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Smooth muscle cell progenitors are primed to muscularize in pulmonary hypertension

Abdul Q. Sheikh¹, Ashish Misra¹, Ivan O. Rosas², Ralf H. Adams³, and Daniel M. Greif^{1,*}

¹Yale Cardiovascular Research Center, Section of Cardiovascular Medicine, Department of Internal Medicine, Yale University School of Medicine, 300 George Street, Room 773J, New Haven, CT 06511, USA

²Division of Pulmonary and Critical Care, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

³ Department of Tissue Morphogenesis, Max Planck Institute for Molecular Biomedicine, and Faculty of Medicine, University of Münster, 48149 Münster, Germany

Abstract

Excess and ectopic smooth muscle cells (SMCs) are central to cardiovascular disease pathogenesis, but underlying mechanisms are poorly defined. For instance, pulmonary hypertension (PH) or elevated pulmonary artery blood pressure is a devastating disease with distal extension of smooth muscle to normally unmuscularized pulmonary arterioles. We identify novel SMC progenitors that are located at the pulmonary arteriole muscular-unmuscular border and express both SMC markers and the undifferentiated mesenchyme marker platelet-derived growth factor receptor- β (PDGFR- β). We term these cells “primed” because in hypoxia-induced PH, they express the pluripotency factor Kruppel-like factor 4 (KLF4), and in each arteriole, one of them migrates distally, dedifferentiates, and clonally expands, giving rise to the distal SMCs. Furthermore, hypoxia-induced expression of the ligand PDGF-B regulates primed cell KLF4 expression, and enhanced PDGF-B and KLF4 levels are required for distal arteriole muscularization and PH. Finally, in PH patients, KLF4 is markedly up-regulated in pulmonary arteriole smooth muscle, especially in proliferating SMCs. In sum, we have identified a pool of SMC progenitors that are critical for the pathogenesis of PH, and perhaps other vascular disorders, and therapeutic strategies targeting this cell type promise to have profound implications.

INTRODUCTION

Cardiovascular disorders and their sequelae are responsible for ~30% of all deaths worldwide (1). A critical pathological component of many of these diseases is excessive

*Corresponding author. daniel.greif@yale.edu.

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smooth muscle. One such devastating disease is pulmonary hypertension (PH) in which normally unmuscularized distal pulmonary arterioles become muscularized (2). PH is classified by the World Health Organization (WHO) into five major groups based on disease etiology (3), and most therapeutic approaches are geared at combating the underlying etiology. In pulmonary artery hypertension (PAH; WHO group 1 PH), formerly classified as primary PH, decreased pulmonary arterial compliance predicts mortality (4), and pathologically muscularized distal arterioles contribute to this reduced compliance. Current treatments for PAH induce vascular dilation but do not ameliorate excess smooth muscle cell (SMC) accumulation and have modest clinical efficacy (5). A key factor limiting therapeutic strategies for cardiovascular diseases, such as PH in general, is that the key molecular and cellular events and signaling pathways underlying vessel hypermuscularization are not well understood.

Hypoxia is a common cause of PH (WHO group 3) and also exacerbates PH due to other etiologies. Mice treated with chronic hypoxia develop PH and enhanced muscularization of distal pulmonary arterioles (6–8). Under normoxic conditions, markers α -smooth muscle actin (SMA) and smooth muscle myosin heavy chain (SMMHC) (9) are coexpressed in SMCs of the proximal pulmonary arterioles (10). We recently established that the vast majority of distal arteriole SMCs in hypoxia-induced PH derive from preexisting proximal SMCs, and this pathological muscularization encompasses a stereotyped program of SMC dedifferentiation, distal migration, proliferation, and then differentiation (10). Herein, we hypothesized that these pathological distal arteriole SMCs derive from a prespecified progenitor pool in the vascular wall, and understanding how such cells are mobilized to give rise to excess smooth muscle in pulmonary vascular disease could have important implications for novel therapeutic strategies to combat human pathology.

Using immunohistochemical, clonal, and genetic analysis in the current study, we identify one to three SMC marker⁺ cells at each pulmonary arteriole muscular-unmuscular transition zone that express the undifferentiated mesenchyme marker platelet-derived growth factor receptor- β (PDGFR- β), and we determine the role of these SMA⁺SMMHC⁺PDGFR- β ⁺ cells in PH. Our findings indicate that these cells are SMC progenitors and are “primed” to muscularize the distal arteriole in response to an insult, such as hypoxia. With hypoxic exposure, these rare primed cells express the pluripotency factor Kruppel-like factor 4 (KLF4) (11), and for each distal arteriole, one primed cell migrates distally and clonally expands. We also show that hypoxia stimulates polarized lung expression of the ligand PDGF-B, which induces primed cell KLF4 expression, and enhanced levels of PDGF-B and KLF4 are required for distal arteriole muscularization and PH. Finally, our studies in human lung samples indicate that KLF4 is up-regulated in pulmonary arteriole smooth muscle from PH and PAH patients and especially in proliferating SMCs. Thus, we have identified a novel SMC progenitor pool that is critical in pulmonary vascular pathology in mice and demonstrated mechanisms underlying clonal progenitor expansion, which may be integral to human disease.

RESULTS

Distal pulmonary arteriole SMCs are monoclonal in PH Distal pulmonary arteriole muscularization is a hallmark of human PH (12). The branching patterns of the lung airways and arteries are similar and stereotyped in the adult mouse (10, 13), and we recently established an approach (10) to study distal arteriole muscularization by reproducibly identifying three specific arteriole beds in the left lung that are unmuscularized under normal conditions and become coated with smooth muscle upon hypoxia exposure (fig. S1). In this previous work, we divided the pulmonary arterioles based on their position in the vascular tree and lumen diameter into proximal (P; >75 μ m), middle (M; 25 to 75 μ m), and distal (D; <25 μ m), and determined that preexisting pulmonary artery (PA) SMCs give rise to the vast majority of hypoxia-induced distal arteriole SMCs (10).

Here, we analyzed the clonal relationship of hypoxia-induced distal arteriole SMCs. These studies used the multicolor Rainbow (Rb) Cre reporter, which upon Cre-induced recombination randomly expresses Cerulean, mCherry, or mOrange in a mutually exclusive manner (14, 15). Thus, a *ROSA26R*^(Rb/+) cell that has undergone Cre-induced recombination will permanently express only one of these fluorophores, and all of the cell's progeny will also express the same fluorophore. Mice carrying *SMA-CreER^{T2}* (16) and *ROSA26R*^(Rb/+) were induced with tamoxifen, rested, and exposed to normoxia or hypoxia (FiO₂ 10%) for 7 or 21 days, and then pulmonary arterioles were imaged for the three Rb colors (Fig. 1). Because SMCs of the proximal and middle pulmonary arterioles are present at the time of tamoxifen induction, they are a mixture of cells marked by Cerulean, mOrange, or mCherry (normoxia in Fig. 1). The hypoxia-induced distal arteriole SMCs could potentially either derive from multiple preexisting PA SMCs and thus be of multiple colors (that is, polyclonal) or instead derive from expansion of a single PA SMC and be one color (Fig. 1A). Hypoxia-induced SMCs of each distal arteriole were almost all of a single color, indicating monoclonality (Fig. 1, B and C).

Primed SMCs are the source of distal arteriole smooth muscle

We next sought to identify the parent preexisting SMC that gives rise to the hypoxia-induced distal SMCs in a given arteriole. We determined that each arteriole in the aforementioned vascular beds contained an average of 2.4 ± 0.7 PDGFR- β ⁺SMA⁺SMMHC⁺ cells (range, 1 to 3 cells; $n = 16$ arterioles from six lungs), and each of these cells was located at the middle-distal (M-D) arteriole border (Fig. 2, A to C), which under normoxic conditions coincides with the transition from the muscularized to unmuscularized blood vessel (10).

We hypothesized that this novel SMC subtype is primed to migrate distally and clonally expand in response to hypoxia and therefore fate-mapped primed SMCs during hypoxia-induced PH. Mice carrying PDGFR- β -*CreER^{T2}* and the *ROSA26R*^(mTmG/+) Cre reporter (17) were induced with tamoxifen to label primed cells, rested, and then analyzed immediately (Fig. 2C) or after exposure to normoxia or hypoxia for 3 weeks (Fig. 2, D to G). The GFP lineage tag was detected in $95 \pm 2\%$ of distal arteriole SMCs ($n = 205$ cells scored in eight arterioles from three lungs) in hypoxia. Together with the clonal analysis findings (Fig. 1), these data indicate that a single specialized arteriole SMC present at the muscular-

unmuscular border under normoxic conditions is the source of almost all hypoxia-induced distal arteriole SMCs.

Pulmonary arteriole SMCs express KLF4 in PH

We recently showed that during hypoxia-induced distal muscularization in mice, pulmonary arteriole SMCs undergo stereotyped steps of dedifferentiation (SMMHC down-regulation), distal migration, proliferation, and finally, differentiation (SMMHC expression and PDGFR- β down-regulation) (10); however, the underlying molecular mechanisms have not been defined. In cultured rat aortic SMCs, knockdown of the pluripotency factor *KLF4* attenuates PDGF-BB-induced dedifferentiation (18). The function of any pluripotency factor in SMCs in PH pathogenesis or with hypoxia has not been investigated, and under basal conditions, *KLF4* has not been identified in SMCs (19–21).

Thus, we initially evaluated *KLF4* expression in human PA SMCs in lung sections and in culture under pathological conditions. In comparison to control patients, lung sections from PH and PAH patients demonstrated up-regulation of PDGFR- β in the pulmonary arteriole wall and increased muscularization (Fig. 3A), although some arterioles in diseased samples were only coated by a single smooth muscle layer (Fig. 3B). Moreover, there was a marked increase in the numbers of SMCs that were *KLF4*⁺ (Fig. 3, A to D)—where 92.0% of muscularized arterioles from PH and PAH samples had at least one *KLF4*⁺ SMC versus 1.5% in controls—or proliferative, as indicated by Ki67 expression (Fig. 3, C and E). Furthermore, *KLF4* expression and proliferation were highly correlated in pulmonary arteriole SMCs of PH or PAH patients, respectively: 67 or 79% of *KLF4*⁺ SMCs were also Ki67⁺, and 62 or 35% of Ki67⁺ SMCs were also *KLF4*⁺ (Fig. 3, F and G). In addition, hypoxia treatment induced *KLF4* expression in cultured primary human pulmonary arteriole SMCs (Fig. 3, H and I).

We next evaluated the spatiotemporal expression pattern of *KLF4* in pulmonary arteriole SMCs during PH onset in the hypoxia mouse model. Under normoxic conditions, the number of SMCs expressing *KLF4* throughout the pulmonary arterioles was negligible (Fig. 4, A to C). Hypoxia day 3 is the first time at which SMA⁺ cells are located distal to the M-D border (10), and at this time point, many of these cells expressed *KLF4* as did middle arteriole SMCs in proximity to the M-D border (the “M_b region”; in contrast, the “M_a region” is the more proximal portion of the middle arteriole) (Fig. 4, A and B). By hypoxia day 7, *KLF4* expression peaked with ~60% of distal pulmonary arteriole SMCs expressing *KLF4* and then subsequently decreased by days 14 to 21 (Fig. 4, A to C, and fig. S2), which coincides with the distal arteriole SMC differentiation phase (10).

Because primed cells are the source of distal arteriole smooth muscle, we then evaluated *KLF4* expression in primed cells. At day 2 of hypoxia, SMA⁺ cells had not yet breached the M-D border (Fig. 4C and fig. S2), and primed cells remained at this border, uniformly *KLF4*⁺ ($n = 40$ primed cells in 16 arterioles) but not proliferative [no bromodeoxyuridine (BrdU)⁺ primed cells detected; $n = 6$ arterioles from two lungs]. Furthermore, 85% of *KLF4*⁺ SMCs in the M_b region were primed cells (Fig. 4D).

SMCs require KLF4 cell autonomously to muscularize the distal arteriole in PH

Given the early, robust, and specific upregulation of KLF4 in primed SMCs with hypoxia exposure, we next evaluated the role of smooth muscle KLF4 in distal arteriole muscularization. To delete *Klf4* in SMA⁺ cells, *SMA-CreER^{T2}* mice also carrying *Klf4^(flox/flox)* (22) were induced with tamoxifen, and then these mice were rested and exposed to hypoxia for up to 21 days. Tamoxifen treatment prevented hypoxia-induced KLF4 expression in middle pulmonary arteriole SMCs and distal arteriole muscularization (Fig. 5A and fig. S3). Furthermore, *Klf4* deletion prevented PH and right ventricle (RV) hypertrophy (Fig. 5, B and C). In the absence of tamoxifen, mice exposed to 3 days of hypoxia demonstrated rare PDGFR-β⁺SMA⁺ cells that breached the M-D border (Fig. 5D). Additionally, consistent with our previous results (10), without tamoxifen pretreatment, 21 days of hypoxia induced redistribution of PDGFR-β⁺SMA⁺SMMHC⁺ cells to the new distally located muscular-unmuscular transition zone (Fig. 5E and fig. S4). In contrast, with tamoxifen-induced *Klf4* deletion in SMCs, primed cells remain localized to the muscular-unmuscular M-D border under normoxic or hypoxic conditions (Fig. 5, D and E). These data as well as experiments with cultured human PA SMCs (fig. S5, A to C) suggest that KLF4 is a key factor in hypoxia-induced SMC migration and proliferation.

Clonal analysis and primed cell fate mapping collectively suggest that a single primed cell gives rise to almost all hypoxia-induced distal pulmonary arteriole SMCs in mice. Primed cells expressed KLF4 in response to hypoxia, and SMC *Klf4* was a requisite for hypoxia-induced distal muscularization. Thus, we postulated that there is competition between preexisting arteriole SMCs to give rise to hypoxia-induced distal arteriole SMCs and that KLF4 expression conveys an advantage in this competition.

To test this hypothesis, we conducted a mosaic analysis to compare the contribution to distal arteriole muscularization of PA KLF4⁺ SMCs and *Klf4*-deficient SMCs (Fig. 5F). *SMA-CreER^{T2}*, *ROSA26R^(mTmG/+)*, and *Klf4^(flox/flox)* mice were injected with a single, moderate tamoxifen dose (1 mg) and then allowed to rest for 3 days to generate proximal and middle pulmonary arterioles containing both *Klf4-null* and *Klf4^(flox/flox)* SMCs (fig. S6A) before exposure to normoxia or hypoxia for 21 days. Under normoxic conditions, 49 ± 5% of proximal and 52 ± 4% of middle arteriole SMCs were GFP⁺, confirming mosaicism (fig. S6). After 21 days of hypoxia, distal arteriole SMCs were exclusively GFP⁻, indicating that KLF4⁺ PA SMCs have a distinct advantage for producing distal pulmonary arteriole SMCs (Fig. 5F and fig. S6B).

Hypoxia-induced PDGF-B up-regulation is required for distal arteriole muscularization, PH, and primed cell KLF4 expression

Because PDGFR-β is expressed by primed cells, we characterized lung expression of its ligand PDGF-B in mice exposed to normoxia or hypoxia. Immunohistochemistry detected minimal PDGF-B in the normoxic lung but substantial up-regulation diffusely in the lung within 2 days of hypoxia (Fig. 6A and fig. S7). In addition, analysis of isolated cells at this time point demonstrated marked up-regulation of *Pdgfb* transcript in ECs of the hypoxic lung (fig. S8 and table S1). Subsequently, PDGF-B protein expression was polarized, enriched in peripheral regions of the lung at hypoxia day 7, and by day 21, anti-PDGF-B

staining intensity was decreased with the remaining PDGF-B predominantly limited to the lung periphery (Fig. 6A). Such hypoxia-induced polarized PDGF-B expression may be important for directional distal pulmonary arteriole muscularization.

We then exposed *PDGF-B*^(+/-) mice (23) to hypoxia for 21 days, and these mice demonstrated unmuscularized distal pulmonary arterioles as well as normal PA blood pressure and RV mass (Fig. 6, B to D). In contrast, hypoxia-exposed wild-type mice have muscularized distal arterioles, PH, and RV hypertrophy (Fig. 6, B to D). Notably, there were equivalent numbers of primed SMCs in normoxic *PDGF-B*^(+/-) and wild-type mice (2.7 ± 0.6 per arteriole versus 2.4 ± 0.7 per arteriole, respectively), but exposure to 2 days of hypoxia resulted in $81 \pm 3\%$ reduction in KLF4⁺ primed cells in *PDGF-B*^(+/-) compared to wild-type mice (Fig. 6, E and F). Related studies with cultured primary human PA SMCs indicate that PDGF-B-induced migration is markedly attenuated by small interfering RNA (siRNA)-mediated knockdown of *KLF4* (fig. S5, D and E).

DISCUSSION

Here, we primarily focus on the pathological muscularization of normally unmuscularized distal pulmonary arterioles, which occurs in diverse forms of PH and is important to disease pathogenesis (4, 6–8, 12, 24). PH is generally also characterized by the thickening of the adventitial, medial, and intimal compartments, whereas changes typical of advanced PAH include obliterative plexiform lesions and a hypercellular and fibrotic neointima (6, 7, 12, 24). The classical smooth muscle phenotypic modulation model purports that in response to pathological insults, numerous differentiated vascular SMCs dedifferentiate, migrate, and proliferate, and then subsequently redifferentiate and become quiescent (25). However, in the current study, we identify a pool of select pulmonary arteriole SMC progenitors that have a head start on other SMCs in disease given their position and expression of the undifferentiated marker PDGFR- β under basal conditions (Fig. 7A). We termed these PDGFR- β ⁺SMA⁺SMMHC⁺ cells at the M-D pulmonary arteriole border (which coincides with the muscular-unmuscular transition in normoxia) as primed cells because they are poised to muscularize the distal arteriole in response to hypoxia. Each M-D arteriole border has one to three primed cells, and upon hypoxic exposure, primed cells are induced (Fig. 7B), migrate beyond this border, and dedifferentiate (Fig. 7C); one of these cells clonally expands (Fig. 7D), giving rise to almost all of the differentiated pathological distal arteriole SMCs (Fig. 7E).

In contrast to our findings, a previous study using X-chromosome inactivation for clonal analysis of sectioned human pulmonary arterioles suggests that there is monoclonal EC accumulation in idiopathic PAH, but pathological SMCs in idiopathic PAH or PH due to secondary causes, such as congenital heart disease, generally do not arise by clonal expansion (26). This difference in SMC clonality between our studies in mice and the human data perhaps reflects differences between species or the intrinsically crude nature of clonal analysis of sectioned human tissue. In addition, the hypoxia mouse model mimics human PH (WHO group 3) but does not recapitulate many facets of PAH (WHO group 1), such as intimal fibrosis and proliferation as well as plexiform lesions (7); however, distal pulmonary arteriole muscularization is a key pathological characteristic of PH in general.

Related studies in human aortic and coronary artery atherosclerotic lesions as well as an atherosclerotic mouse model suggest that patch size of monoclonal SMCs in atherosclerotic lesions may be large (27–29). Thus, it is intriguing to consider whether blood vessels prone to atherosclerosis have specialized cells with similar properties to pulmonary arteriole primed cells and whether such cells clonally expand in disease.

SMC dedifferentiation is an integral component of vascular disease in general (25, 30), and PH in particular (10), and the pluripotency factor KLF4 is a key player in cell dedifferentiation (31, 32). KLF4 protein has not been identified in SMCs under basal conditions (19–21); however, in mouse models, KLF4 is expressed in the tunica media after carotid artery ligation (19) and in SMC-derived cells in atherosclerosis (33). Furthermore, smooth muscle–specific *Klf4* deletion has been shown to attenuate the severity of vascular disease (atherosclerosis and aortic aneurysm) in mice (33, 34). Here, we examined the in vivo role of smooth muscle KLF4 in PH: hypoxia-induced KLF4 expression is required cell autonomously for primed cell migration beyond the M-D border and hence facilitates clonal expansion, distal arteriole muscularization, and PH. Furthermore, in humans with PH and PAH, KLF4 expression is markedly up-regulated in proliferative pulmonary arteriole SMCs. In cultured rat aortic SMCs, KLF4 has previously been shown to play a key role in PDGF-B–induced down-regulation of differentiation markers (35). Here, we demonstrated that hypoxia-induced PDGF-B expression is required for primed cell KLF4 expression, distal pulmonary arteriole muscularization, and PH.

In addition to SMCs, KLF4 is also expressed in a number of other cell types in the lung, including ECs (20, 36, 37). Constitutive *Klf4* deletion in ECs exacerbates hypoxia-induced PH, and lungs isolated from these mice have altered levels of gene products implicated in PH pathogenesis, including reduced endothelial nitric oxide synthase and prostacyclin synthase and increased endothelin 1 (37, 38). Thus, in the absence of smooth muscle targeting, therapeutic strategies that broadly inhibit KLF4 function in the lung may be suboptimal. In an effort to delineate cell type–specific targets, future studies should compare the regulation of and by KLF4 in PA ECs and SMCs under basal and pathological conditions.

We have identified previously undescribed SMC progenitors that are present in normal PAs and give rise to almost all hypoxia-induced distal pulmonary arteriole SMCs in PH. The high fidelity of the normal pulmonary vasculature with regard to the discreet transition from unmuscular to muscular arteriole and the location of primed cells at this transition are striking. The initial migration of primed cells beyond this M-D border appears to be critical to PH pathogenesis and is facilitated by a signaling pathway of hypoxia-induced expression of lung PDGF-B, which induces KLF4 expression in primed cells. With continued hypoxic exposure, differentiated SMCs populate the distal arteriole, but rare cells with a similar molecular signature to primed cells are positioned at the new distally localized muscular-unmuscular border (Fig. 7E). Thus, the muscular-unmuscular vascular transition zone may generally be a niche that is permissive or inductive to primed cells. Furthermore, in the future, it will be important to determine the fate of primed cells during pulmonary arteriole reverse remodeling when hypoxia-treated mice return to normoxic conditions.

Improved therapies are desperately needed for PH and PAH because almost one-half of patients die within 3 years of the initial PAH diagnosis (5). Targeting primed cells in the early stages of PH pathogenesis is an attractive therapeutic strategy. By characterizing the gene expression profiles of primed cells from the human lung, molecular pathways may be identified that can be used to specifically target and/or manipulate the biology of these cells. In sum, primed cells are critical players in the pathogenesis of PH and perhaps other cardiovascular diseases as well, and intense study of this specialized SMC progenitor pool is warranted.

MATERIALS AND METHODS

Study design

The overall objectives of this study were to identify and characterize a novel SMC progenitor pool in the pulmonary arterioles and to determine the molecular and cellular processes underlying the contribution of these progenitors to distal arteriole muscularization in disease. To this end, we studied a hypoxia-induced mouse model of PH as well as lung samples from humans with PH and PAH. For mouse studies, a power analysis with 95% power and $\alpha = 0.05$ indicated that a sample size of a minimum of four mice per group was required for expression analysis and gene deletion experiments. A negative binomial with one success analysis of the mouse clonal analysis experiments indicated that if SMCs of $n = 4$ newly muscularized distal arterioles are of the same color, the type 1 error (or α) for incorrectly claiming monoclonality was $<1\%$. For studies with human samples, a power analysis based on our initial data determined that samples from at least two distinct patients per group would provide 95% power to detect a significant difference with $\alpha = 0.05$. The number of repeats for each experiment is included in the figure legends. Endpoints and hypoxia duration and magnitude were prospectively selected on the basis of previous studies of hypoxia-induced PH (6, 10). All data are included (no outliers were excluded).

Animals and tamoxifen treatment

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Yale University. C57BL/6 mice (Jackson Laboratory) were used for wild-type analysis. *SMA-CreER^{T2}*, *Klf4(flox/flox)*, *PDGF-B^(+/-)*, and Cre reporters *ROSA26R^(Rb)*, *ROSA26R^(mTmG)*, and *ROSA26R^(ZsGreen1)* have been described (14–17, 22, 23, 39), and *PDGFR- β -CreER^{T2}* mice were generated as described in the Supplementary Materials and Methods. For CreER-catalyzed recombination, unless otherwise noted, mice were injected intraperitoneally with tamoxifen (1 mg/day for 5 days), rested for 5 days, and then exposed to normoxia or hypoxia. For cell proliferation studies, mice were injected with BrdU, and lungs were analyzed as previously described (10).

Hypoxia treatment and hemodynamic measurements

Adult mice were exposed to hypoxia (FiO₂ 10%) for up to 21 days in a rodent hypoxia chamber with a calibrated oxygen controller and sensor (BioSpherix). RVSP (equivalent to PA systolic pressure) and the weight ratio of the RV to that of the sum of the LV and septum were measured (10).

Lung preparation

As described previously (10), after euthanasia by isoflurane inhalation, the pulmonary vasculature was flushed with phosphate-buffered saline (PBS), and lungs were inflated with 2% low-melt agarose. Solidified agarose-filled lobes were immersed in Dent's fixative (4:1 methanol/dimethyl sulfoxide) at 4°C overnight and then washed and stored in 100% methanol. In preparation for staining, lungs were bleached in 5% H₂O₂, rehydrated into PBS, vibratome-sectioned at a thickness of 150 µm, and then analyzed by immunohistochemistry. For clonal analysis, *SMA-CreER^{T2}*, *ROSA26R^(Rb/+)* lungs were fixed in 4% paraformaldehyde over-night, vibratome-sectioned, and directly imaged for the three Rb colors.

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 to 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 medium (Dako). Primary antibodies used were rat anti-MECA32 (1:15; Developmental Studies Hybridoma Bank), rabbit anti-GFP (1:250; Invitrogen), rabbit anti-SMMHC (1:250; Biomedical Technologies), rabbit anti-PDGF-B (1:100; Abcam), rabbit anti-KLF4 (1:100; R&D Systems), rat anti-BrdU (1:150; Jackson ImmunoResearch), mouse anti-human CD31 (1:50; Dako), rabbit anti-human von Willebrand factor (1:100; Dako), rabbit anti-human PDGFR-β (1:100; Cell Signaling), mouse anti-human Ki67 (1:100; Dako), directly conjugated Cy3 or fluorescein isothiocyanate mouse anti-SMA clone 1A4 (1:250; Sigma), and goat biotinylated anti-PDGFR-β (1:10; R&D Systems). Elite ABC reagents (Vector Laboratories) and fluorescein tyramide system (PerkinElmer) were used to amplify the biotinylated PDGFR-β staining as described previously (13, 15). Secondary antibodies were conjugated to either Alexa 488, Alexa 564, Alexa 647 (Invitrogen), or DyLight 649 (Jackson ImmunoResearch Laboratories) fluorophores (1:500). Nuclei were stained with DAPI (1:500).

Studies of deidentified human samples were deemed by the Yale University Human Investigation Committee as not constituting human subjects research. The human lung samples from patients with PH (WHO PH group 3, idiopathic pulmonary fibrosis and alveolar hypoventilation/sleep apnea; group 5, sarcoidosis), PAH (group 1, idiopathic PAH and connective tissue disorder), and controls (normal lungs and no cardiopulmonary illness) were obtained from the National Disease Research Interchange, Yale University Pathology, and Brigham and Women's Hospital, and were fixed in formalin, paraffin-embedded, and sectioned. Paraffin was removed from sections with xylene, and after ethanol washes, sections were rehydrated into water. Rehydrated sections were washed with TNT [10mM tris-HCl (pH 8.0), 150 µM NaCl, and 0.2% Tween 20] and subjected to antigen retrieval by incubating in boiling 10 µM sodium citrate (pH 6.0) for 20 min. Sections were then immersed in cold water and immunostained as described above for vibratome sections, except washes were done in TNT.

In vivo cell quantification

For mouse studies, the number of SMCs was determined by counting the DAPI-marked nuclei of cells staining for SMA on confocal sections of the proximal, middle, and/or distal regions of the specific arterioles. The middle arteriole was divided into M_b , the middle region within 35 μm of the M-D border, and M_a , the remaining proximal region of the middle arteriole (generally ~ 70 μm in length). Similarly, the number of primed cells was determined by counting the DAPI-marked nuclei of $\text{PDGFR-}\beta^+\text{SMA}^+\text{SMMHC}^+$ pulmonary arteriole cells. SMCs and primed cells were scored for KLF4 staining. For the Rb Cre reporter, the numbers of mOrange, mCherry, or Cerulean marked cells in the proximal, middle, and distal regions were scored. For human studies, the number of SMCs coating arterioles of diameter ~ 25 to 35 μm was determined as noted above for mice. SMCs were similarly scored for KLF4 and/or Ki67 staining.

Human PA SMC culture and hypoxia exposure

Primary human PA SMCs (American Type Culture Collection) were cultured in M199 medium supplemented with 10% fetal bovine serum (Invitrogen), 5% penicillin/streptomycin (Life Technologies), recombinant human fibroblast growth factor, and epidermal growth factor (R&D Systems). In select experiments, cells were subjected to KLF4 knockdown. Subsequently, cells were either exposed to normoxia or 1% hypoxia for up to 24 hours in M199 supplemented with 1% fetal bovine serum and 5% penicillin/streptomycin.

siRNA-mediated KLF4 knockdown

Human PA SMCs were transfected with Lipofectamine (Life Technologies) containing siRNAs (OriGene) targeted against *KLF4* (30 nM) or scrambled RNA for 6 hours. Cells were then washed in M199 and cultured in normal cell culture medium for 72 hours.

Western blot

For protein analysis, PA SMCs were serum-starved in M199 containing 5% penicillin/streptomycin overnight before exposure to normoxia or hypoxia. Cells were then lysed in radioimmunoprecipitation assay buffer containing cOmplete protease inhibitor and PhosSTOP phosphatase inhibitor cocktails (Roche Applied Science). Lysates were centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatants were collected. Protein concentrations were determined with the bicinchoninic acid kit (Pierce). For each protein sample, 40 μg was separated by SDS–polyacrylamide gel electrophoresis and transferred to nitro-cellulose membranes (Millipore) overnight. Membranes were blocked with 5% bovine serum albumin in PBS containing 0.05% Tween 20 for 2 hours and incubated with rabbit anti-KLF4 and rat anti-GAPDH antibodies (1:500; Cell Signaling Technology) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (1:2000; Cell Signaling Technology) for 1 hour. Detection was performed with the Western Blotting Substrate Plus (Pierce) and G:BOX imaging system (Syngene).

Proliferation assay of cultured cells

Primary human PA SMCs were treated with scrambled or KLF4-targeted siRNA and then exposed to normoxia or hypoxia (1% O₂) for up to 48 hours. Four hours before harvesting, BrdU (10 nM) was added to the cells. Cells were then treated with 1 M HCl for 15 min and stained for BrdU, SMA, and nuclei (DAPI).

Imaging

Lung sections were imaged on confocal microscopes (PerkinElmer UltraView Vox Spinning Disc or Leica SP5 point-scanning). Volocity software (PerkinElmer) and Adobe Photoshop were used to process images.

Statistical analysis

Student's *t* test, multifactor ANOVA, and post hoc test with Bonferroni corrections were used to analyze the data (StatPlus software). Statistical significance threshold was set at *P* 0.05. All tests assumed normal distribution and were two-sided, and all data are presented as means ± SD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Hypoxia-induced SMCs in distal pulmonary arterioles derive from a single preexisting SMC

SMA-CreER^{T2}, *ROSA26R^(Rb/+)* mice were induced with tamoxifen (1 mg/day for 5 days), rested for 5 days, and then exposed to normoxia or hypoxia (FiO₂ 10%). **(A)** Potential patterns of Rb colors in pulmonary arteriole SMCs. Middle (M) arteriole SMCs are present under normoxia and thus marked by different Rb colors [Cerulean (Cer), mOrange (mOr), or mCherry (mCh)]. Hypoxia-induced distal (D) arteriole SMCs will be multiple colors if they derive from multiple polyclonal preexisting SMCs or one color if they derive from a single preexisting SMC. **(B)** Vibratome sections of lungs isolated from one normoxic mouse and three mice exposed to hypoxia for 7 or 21 days as indicated and imaged with direct fluorescence of Rb color channels. Alveolar SMCs are marked by arrowheads. Scale bar, 25 μ m. **(C)** Quantification of SMCs in the proximal (P), middle (M), or distal (D) arterioles by color. Data are averages $\pm$ SD [$n=4$ lungs, two to three arterioles per lung; number of cells scored per arteriole by arteriole type were as follows: proximal (each condition, 165 to 230 cells), middle (each condition, 140 to 206), and distal (normoxia, 0; hypoxia, 70 to 108)].

Fig. 2. Primed cells are the major source of hypoxia-induced distal arteriole SMCs
(A and B) In wild-type (WT) mice, arterioles in proximity to airway branches L.L1.A1 (left bronchus–first lateral secondary branch–first anterior branch) were stained for SMA and PDGFR- β as well as for mouse pan-endothelial cell antigen-32 (MECA-32) [endothelial cell (EC) marker] (A) or SMMHC (B), as indicated. **(C)** *PDGFR- β -CreER^{T2}, ROSA26R^(mTmG/+)* mice were induced with tamoxifen and rested for 2 days. After the rest period, lungs were stained for PDGFR- β , green fluorescent protein (GFP) lineage tag, and SMMHC. **(D to G)** *PDGFR- β -CreER^{T2}, ROSA26R^(mTmG/+)* mice were induced with tamoxifen and rested for 5 days. Mice were then exposed to 21 days of normoxia or hypoxia before staining lungs for GFP, SMA, and MECA. The boxed regions in (D) and (F) are shown as close-ups in (E) and (G), and further magnifications of the boxes in (E) are displayed as insets. Results are representative of three mice and two to three arterioles per mouse. Scale bars, 25 μ m.

Fig. 3. KLF4 is up-regulated with PA SMC dedifferentiation and proliferation in PH and PAH patients

(A to C) Paraffin sections of pulmonary arterioles from human controls and PH and PAH patients, which are representative of three control patients (normal lungs and no cardiopulmonary illness), three PH patients (WHO PH group 3, idiopathic pulmonary fibrosis and alveolar hypoventilation/sleep apnea; group 5, sarcoidosis), and two PAH patients (group 1, idiopathic PAH and connective tissue disorder). Sections were stained for KLF4, SMA, nuclei [4',6-diamidino-2-phenylindole (DAPI)], and either PDGFR- β (A), von Willebrand factor (vWF) (B), or the proliferation marker Ki67 (C). Arrowheads in (C) indicate KLF4⁺Ki67⁺ SMCs. Scale bars, 25 μ m. (D to G) Quantification from images as shown in (A) to (C), indicating the percent of SMCs that are KLF4⁺ (D) or Ki67⁺ (E) as well as the percent of KLF4⁺ SMCs (F) or Ki67⁺ SMCs (G) that also express the other marker. Per each patient classification, at least 384 arterioles were scored (D to G); **P* < 0.01 versus control. (H and I) Primary human PA SMCs were treated with normoxia or hypoxia (1% O₂) for indicated times, and KLF4 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein levels were assayed by Western blot with densitometric analysis; *n* = 4 distinct samples in duplicate. Data are averages [relative to GAPDH in (I)] $\pm$ SD. Single-factor analysis of variance (ANOVA) was used in (D) to (G), and two-factor ANOVA was used in (I).

Fig. 4. Mice exposed to hypoxia have enhanced KLF4 expression in pulmonary arteriole SMCs
Mice were exposed to normoxia or indicated days of hypoxia (FiO₂ 10%), and then arterioles in proximity to L.L1.A1 airway branches were analyzed. **(A)** Lung vibratome sections stained for KLF4, SMA, MECA32, and nuclei (DAPI). **(B)** Quantification of KLF4⁺ SMCs in proximal (>75 μm diameter), middle (25 to 75 μm) and distal (<25 μm) arterioles. The middle arterioles were further classified into proximal (M_a) and distal (M_b) subdivisions (Materials and Methods). Images are representative of *n* = 4 lungs, two to three arterioles per lung; number of SMA⁺ cells scored per arteriole by arteriole type were as follows: proximal (each condition, 170 to 220 SMA⁺ cells), M_a (each condition, 100 to 120), M_b (each condition, 50 to 65), or distal (normoxia, 0; hypoxia 3 days, 25; hypoxia 7 days, 42; hypoxia 21 days, 110). **P* < 0.001 versus normoxia, distal arterioles; ^Δ*P* < 0.01 versus normoxia middle arteriole, M_b subdivision. **(C)** Arterioles from mice at normoxia or hypoxia days 2 and 7 stained for SMA, PDGFR-β, and KLF4. Images are representative of 13 arterioles. **(D)** Quantification of KLF4⁺ SMCs in proximity to M-D border (M_b region) at hypoxia day 2. Data are averages ± SD (*n* = 5 lungs, two to three arterioles per lung; total KLF4⁺ cells were 43). Statistical tests used were two-factor ANOVA (B) and Student's *t* test (D). Scale bars, 25 μm.

Fig. 5. KLF4 is required cell autonomously in SMCs for distal pulmonary arteriole muscularization and PH

(A to F) Mice were injected with tamoxifen [1 mg/day for 5 days in (A) to (E) for complete *Klf4* deletion or a single 1-mg injection in (F) for mosaic analysis], rested for 5 days (A to E) or 3 days (F), and then exposed to normoxia or hypoxia (FiO₂ 10%) for 3 days (D) or 21 days (A to C, E, and F). In *SMA-CreER*^{T2}, *Klf4*^(flox/flox) mice, arterioles in proximity to L.L1.A1 (A and E) or L.M1 (left bronchus–first medial branch) (D) airway branches were stained for SMA and MECA-32 and also for PDGFR-β (D and E) as indicated. Arrowheads in (D) indicate primed cells that migrated beyond the M-D border. Measurements of RV systolic pressure [RVSP; equivalent to PA systolic pressure] and the ratio of the weight of the RV to that of the sum of the left ventricle (LV) and septum (S) are shown (B and C). Data are averages ± SD (*n* = 4 mice). Statistical test used in (B) and (C) was two-factor ANOVA. Distal arterioles of the L.L1.A1 regions in *SMA-CreER*^{T2}, *Klf4*^(flox/flox), *ROSA26R*^(mTmG/+) mice were stained for SMA, GFP lineage tag, and MECA32 (for all immunohistochemistry experiments; *n* = 4 lungs for each condition and two to three arterioles per lung). Scale bars, 25 μm.

Fig. 6. Hypoxia induces polarized PDGF-B lung expression, and reduced PDGF-B prevents distal arteriole muscularization, PH, and primed cell KLF4 expression

(A) Adult WT mice were exposed to normoxia or to 2, 7, or 21 days of hypoxia (FiO₂ 10%), and for each time point, coronal left lung sections are shown as large panels on the left with staining for PDGF-B and SMA. Arrows indicate boxed regions, which contain an arteriole in proximity to the L.L1.A1.M1 airway and are displayed as two closeups (without or with staining for MECA-32) on the right for each time point ($n = 3$ lungs, two to three arterioles per lung). (B to D) *PDGF-B*^(+/-) or WT mice were exposed to normoxia or hypoxia for 21 days, and then RVSP and ventricle mass ratio were measured, and left lungs were stained for PDGF-B, SMA, and MECA-32. Data in (C) and (D) are averages $\pm$ SD. [$n = 4$ lungs for (B) to (D), and two to three arterioles were stained as in (B) per lung]. (E) The M-D border of arterioles in proximity to the L.M1 airway is shown for WT and *PDGF-B*^(+/-) mice exposed to hypoxia for 2 days. Sections were stained for KLF4, SMA, and PDGFR- β . (F) Quantification of the percentage of primed cells that express KLF4 from images, such as those shown in (E), of hypoxic WT and *PDGF-B*^(+/-) mice. Data are averages $\pm$ SD [$n = 4$ lungs, two to three arterioles per lung, and total scored primed cells were 28 in WT and 32 in *PDGF-B*^(+/-)]. Statistical tests used were two-factor ANOVA (C and D) and Student's *t* test (F). Scale bars, 20 μ m (A) and 25 μ m [(B) and (E)].

Fig. 7. Summary of molecular and cellular events in hypoxia-induced distal pulmonary arteriole muscularization

(A) Under normoxic conditions, SMCs (red outline) coat proximal and middle, but not distal, pulmonary arteriole EC tubes and express SMA and SMMHC (no fill). In addition, rare PDGFR-β⁺SMA⁺SMMHC⁺ primed SMCs (pink fill) are located at each M-D border, which coincides with the transition from muscularized to unmuscularized arteriole. (B) Upon initial hypoxic exposure, lung PDGF-B expression is markedly increased, which is required for KLF4 induction in primed cells (pink fill with red dot). (C) Within a day after KLF4 expression, an induced primed SMC (a KLF4⁺ PDGFR-β⁺ SMA⁺ SMMHC⁺ cell) migrates distally across the M-D border and, on the basis of our previous work (10), dedifferentiates, as indicated by down-regulating SMMHC expression (yellow fill with red dot). (D) Subsequently, the dedifferentiated cell clonally expands, giving rise the vast majority of distal arteriole SMCs. (E) These cells then reexpress SMMHC (10), and again, rare primed SMCs are localized at the now distally located muscular-unmuscular vascular border.