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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^+ (PDGFR-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.

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.

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SUPPLEMENTAL INFORMATION

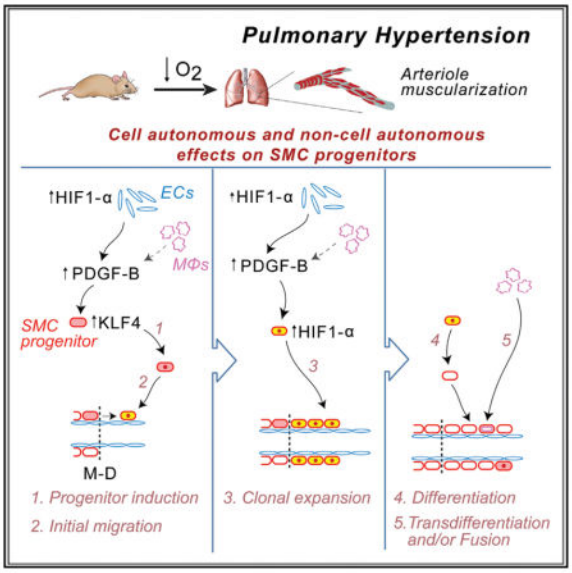
Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.043.

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.



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 (PDGFR-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; Žaloudíková 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.

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 [L.L1.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-CreER^{T2}-mediated deletion of *Hif1a* attenuates hypoxia-induced pulmonary vascular remodeling (Ball 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% FiO_2) 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). *Pdgfrb-CreER^{T2}, 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-CreER^{T2}, 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-CreER^{T2}, Hif1a$^{(flox/flox)}$, ROSA26R$^{(mTmG/+)}$* 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-CreER^{T2}, 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 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^{T2}, Klf4$^{(flox/flox)}$* 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 $^{+}$ cells does not alter primed cell expression of the other transcription factor (Figures 1F, 1G, 2D, and 2E).

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-CreER^{T2}, Hif1a$^{(flox/flox)}$* 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-CreER^{T2}, Hif1a$^{(flox/flox)}$* 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-CreER^{T2}, Hif1a$^{(flox/flox)}$* 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, 3I, and S4C). 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-CreER^{T2}, Vhl$^{(flox/flox)}$* mice with tamoxifen and then allowed them to rest for up to 47 days under normoxic conditions. HIF1-α and PDGF-B 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 PDGF-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 PDGF-B in lung ECs (Sheikh et al., 2015), 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-CreER^{T2}, Pdgfb$^{(flox/flox)}$* 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-CreER^{T2}, Pdgfb$^{(flox/flox)}$* 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 S4F). 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-CreER^{T2}* mice carrying *Hif1a$^{(flox/flox)}$* or *Pdgfb$^{(flox/flox)}$* (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$^{(mTmG/+)}$*. Under normoxic conditions, tamoxifen treatment of *Csf1r-Mer-iCre-Mer, ROSA26R$^{(mTmG/+)}$* mice results in marking of CD68$^+$ cells, but not arterial SMCs (A. Misra, Z. Feng, R. Chandran, I. Kabir, N. Rottlan, B. Aryal, A.Q.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-CreER^{T2}, 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-CreER^{T2}* 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).

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 arteriole border of the normal lung that give rise to most hypoxia-induced distal arteriole 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-CreER*^{T2}*, 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 arteriole 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 arteriole 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-CreER*^{T2} (Ball et al., 2014; Kim et al., 2013), either of which would be expected to delete *Hif1a* in primed cells. Tamoxifen-induced *Myh11-CreER*^{T2}*, Hif1a*$^{(flox/flox)}$ mice develop hypoxia-induced RVH (Ball et al., 2014), whereas *Pdgfrb-CreER*^{T2}*, 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 (Izikki 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 *Hif2a* 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 arteriole muscularization (Figure 4). Specific and conditional deletion of *Hif1a* in ECs attenuates hypoxia-induced primed cell KLF4 expression and distal arteriole accumulation and proliferation, and in contrast to the stereotyped pattern of distal muscularization in controls, distal arteriole SMCs in mutants have perturbed expression of differentiation markers and form clumps, although not all SMCs are directly apposed 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 arteriole muscularization (Sheikh et al., 2015), $Cdh5\text{-}CreER^{T2}$, $Pdgfb^{(flox/flox)}$ 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% O_2 for 48 hr) (Vergadi et al., 2011) or obtained from mice exposed to hypoxia (9% FiO_2) (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 CXCRL1, 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 arteriole SMA^+SMMHC^+ SMCs 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 $Apoe^{(-/-)}$ 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 $CSF1R^+$ cells substantially abrogates hypoxia-induced distal pulmonary arteriole muscularization.

Altogether, our findings emphasize the importance of primed cell autonomous and non-cell autonomous pathways in the context of hypoxia-induced distal arteriole muscularization and PH. Primed cell expression of KLF4 is requisite for initial breaching of the muscular-unmuscular border, and subsequently, primed cell HIF1-α is integral for the expansion of early distal arteriole 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 arteriole muscularization through both trans-differentiation to $SMMHC^+CD68^-$ 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% FiO_2) 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-melt 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% H_2O_2, 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-SMMHC (1:250, Biomedical Technologies), rabbit anti-PDGF-B (1:100, Abcam), goat anti-KLF4 (1:100, R&D Systems), rabbit anti-HIF1-α (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 (Greif 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 $^+$SMA$^+$DAPI$^+$ cells) at the muscular-unmuscular border of specific pulmonary arterioles or distal arteriole SMCs (DAPI$^+$ cells expressing SMA or SMMHC) 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 SP5 point scanning). Volocity 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 $p \leq 0.05$. All tests assumed normal distribution and were two sided.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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

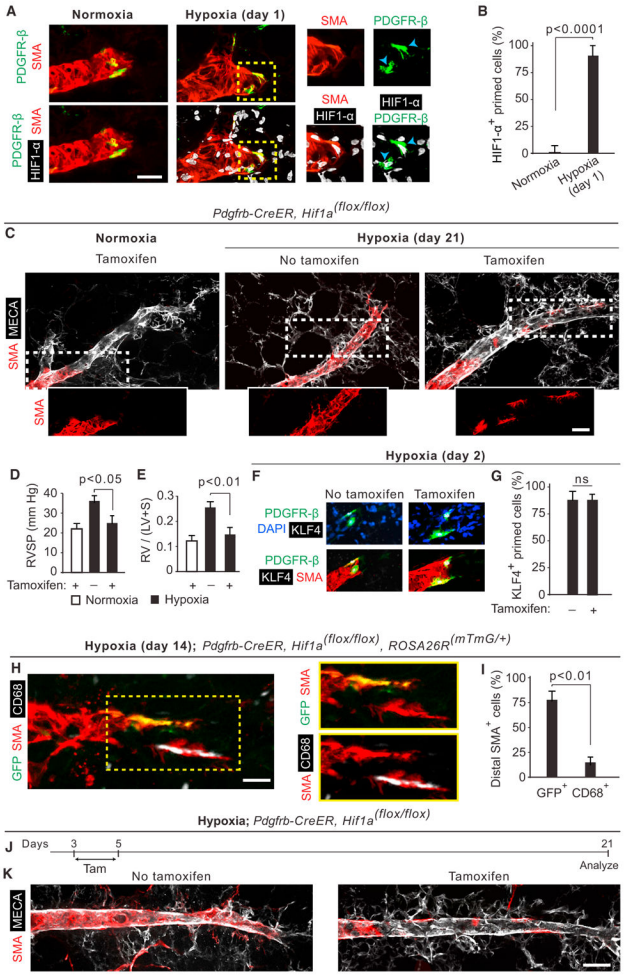

Figure 1. HIF1-α Is Required for Distal Arteriole Muscularization and PH

(A) Wild-type mice were exposed to normoxia or hypoxia (10% FiO_2) 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 L.L1.A1 (left bronchus-first lateral secondary branch-first anterior branches) 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-CreER^{T2}, 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 antibody (MECA-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-CreER^{T2}, 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. 35 SMA$^+$ cells were scored.

(J) Experimental strategy for (K), in which *Pdgfrb-CreER^{T2}, 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.

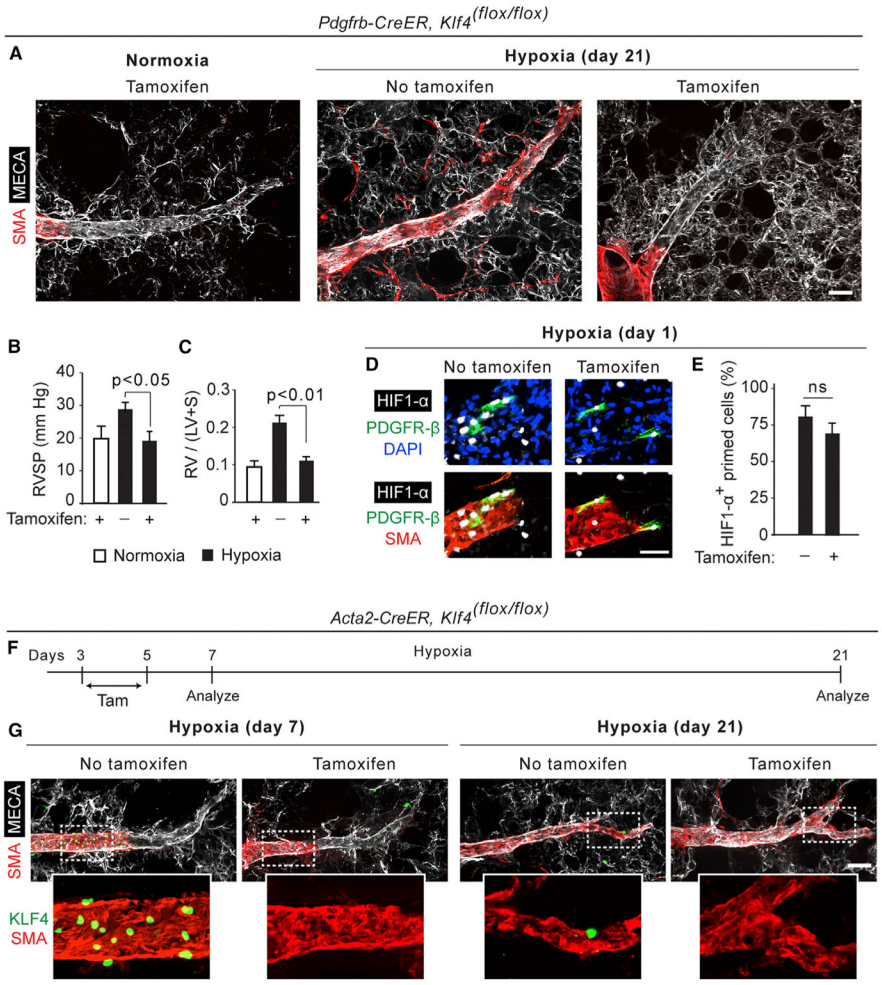

Figure 2. Primed Cell KLF4 Is Required for Distal Muscularization and PH

(A–E) *Pdgfrb-CreER^{T2}, Klf4$^{(flox/flox)}$* 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 $^{+}$ 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^{T2}, Klf4$^{(flox/flox)}$* mice are induced with tamoxifen (1.5 mg/day) from hypoxia days 3–5.

(G) Arterioles of *Acta2-CreER^{T2}; Klf4$^{(flox/flox)}$* 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.

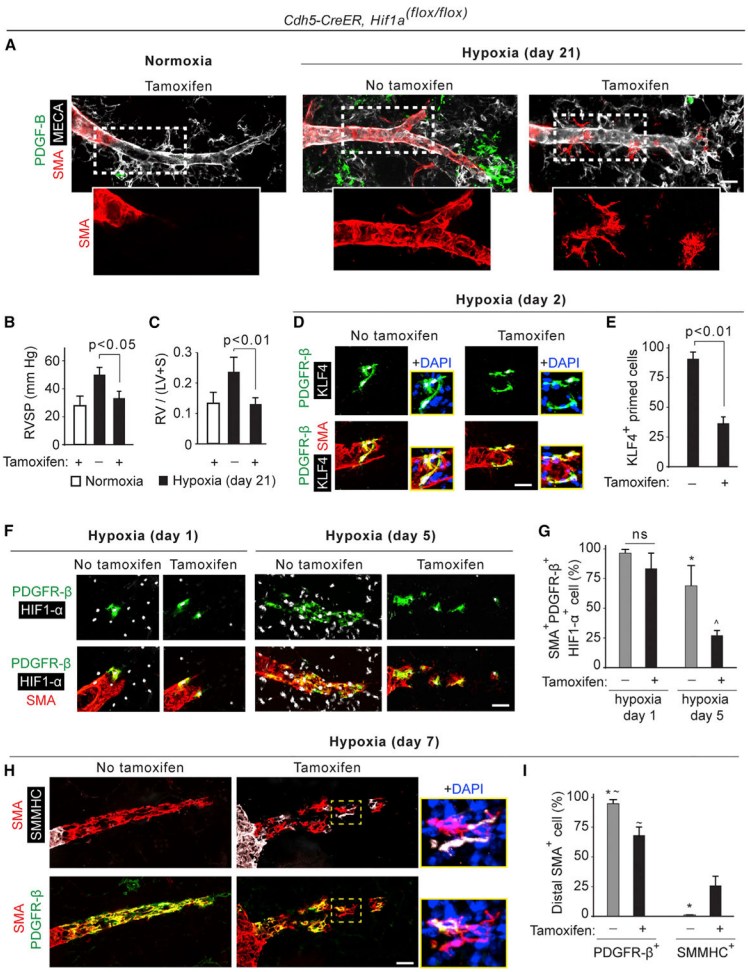

Figure 3. Endothelial *Hif1a* Deletion Attenuates Distal Muscularization and PH and Perturbs SMC Differentiation

Cdh5-CreER^{T2}*, Hif1a*$^{(flox/flox)}$ 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^{+} 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 $^{+}$ cells at hypoxia day 1 (primed cells) or hypoxia day 5 (middle or distal arteriole SMCs) that are also HIF1-α $^{+}$ 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 tamoxifen group, whereas 152 cells were scored at hypoxia day 5 for the no-tamoxifen group and 160 cells were scored for the tamoxifen group. The percentage of distal arteriole SMA^{+} cells that are PDGFR-β $^{+}$ and/or SMMHC^{+} at hypoxia day 7 is shown in (I). n = 5 mice. 235 cells with and 219 cells without tamoxifen were scored for PDGFR-β, and 72 cells with and 53 cells

without tamoxifen were scored for SMMHC. *$p < 0.05$ versus tamoxifen, ^$p < 0.05$ versus hypoxia day 1, ~$p < 0.01$ versus $SMMHC^+$ cells. ns, not significant.
All error bars indicate SD. Scale bars, 20 μm. See also Figures S2 and S4.

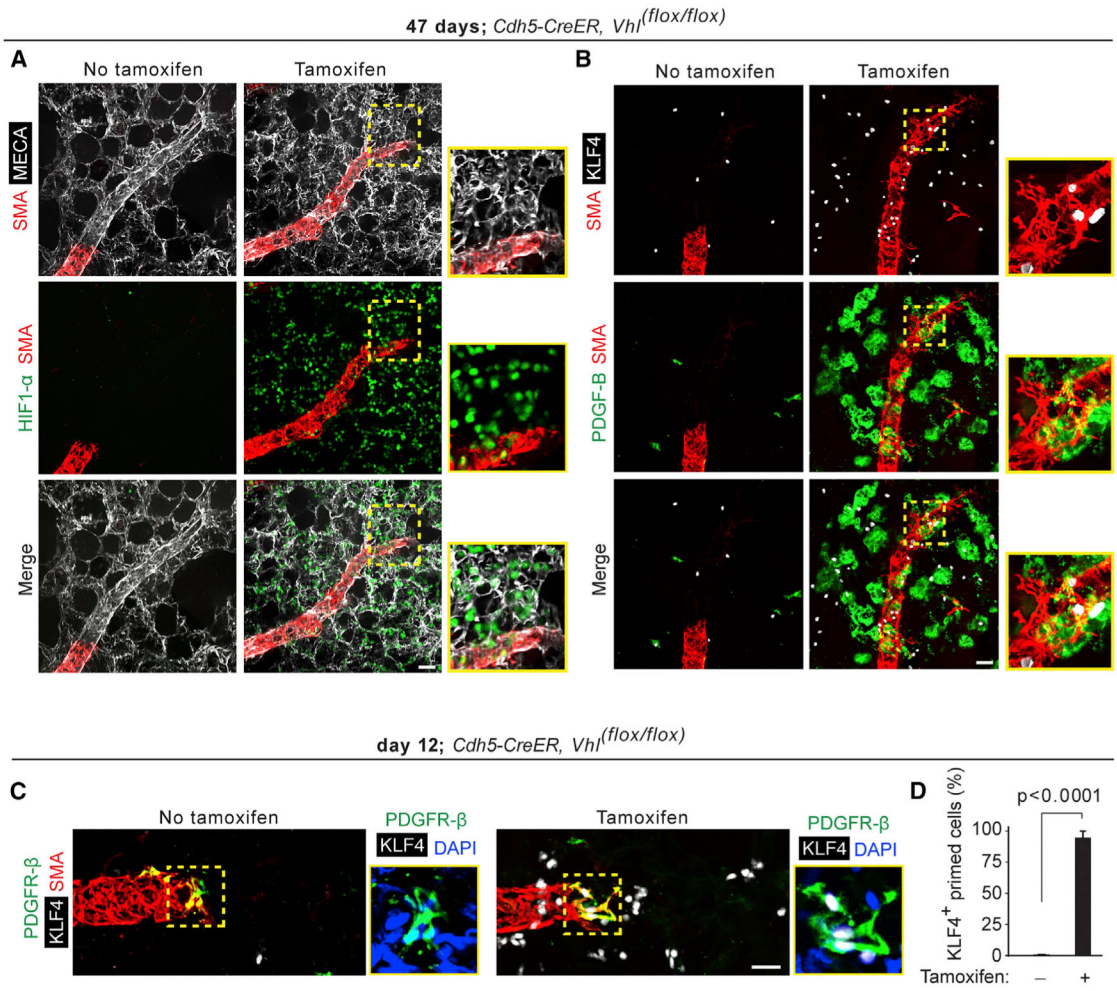

Figure 4. Endothelial-Specific *Vhl* Deletion Induces HIF1-α and PDGF-B Expression and Distal Arteriole Muscularization under Normoxia

Cdh5-CreER^{T2}*, Vhl*$^{(flox/flox)}$ mice were injected with tamoxifen (1 mg/day for 5 days) and then analyzed for 12 days (A and B) or 47 days (C and D) thereafter.

(A–C) Arterioles near L.L1.A1 airways were analyzed in vibratome sections stained for SMA, as well as for HIF1-α and MECA-32 in (A), PDGF-B and KLF4 in (B), or PDGFR-β, KLF4, and nuclei (DAPI) in (C). Boxed regions are shown as close-ups. n = 4 mice, with 2–3 arterioles per mouse.

(D) Quantification of the percentage of KLF4^{+} primed cells 1 week after tamoxifen induction from images shown in (C). n = 5 mice, with 2–3 arterioles per mouse. Total primed cells scored were 45 and 41 in no-tamoxifen and tamoxifen groups, respectively. All error bars indicate SD. Scale bars, 20 μm. See also Figure S2.

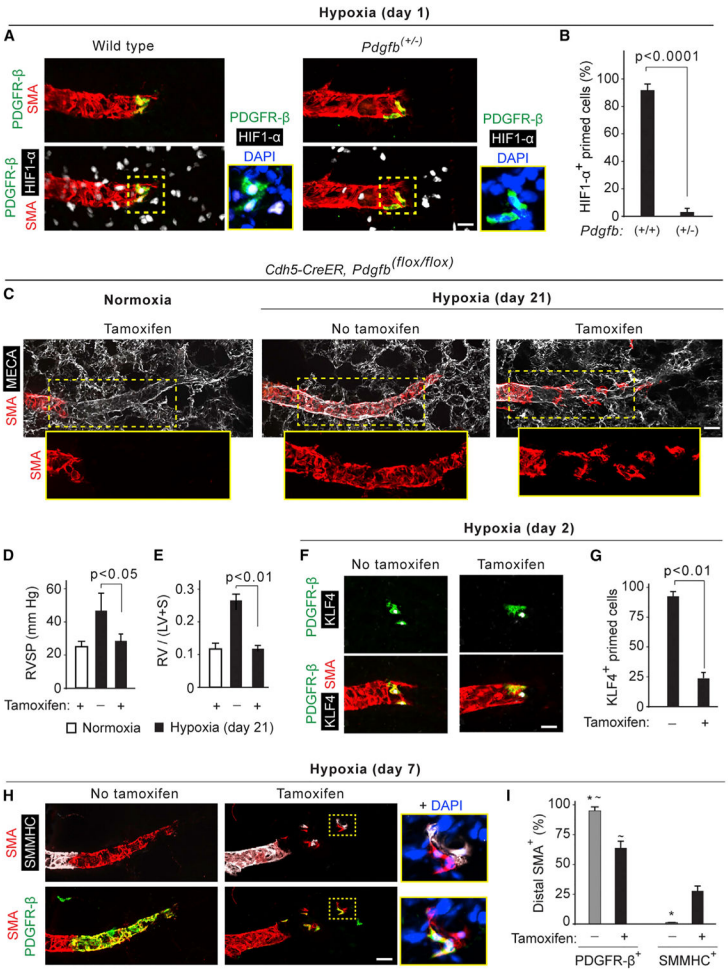

Figure 5. Endothelial-Specific *Pdgfb* Deletion Attenuates Distal Muscularization, KLF4 Expression, and PH and Perturbs SMC Differentiation

(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-α, PDGFR-β, and DAPI.

(B) Quantification of primed cells that express HIF1-α 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–I) *Cdh5-CreER*^{T2}, *Pdgfb*$^{(flox/flox)}$ mice were injected with tamoxifen (1 mg/day for 5 days), rested for 5 days, and then exposed to normoxia or hypoxia for 2, 7, or 21 days as indicated. Distal arterioles near L.L1.A1 airway branches were stained with SMA, as well as for MECA-32 in (C), KLF4 and PDGFR-β in (F), or SMMHC and PDGFR-β in (H). RVSP and RV weight ratio are quantified in (D) and (E), respectively. n = 5 mice. In (G), the percentage of KLF4^{+} primed cells at hypoxia day 2 was quantified. n = 5 mice, with 2–3 arteriole per lung. Total primed cells scored were 37 and 41 in no-tamoxifen and tamoxifen groups, respectively. In (I), the percentage of SMA^{+} distal arteriole cells at hypoxia day 7 that are PDGFR-β $^{+}$ and/or SMMHC^{+} are quantified. n = 5 mice. In the distal arteriole, 233 cells with and 217 cells without tamoxifen were scored for PDGFR-β, and 78 cells with and

71 cells without tamoxifen were scored for SMMHC. *$p < 0.05$ versus tamoxifen treatment, ~$p < 0.01$ versus $SMMHC^+$ cells.

All error bars indicate SD. Scale bars, 20 μm. See also Figures S2, S4, and S5.

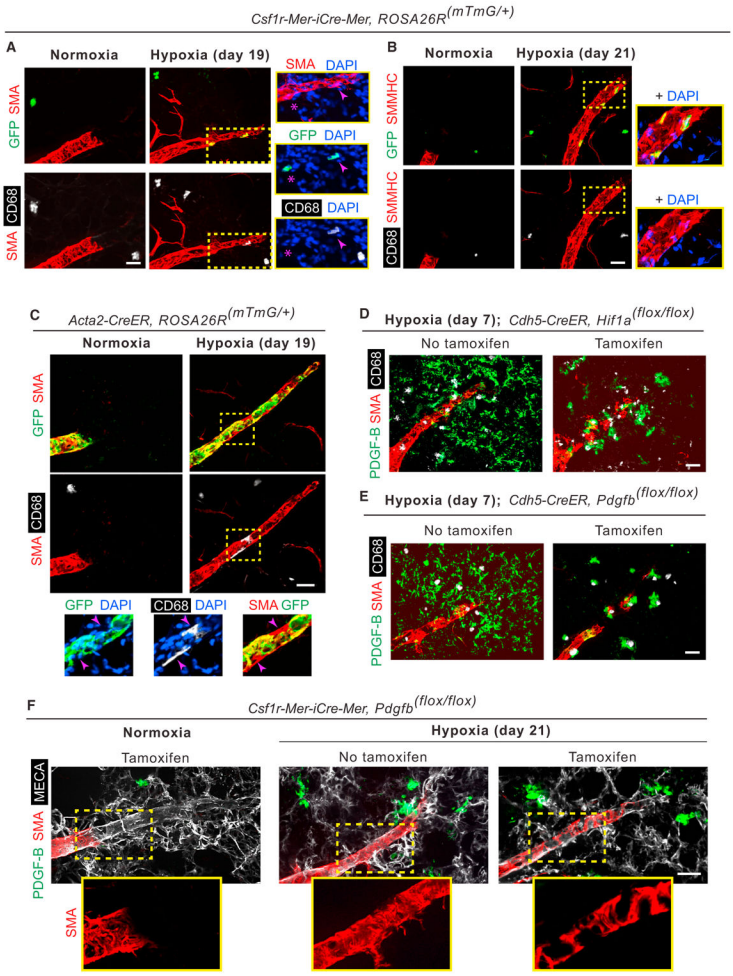

Figure 6. Macrophages Give Rise to Distal Arteriole SMC Marker$^+$ Cells, and Macrophage-Derived PDGF-B Is Integral for Distal Muscularization

Tamoxifen (1 mg/day) was injected for 5 days in *Acta2-CreER^{T2}* (C) or *Cdh5-CreER^{T2}* (D and E) mice or for 15 days in *Csf1r-Mer-iCre-Mer* mice (A, B, and F). Mice were rested and exposed to normoxia or hypoxia for 7, 19, or 21 days as indicated, and then vibratome sections containing distal arterioles near L.L1.A1 airway branches were immunostained.

(A–C) Sections from *ROSA26R$^{(mTmG/+)}$* mice carrying *Csf1r-Mer-iCre-Mer* (A and B) or *Acta2-CreER^{T2}* (C) were stained for GFP (lineage tag), CD68, nuclei (DAPI), and either SMA (A and C) or SMMHC (B). Boxed regions are shown as close-ups. In (A), arrowhead points to a SMA$^+$ cell expressing GFP and CD68, and the asterisk indicates the SMA$^+$GFP$^+$CD68$^-$ cell. Close-ups in (B) show SMMHC$^+$GFP$^+$CD68$^-$ cells and in (C) show SMA cells that are GFP$^-$CD68$^+$.

(D and E) Sections from *Cdh5-CreER^{T2}* mice that are homozygous for floxed *Hif1a* (D) or *Pdgfb* (E) alleles were stained for PDGF-B, SMA, and CD68.

(F) Sections from *Csf1r-Mer-iCre-Mer, Pdgfb$^{(flox/flox)}$* mice were stained for PDGF-B, SMA, and MECA-32. Boxed regions are shown as close-ups below. n = 3 mice, with 2–3 arterioles per mouse.

Scale bars, 20 μm. See also Figures S2 and S6.

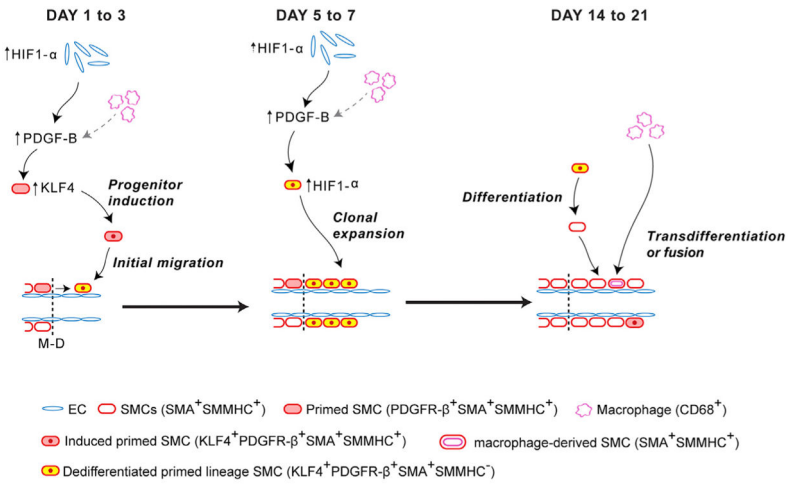

Figure 7. Summary of Cell Autonomous and Non-cell Autonomous Regulation of Primed Cells in Hypoxia-Induced Distal Pulmonary Arteriole Muscularization

During hypoxia days 1–3, EC expression of HIF1-α and PDGF-B induces primed cell upregulation of KLF4 (progenitor induction). This progenitor induction results in initial migration of a primed cell beyond the M (middle)-to-D (distal) arteriole border. In contrast to EC HIF1-α, primed cell HIF1-α is expendable for progenitor induction and initial migration. Distal arteriole SMC proliferation peaks at hypoxia day 7 (Sheikh et al., 2014), and at hypoxia days 5–7, lung PDGF-B expression by ECs, and perhaps by macrophages, stays at high levels, resulting in HIF1-α expression in distal arteriole SMCs. Expansion of the primed cell lineage in the distal arteriole requires this lineage to express HIF1-α, but not KLF4. During hypoxia days 14–21, PDGF-B expression in the lung wanes and newly derived distal arteriole cells differentiate into mature SMCs. Furthermore, pre-existing macrophages transdifferentiate into or fuse with distal arteriole SMCs.