, not significant. G, H, In tamoxifen-induced Csf1r-Mer-iCre-Mer, ROSA26R^mTmG/+^ mice, the percent of distal arteriole SMA^+^ cells at hypoxia day 19 or SMMHC^+^ cells at hypoxia day 21 that are GFP^+^ are shown as determined from images as displayed in Figures 6A or B, respectively. In (G), 46 GFP^+^ SMA^+^ cells from n=3 mice exposed to hypoxia, 2-3 arterioles per mouse were further scored as to whether or not they express CD68; ~ vs. normoxia, p<0.05; * vs. CD68^+^ cells, p<0.01. In (H), 42 cells were scored for hypoxia in 3 mice, 3 arterioles per mouse.
Table S1. Primer pair sequences for quantitative reverse transcription polymerase chain reaction. (Related to Figures 3-6.) This table shows primer pairs used for qRT-PCR of lungs cells isolated from mice.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse strains

\( Pdgfrb^{-/}\text{CreER}\textsuperscript{T2}, \text{Acta2-CreER}\textsuperscript{T2}, \text{Cdh5-CreER}\textsuperscript{T2}, \text{Csf1r-Mer-iCre-Mer}, \text{Hif1a}\textsuperscript{flox/flox}, \text{Klf4}\textsuperscript{flox/flox}, \text{Vhl}\textsuperscript{flox/flox}, \text{Pdgfb}\textsuperscript{flox/flox}, \text{Pdgfb}\textsuperscript{+/−} \) and \( \text{ROSA26R}\textsuperscript{mTmG/mTmG} \) Cre reporter mice have been described \(^1\text{−}^8\) (and references in main text: Greif et al., 2012 and Qian et al., 2011). C57BL/6 mice (Jackson Laboratory) were utilized for wild type analysis.

EC isolation from murine lung

Lung ECs were isolated from mice as described previously (Sheikh et al., 2015 – reference in main text) with minor modifications. The lung vasculature was flushed with PBS as noted above, and lobes were dissected from the bronchi and mediastinal connective tissue. Lobes were minced and digested in collagenase I (100-150 U/ml) for 45 minutes at 37°C. The digested tissue was mechanically dissociated by triturating, filtering through a 70 µm disposable cell strainer and centrifugation for 8 minutes at 4°C. The pellet was resuspended in cold PBS containing 0.1% BSA to generate a single cell suspension. Sheep anti-rat IgG Dynal magnetic beads (Invitrogen) were resuspended in PBS containing 0.1% BSA and incubated with rat anti-mouse CD31(1:250, BD Biosciences) monoclonal antibody overnight at 4°C. The lung single cell suspension was then incubated with these anti-CD31-coated beads for 10-20 minutes at room temperature. A magnet was used to separate the cells bound to beads and unbound cells. Cells were then subjected to qRT-PCR.

Fluorescent activated cell sorting (FACS)
Mice were euthanized, and the lung vasculature was perfused with PBS as described above. Lungs were inflated with dispase (1 U/ml, StemCell), harvested and then incubated with dispase for one hour at 37°C. This digestion was stopped by addition of DMEM with 50% fetal calf serum, 2.5% HEPES, 1% penicillin-streptomycin and 2.1% DNase. The digested tissue was mechanically dissociated by trituration, serially filtered through 100 µm and 40 µm filters and then centrifuged for 10 minutes at 4°C. The pellet was resuspended in FACS buffer (BD Biosciences) and incubated with APC directly conjugated anti-CD64 antibody (1:250, BioLegend) for 20 minutes at 4°C. Cells were then washed with FACS buffer and filtered through a 100 µm strainer prior to sorting with a FACSARia II sorter. APC was excited at 640 nm, and emission was collected through a 670 nm/30 bandpass filter. FACSDiva 7 software was used to analyze results. Sorted cells were subjected to qRT-PCR.

Quantitative real time RT-PCR analysis

RNA was isolated from lung cells with the RNeasy Plus Kit (Life Technologies) and reverse transcribed with the iScript cDNA Synthesis Kit (Biorad). Transcript expression levels were determined by qRT-PCR and normalized to 18S or Gapdh. Forward and reverse primer pairs are listed in Table S1.
SUPPLEMENTAL REFERENCES

1. Wendling, O., Bornert, J. M., Chambon, P. & Metzger, D. Efficient temporally-controlled targeted mutagenesis in smooth muscle cells of the adult mouse. *Genesis 47*, 14-18, (2009).

2. Katz, J. P., Perreault, N., Goldstein, B. G., Actman, L., McNally, S. R., Silberg, D. G., Furth, E. E. & Kaestner, K. H. Loss of Klf4 in mice causes altered proliferation and differentiation and precancerous changes in the adult stomach. *Gastroenterology 128*, 935-945, (2005).

3. Leveen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larsson, E. & Betsholtz, C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev 8*, 1875-1887, (1994).

4. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. & Luo, L. A global double-fluorescent Cre reporter mouse. *Genesis 45*, 593-605, (2007).

5. Enge, M., Bjarnegard, M., Gerhardt, H., Gustafsson, E., Kalen, M., Asker, N., Hammes, H. P., Shani, M., Fassler, R. & Betsholtz, C. Endothelium-specific platelet-derived growth factor-B ablation mimics diabetic retinopathy. *EMBO J 21*, 4307-4316, (2002).

6. Wang, Y., Nakayama, M., Pitulescu, M. E., Schmidt, T. S., Bochenek, M. L., Sakakibara, A., Adams, S., Davy, A., Deutsch, U., Luthi, U., Barberis, A., Benjamin, L. E., Makinen, T., Nobes, C. D. & Adams, R. H. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature 465*, 483-486, (2010).

7. Haase, V. H., Glickman, J. N., Socolovsky, M. & Jaenisch, R. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. *Proc Natl Acad Sci U S A 98*, 1583-1588, (2001).
Ryan, H. E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J. M. & Johnson, R. S. Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. 

*Cancer Res* **60**, 4010-4015, (2000).