Endothelial Fate Mapping in Mice With Pulmonary Hypertension

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Background—Pulmonary endothelial injury triggers a reparative program, which in susceptible individuals is characterized by neointima formation, vascular narrowing, and the development of pulmonary arterial hypertension. The neointimal cells in human pathological plexiform lesions frequently coexpress smooth muscle α-actin and the endothelial von Willebrand antigen, creating a question about their cellular lineage of origin.

Methods and Results—Experimental pulmonary hypertension with neointima formation develops in C57Bl/6 mice subjected to left pneumonectomy followed 1 week later by jugular vein injection of monocrotaline pyrrole (20 μg/μL and 1 μL/g; group P/MCTP). Compared with the group vehicle, by day 35, group P/MCTP developed higher right ventricular systolic pressure (54±5 versus 25±2 mm Hg; P<0.01) and right ventricular hypertrophy (0.58±0.16 versus 0.26±0.05; P<0.01). Transgenic vascular endothelial-cadherin Cre recombinase or Tie-2 Cre mice were intercrossed with mTomato/mGreen fluorescent protein double-fluorescent Cre reporter mice to achieve endothelial genetic lineage marking with membrane-targeted green fluorescent protein. In control mice, few endothelial lineage–marked cells lining the lumen of small pulmonary arteries demonstrate expression of smooth muscle α-actin. Concurrent with the development of pulmonary hypertension, endothelial lineage–marked cells are prominent in the neointima and exhibit expression of smooth muscle α-actin and smooth muscle myosin heavy chain. Human pulmonary arterial hypertension neointimal lesions contain cells that coexpress endothelial CD31 or von Willebrand antigen and smooth muscle α-actin.

Conclusion—Neointimal cells in pulmonary hypertension include contributions from the endothelial genetic lineage with induced expression of smooth muscle α-actin and smooth muscle myosin heavy chain. (Circulation. 2014;129:692-703.)

Key Words: cell lineage ▪ monocrotaline pyrrole ▪ pneumonectomy ▪ vascular diseases

Idiopathic pulmonary arterial hypertension (PAH) is characterized by pathological neointima formation within small pulmonary arteries, leading to increased pulmonary vascular resistance and arterial pressure, which in turn causes right ventricular congestive failure and eventual death.1 In susceptible individuals, diverse endothelial injuries trigger vascular dysfunction, including insufficient vasodilation, excessive vasoconstriction, enhanced growth of smooth muscles, and prominent perivascular inflammation.2

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The pathological lesions of pulmonary hypertension demonstrate features of intimal proliferation, medial hypertrophy, and adventitial fibrosis.3,4 Small pulmonary arteries exhibit greater luminal narrowing associated with neointima formation than musculization of the medial wall. Chronic severe pulmonary vascular disease is associated with plexiform lesions that demonstrate "a central zone of proliferative endothelial tissue," often associated with a thrombus and eventual replacement by fibrous tissue.5

The expression of smooth muscle α-actin (SMA) within neointimal cells has long raised questions for pathologists about what cell lineages contribute to the neointima. The most likely cell lineages that may contribute to the pathological neointima include, vascular smooth muscle cells undergoing dedifferentiation, myofibroblasts derived from migrating adventitial fibroblasts, and endothelial cells transitioning into mesenchymal cells.6,7 Expression of the endothelial von Willebrand antigen in plexiform lesions and microsatellite analyses was used to infer that individual plexiform lesions represent monoclonal expansions of endothelial cells.8 Experimental models of pulmonary hypertension that have used combinations of chronic hypoxia, pneumonectomy, and endothelial injury by monocrotaline or the vascular endothelial growth factor receptor antagonist SUGEN 5416 have been reviewed.9 Whereas models based on hypoxia demonstrate...

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predominantly hypertrophy of medial smooth muscles, models based on endothelial injury after exposure to SU5416 or monocrotaline trigger more local inflammation and proliferative neointimal lesions that exhibit pathologies similar to human idiopathic PAH. Endothelial injury models of experimental pulmonary hypertension are augmented by combination with a second stimulus such as hypoxia exposure after SU5416 and pneumonectomy before monocrotaline.

The *Crotalaria* plant alkaloid monocrotaline undergoes hepatic metabolism in many species (except mice) into monocrotaline pyrrole (MCTP), which reacts covalently with pulmonary endothelial cells, triggering megalocytosis, microvascular leak, mononuclear cell infiltration, and alterations in muscular walls that lead to pulmonary hypertension.10-14 The addition of surgical pneumonectomy to monocrotaline injection (60 mg/kg, single injection subcutaneously) produced greater experimental pulmonary hypertension with severe neointimal formation in rats compared with monocrotaline alone.15,16 Surgical pneumonectomy increases blood flow and triggers compensatory lung growth in the remaining lung. We tested the hypothesis that increased blood flow contributes to the pathogenesis of experimental pulmonary hypertension by surgical creation of an aortocaval fistula to further increase pulmonary blood flow in pneumonectomized rats injected with monocrotaline.16 Unexpectedly, we discovered that increased pulmonary blood flow in rats with an aortocaval fistula reduced the severity of pulmonary hypertension. We proposed that increased oxygen concentration in the mixed venous blood of animals with an aortocaval fistula conferred protection against neointima formation and pulmonary hypertension. In this rat model of experimental pulmonary hypertension, we concluded that pneumonectomy amplifies the pathophysiology of disease through induction of compensatory lung growth. We and others used rat models of experimental pulmonary hypertension triggered by endothelial injury to characterize novel antiproliferative strategies to reverse established pulmonary vascular disease such as simvastatin.17-20

In contrast to rats, mice are relatively resistant to the induction of pulmonary hypertension by monocrotaline.21 Mice administered monocrotaline at 24 mg·kg⁻¹·d⁻¹ in the drinking water for 6 weeks (10 times higher dose than required in rats to produce an equivalent degree of pulmonary endothelial dysfunction) demonstrated lung edema and inflammation but did not develop significant occlusive wall thickening of pulmonary arteries. Recently, several groups reported that injection of 300 or 600 mg/kg monocrotaline (10 times higher dose than used in rats) subcutaneously once per week for 4 to 10 weeks produced mild increases in right ventricular systolic pressures (RVSPs), right ventricular hypertrophy, and vascular narrowing.22-25 As in rats, monocrotaline induction of pulmonary hypertension in mice is characterized by perivascular accumulation of inflammatory cells.

MCTP is the active metabolite of monocrotaline and is 200 to 400 times more potent in producing cell injury and triggering the development of pulmonary hypertension.26,27 We synthesized MCTP by chemical dehydration as described by Mattocks et al28 for direct injection through the jugular vein into the pulmonary circulation to trigger endothelial injury and pulmonary hypertension. From our experience in rats,16 we elected to combine pneumonectomy with injection of MCTP into the pulmonary circulation with the intention of producing a 2-hit mouse model that demonstrated substantial pulmonary hypertension with neointima formation.

Genetic lineage marking achieved through Cre-directed recombination at loxP sites permanently alters the genome so that a given cell and all its progeny are indelibly labeled.29 Analyses of cell fates during normal development and lineage transitions that occur during disease pathogenesis become feasible. Here, we test the hypothesis that the endothelial genetic lineage contributes to the neointima in experimental pulmonary hypertension. We demonstrate that a fraction of neointimal cells exhibit an endothelial lineage of origin while actively expressing smooth muscle genes.

**Methods**

**MCTP Synthesis**

Dehydromonocrotaline, which is MCTP, was synthesized from monocrotaline (Sigma) by the method of Mattocks et al.28 With the use of mass spectrometry and nuclear magnetic resonance spectroscopy, the conversion of monocrotaline to MCTP was found to be complete. For the starting product, the parent peak is at m/e 326, and the desired peak for MCTP is at m/e 324. The mass spectra of the product contained daughter ions characteristic of MCTP. The MCTP was dissolved in anhydrous acetonitrile to a final concentration of 20 μg/μL; the product was divided into aliquots and placed in vials (500 μL); the solvent was evaporated; and the lyophilized MCTP was stored at −80°C, shielded from light, until just before use. Five minutes before injection into mice, MCTP was dissolved into dimethylformamide (DMF).

The study was approved by the Stanford Administrative Panel on Laboratory Animal Care, and all animals received humane care.

**Left Pneumonectomy**

Mice were anesthetized in a plastic box by exposure to 2.5% isoflurane for 5 minutes. Endotracheal intubation was performed by use of a 20-gauge Teflon catheter over a fiberoptic light and stylus (Kent Scientific Corp, Torrington, CT). After intubation, mice were mechanically ventilated with a MiniVent Type 845 (stroke volume, 200 μL; 110 strokes per minute; Hugo Sachs Electronic/Harvard Apparatus, Holliston, MA), and anesthesia was maintained with 1.5 to 2.0% isoflurane. Mice were positioned supine; the forelimbs were gently retracted with tape; and the sternum and left side of the chest were cleaned by aseptic technique. Left thoracotomy was performed in the fourth intercostal space with atraumatic technique, and the ribs were retracted with 2 hooks fashioned from large paper clips. The left lung was gently lifted out of the chest; the hilum was clamped with an atraumatic mosquito clamp and ligated with 4-0 silk suture; and the left pneumonectomy was completed with aseptic technique. Isoflurane was turned to 0% while the ribs were closed with a single 4-0 suture and the skin was closed with two 4-0 sutures. When mice were confirmed awake by spontaneous ventilation and appropriate movement in response to stimulation, the mice were extubated and transferred into a warm recovery cage (30°F–35°F, 2 L/m oxygen) for 2 hours. Survival 1 day after pneumonectomy was 90% to 100%.

**MCTP Administration**

One week after left pneumonectomy, mice were anesthetized in a plastic box using 2.0% isoflurane and then positioned supine in a plastic box using 2.0% isoflurane and then positioned supine, and anesthesia was maintained with 1.5 to 2.0% isoflurane. Mice were positioned supine; the forelimbs were gently retracted with tape; and the sternum and left side of the chest were cleaned by aseptic technique. Left thoracotomy was performed in the fourth intercostal space with atraumatic technique, and the ribs were retracted with 2 hooks fashioned from large paper clips. The left lung was gently lifted out of the chest; the hilum was clamped with an atraumatic mosquito clamp and ligated with 4-0 silk suture; and the left pneumonectomy was completed with aseptic technique. Isoflurane was turned to 0% while the ribs were closed with a single 4-0 suture and the skin was closed with two 4-0 sutures. When mice were confirmed awake by spontaneous ventilation and appropriate movement in response to stimulation, the mice were extubated and transferred into a warm recovery cage (30°F–35°F, 2 L/m oxygen) for 2 hours. Survival 1 day after pneumonectomy was 90% to 100%.

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mouse weight (1 μL/g). A dissecting microscope was used to guide
the needle into the right internal jugular vein, and the MCTP
was injected slowly over ≈1 minute. After removal of the needle, pressure
was applied to the injection site indirectly through a layer of fatty
tissue until hemostasis was complete. The skin was then closed with
two 4-0 sutures. Survival at 3 days after MCTP injection was ≈75%.

Hemodynamic Studies
For hemodynamic measurements, mice were anesthetized in a
plastic box using 2% isoflurane and then placed in a supine posi-
tion. Anesthesia was maintained with 1% isoflurane delivered by
hood. RVSP measurements were obtained by percutaneous needle
(30 gauge) puncture of the right ventricle or through use of a Millar
catheter (SPR-1000) inserted through the right internal jugular vein
and a PowerLab data acquisition system (AD Instruments, Colorado
Springs, CO).

Tissue Preparation and Histology
Anesthetized mice were euthanized by exsanguination. The heart
and lungs were perfused with PBS followed by 10% neutral buffered
formalin. The heart was weighed, and right ventricular hypertrophy
was determined by the Fulton Index: right ventricle/(left ventricle
and septum). The formalin-fixed lungs were embedded in paraffin
and 5-μm sections were stained with hematoxylin and eosin and with
elastin–van Gieson to mark internal elastic lamina. Vascular narrow-
ing associated with neointima formation was evaluated as described
previously18,30,31: Absence of neointima formation was scored 0; neo-
intima formation causing <50% luminal narrowing was scored 1; and
luminal narrowing >50% was scored 2. The scores of 15 consecu-
tive interacinar pulmonary arteries were assessed to determine the
relative vascular occlusion score (VOS). Immunohistochemistry was
performed on formalin-fixed lung sections stained with a biotinylated
monoclonal antibody (mAb) against SMA (Clone 1A4, Dako), and
detection was performed with the use of streptavidin-peroxidase and
diaminobenzidine substrate.

Study Design
In the pilot study, 4 male C57Bl/6 mice (body weight, 24–28g) under-
went left pneumonectomy on day 0 and injection of MCTP in DMF
(20 μg/g) on day 7. RVSP measurements were made by percutaneous
needle puncture of the study mice on days 0, 7, 21, 35, and 42. Mice
were euthanized on day 42 for histology.

In the main physiology study, 42 pathogen-free, 12-week-old,
male C57Bl/6 mice (body weight, 24–28g) were studied in 5 groups:
Group C (n=6) served as a reference control; group V (n=6) received
DMF vehicle on day 7 (1 μL/g); group P (n=6) received left pneumo-
nectomy on day 0; group MCTP (n=12) received injection of MCTP
in DMF (20 μg/μL and 1 μL/g) on day 7; and group P/MCTP (n=12)
received left pneumonectomy on day 0 followed by injection of
MCTP in DMF (20 μg/μL and 1 μL/g) on day 7. The mice underwent
hemodynamic measurements and euthanasia on day 35.

Statistical Analysis
Data are presented as means±SD. The pilot study of RVSP was ana-
lyzed with a repeated measures ANOVA followed by the Newman-
Keuls tests. The VOSs of control and P/MCTP mice were compared
by a t test. In the main study, groups V, P, MCTP, and P/MCTP were
analyzed by ANOVA and Newman-Keuls tests and with nonparamet-
ric Kruskal-Wallis ANOVA (StatPlus software). Statistical signifi-
cance was indicated by P<0.05.

Genetic Lineage Marking
Double-transgenic mice with endothelial genetic lineage mark-
ing were generated by intercrossing vascular endothelial-cad-
herin (VE-Cad) Cre29 or Tie-2 Cre32 recombinase driver mice with
mTomato/mGreen fluorescent protein (GFP) floxed dual-fluorescent Cre reporter mice. Cre-mediated excision of the membrane-targeted dTomato gene is accompanied by expression of membrane-targeted GFP in endothelial cells.

Immunofluorescence

After exsanguination, the right lung was perfused with PBS followed by 2% paraformaldehyde. Lung lobes were immersed in 2% paraformaldehyde for 2 hours, followed by dehydration in 30% sucrose overnight and embedding in optimal cutting temperature compound. Cryosections were cut at 40-μm thickness. Immunostaining was performed for CD31 or VE-Cad at 1:50 dilution and for SMA and smooth muscle myosin heavy chain (SM-MHC) antigens at 1:200 dilution (rat anti-mouse CD31 mAb MEC13.3 conjugated to Alexa Fluor 647, BioLegend; rat anti-mouse CD144 VE-Cad mAb BV13, eBioscience; mouse anti-SMA mAb IgG2a 1A4, Sigma; mouse anti–SM-MHC mAb IgG1 1G12, Abcam; secondary antibodies used were Alexa Fluor 647 goat anti-rat IgG at 1:50 dilution, Alexa Fluor 647 goat anti-mouse IgG1 or IgG2a at 1:200 dilution). Human lung samples (deidentified) were obtained from the Pathology Department of Stanford Hospital, fixed in 10% formalin for 2 days, dehydrated in sucrose, and embedded in optimal cutting temperature compound for cryosectioning. Immunostaining was performed for CD31 (mouse mAb IgG1 anti-human CD31 JC70A at 1:30 dilution) or von Willebrand factor (mouse mAb IgG1 F8/86 at 1:30 dilution); secondary antibody was Alexa 488 goat anti-mouse IgG1 at 1:100 dilution or mouse anti-SMA mAb 1A4 conjugated to Cy3 at 1:100 dilution. Nuclei were counterstained with DAPI. Experiments conducted using isotype control antibodies, mouse IgG1, and IgG2a (Abcam) in primary incubations followed by secondary detection with Alexa Fluor 647 goat anti-mouse IgG1 or IgG2a demonstrated no specific labeling on the lung sections (data not shown). Confocal microscopy was performed with a Leica DMI 6000 equipped with BD Carv II confocal imager, Chroma Photofluor metal halide illumination, and Leica Imaging Software. Sequential 1-μm optical sections were acquired at 350-nm (DAPI), 488-nm (GFP), 568-nm (dTomato), and 647-nm (immunostaining) wavelengths, and contrast was enhanced with deconvolution software (Leica). NIH Image J software was then used for analyses of the serial z-stacks of 1-μm optical slices.

Results

Mouse Model of Experimental Pulmonary Hypertension

In the pilot study, 4 wild-type C57Bl/6 male mice underwent left pneumonectomy on day 0 and jugular vein injection of synthetic MCTP in DMF (20 μg/g) on day 7 (P/MCTP). Serial measurements of RVSP showed development of PAH by day 35 (Figure 1A). The pilot study of RVSP was analyzed with a repeated measures ANOVA followed by Newman-Keuls tests.

Figure 2. Histopathology of experimental pulmonary hypertension in pneumonecetomized mice injected with monocrotaline pyrrole. A and B, Normal muscular pulmonary artery (PA) adjacent to bronchiole (Br). A, Hematoxylin and eosin stain (H&E). B, Elastin–van Gieson stain (EVG). C and D, Peribronchiolar pulmonary arteries in pneumonecetomized mice injected with monocrotaline pyrrole (P/MCTP) demonstrate medial hypertrophy and neointima formation (C, H&E; D, EVG; internal elastic lamina [IEL] is marked). E and F, Smooth muscle α-actin immunostaining of peribronchiolar pulmonary arteries (E) and intra-acinar pulmonary artery (F) in P/MCTP mice. Objective magnification ×40.
The RVSP at 35 and 42 days was significantly higher than at 0, 7, and 21 days (P<0.01). Mice were euthanized at day 42 for organ harvest and histopathology. The time course of development of experimental pulmonary hypertension in this mouse model correlates closely with our previous studies in pneumonectomized rats injected with monocrotaline.16,17,30,31,34

**Histopathology**

Histology was characterized on paraffin-embedded sections of lung stained with hematoxylin and eosin or with elastin–Van Gieson to mark the elastic lamina of the pulmonary arteries (Figure 2). Compared with control mice (Figure 2A and 2B), P/MCTP mice demonstrated narrowing of peribronchiolar and intra-acinar pulmonary arteries associated with both medial hypertrophy and neointima formation (Figure 2C and 2D). SMA expression determined by immunohistochemistry was prominent throughout the vascular lesions (Figure 2E and 2F). A quantitative analysis of vascular narrowing in small pulmonary arteries (<40 μm in diameter, 15 consecutive vessels per mouse) was performed in the control and P/MCTP groups (n=4). The average VOSs on a scale from 0 to 2 are presented (Figure 1B). Compared with control mice (VOS=0±0), mice in the P/MCTP group developed significant pulmonary vascular remodeling with neointima formation (VOS=1.12±0.49; P<0.01 by t test).

In the main physiology study, 42 twelve-week-old, male C57Bl/6 mice (body weight, 24–28g) were studied in 5 groups: Group C (n=6) served as a reference control; group V (n=6) received DMF vehicle on day 7 (1 μL/g); group P (n=6) underwent left pneumonectomy on day 0; group MCTP (n=12) received injection of MCTP in DMF (20 μg/μL and 1 μL/g) on day 7; and group P/MCTP (n=12) received left pneumonectomy on day 0 followed by injection of MCTP in DMF (20 μg/μL and 1 μL/g) on day 7. The mice underwent hemodynamic measurements (Figure 1C) and euthanasia on day 35. Mice that underwent pneumonectomy alone, group P, showed RVSP similar to those of groups V and C (28±1, 25±2, and 24±2 mm Hg). Mice that received injection of MCTP alone, group MCTP, showed higher RVSP (41±9 mm Hg). Of the 4 groups, mice in group P/MCTP had the highest RVSP (54±5 mm Hg). ANOVA demonstrated differences between groups (P<0.01), with both the MCTP group and P/MCTP groups having higher RVSPs

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**Figure 3.** Vascular endothelial-cadherin (VE-Cad) endothelial genetic lineage marking correlates with endothelial antigen expression. A, C, D, and F, VE-Cad Cre recombinase transgenic mice intercrossed with dual fluorescent mTomato/ mGreen fluorescent protein (GFP) Cre reporter mice exhibit endothelial cells with expression of membrane-targeted GFP (green). Membrane-targeted dTomato (red) marks nonendothelial cells. Nuclei are labeled with DAPI (blue). B and C, Immunostaining for VE-Cad (cyan) and merged with A. E and F, Immunostaining for CD31 (cyan) and merged with D. Single 1-μm confocal optical sections are presented. Objective magnification ×40.
than the other 3 groups. The development of right ventricular hypertrophy correlates with the severity of pulmonary hypertension and is presented as Fulton’s index, right ventricle/(left ventricle+septum) (Figure 1D). Mice in group MCTP showed greater RV hypertrophy than mice in group V (0.36±0.09 versus 0.29±0.03). Mice in group P/MCTP demonstrated the highest right ventricle/(left ventricle+septum) ratio (0.55±0.07). ANOVA demonstrated differences between groups (P<0.01), with the P/MCTP group having a higher value than the other 4 groups. The 5 groups did not differ significantly in terms of body mass or systemic mean arterial pressure.

**Genetic Lineage Marking and Confocal Microscopy**

Dual-fluorescent Cre recombinase reporter mice, mTomato/mGFP, were intercrossed with transgenic endothelial Cre driver mice, VE-Cad Cre" or Tie-2 Cre," and progeny were genotyped to identify the mice that carried both Cre and GFP. These mice demonstrated strong red fluorescence in unrecombined cells and strong green fluorescence in vascular structures (Figures 3–7). The fidelity of VE-Cad Cre-directed endothelial genetic lineage marking in control mice (indicated by GFP labeling in green, Figure 3A, 3C, 3D, and 3F) was assessed by immunostaining of endothelial antigens, VE-Cad (Figure 3B, 3C, and 3E, cyan), and CD31 (Figure 3E and 3F, cyan). Endothelial immunostaining colocalized over green endothelial genetic lineage–marked cells and did not colocalize over red cells (Figure 3C and 3F). The strong expression of membrane-targeted GFP that outlined the recombined cells provided greater clarity than antigen staining for identifying cells that expressed an endothelial phenotype.
Induction of Experimental Pulmonary Hypertension With Neointima Reveals Contribution by GFP-Marked Cells of Endothelial Genetic Lineage

VE-Cad Cre × mTomato/mGFP mice were analyzed as controls or were subjected to the model of experimental pulmonary hypertension that induces neointima formation (P/MCTP). The MCTP used in the study of fluorescently labeled mice had been stored at −80°C for 7 years, and we found it to be less potent than previously observed in our main physiology study (Figures 1 and 2). The fluorescently labeled pneumonectomized mice injected with MCTP (20 μg/g) developed moderate pulmonary hypertension (RVSP ≈ 40 mm Hg) over a period of 7 to 10 weeks. The confocal images (Figures 3–7) were obtained from representative pulmonary hypertensive mice with endothelial genetic lineage marking.

A representative small pulmonary artery of a control mouse viewed in cross section (Figure 4A) demonstrated thin GFP-labeled cells lining the lumen, consistent with endothelial genetic lineage and phenotype, and adjacent, rectangular dTomato-labeled cells with intracellular fibrillar structures, suggestive of a smooth muscle phenotype. Immunostaining for SMA (Figure 4B, cyan) demonstrated colocalization with a subset of GFP endothelial lineage–marked cells (Figure 4C and Movie I in the online-only Data Supplement).

Mice with pulmonary hypertension exhibited neointima formation with contribution from GFP endothelial genetic lineage–marked cells (Figure 4D). Immunostaining for SMA (Figure 4E, cyan) demonstrated augmented luminal expression of SMA with some globular domains, predominantly colocalizing with GFP endothelial lineage–marked cells in the neointima (Figure 4F and Movie II in the online-only Data Supplement).

Experimental Pulmonary Hypertension Is Associated With Induction of Smooth Muscle Gene Expression, Including SM-MHC, in Neointima

A distinguishing feature of PAH and certain experimental models is the expression of SMA in the neointima. Control and pulmonary hypertensive mice were analyzed for expression of SM-MHC, a gene with expression generally restricted to differentiated smooth muscle cells. Control mice demonstrated thin GFP-labeled cells lining the lumen of a small pulmonary...
artery, consistent with an endothelial lineage and phenotype (Figure 5A). Immunostaining for SM-MHC (Figure 5B, cyan) demonstrated a thin circumferential outline that partially colocalized with GFP endothelial lineage–marked cells (Figure 5C and Movie III in the online-only Data Supplement). Induction of pulmonary hypertension (Figure 5D) strongly augmented expression of SM-MHC (Figure 5E, cyan), which colocalized over neointimal cells, a portion of which demonstrated GFP labeling, indicating endothelial lineage of origin (Figure 5F and Movie IV in the online-only Data Supplement).

Other Organs Do Not Demonstrate Colocalization of SMA With Endothelial Genetic Lineage–Marked Cells
In VE-Cad Cre × mTomato/mGFP control mice, careful examination of aorta (Figure 6A–6C) and kidney (Figure 6D–6F) demonstrated GFP endothelial genetic lineage–marked cells distinctly separated from SMA-immunostained cells.

Endothelial Lineage Marking Directed by Tie-2 Cre Also Labels the Neointima in Experimental Pulmonary Hypertension
Tie-2 Cre × mTomato/mGFP control mice demonstrated green GFP labeling circumferentially outlining a small pulmonary artery (Figure 7A). Immunostaining for SMA (Figure 7B, cyan) demonstrated labeling that predominantly colocalized with GFP endothelial lineage–marked cells (Figure 7C and Movie V in the online-only Data Supplement). Induction of experimental pulmonary hypertension (Figure 7D–7I) was associated with neointima formation that partially occluded the lumens with GFP endothelial lineage–marked cells (Figure 7D and 7G). Immunostaining for SMA (Figure 7E,
Human PAH Involves Neointimal Cells With Colocalization of Endothelial Antigens and SMA Expression

Human lung samples were obtained at lobectomy or autopsy and represented normal histology or PAH pathology. The human samples were processed similarly to the mouse lungs, and double immunostaining and confocal microscopy were used to evaluate colocalization of endothelial antigens and SMA (Figure 8). Compared with normal lung (Figure 8A, 8C, and 8E and Movies VIII and X in the online-only Data Supplement), pulmonary arteries in the PAH lung demonstrated substantial narrowing of the vascular lumen associated with increased number of cells expressing SMA (Figure 8B, 8D, and 8F and Movies IX and XI in the online-only Data Supplement). Individual neointimal cells in the PAH lung demonstrate colocalization of SMA and CD31. Sometimes it is in a collinear pattern and at other times globular intracellular SMA staining is enveloped by membrane-directed CD31 staining (Figure 8D and Movie IX in the online-only Data Supplement). Compared with normal lung (Figure 8E and Movie X in the online-only Data Supplement), the PAH lung demonstrated substantial narrowing of the vascular lumen associated with neointimal cells that coexpressed SMA and endothelial von Willebrand factor (Figure 8F and Movie XI in the online-only Data Supplement). Colocalization of SMA and von Willebrand factor expression is additionally evident in the pulmonary microvasculature (Figure 8F, left periphery). Neointimal cells in PAH lesions expressing SMA more...
frequently demonstrated coexpression of endothelial von Willebrand factor than CD31 (Figure 8F versus Figure 8D).

**Discussion**

We tested the hypothesis that neointima formation in experimental pulmonary hypertension originates from the endothelial genetic lineage. We developed a mouse model of pulmonary hypertension that involves surgical left pneumonectomy followed 1 week later by jugular vein injection of synthetic MCTP. Beginning at 35 days, mice exhibit pulmonary hypertension and neointima formation with vascular narrowing. This model represents an extension of our prior work in rats in which we characterized how the addition of pneumonectomy to a single injection of monocrotaline amplified the severity of disease through the intersection of compensatory lung hypertrophy after pneumonectomy with pulmonary endothelial injury from MCTP formed by hepatic metabolism of monocrotaline. Compared with rats, our mice experienced higher mortality with experimental pulmonary hypertension that was principally related to respiratory distress that developed in the first 2 days after injection of MCTP. The quality of synthetic MCTP is important for the success of this mouse model. Despite these technical challenges, this model enables characterization of cellular and molecular pathogenesis of pulmonary hypertension and neointima formation in genetically modified mice.

During endothelial differentiation, the expression of Tie-2 precedes the expression of VE-Cad. Both VE-Cad Cre and Tie-2 Cre driver mice have been used to mark the endothelial lineage in studies of mouse development. Permanent endothelial lineage marking directed by Tie-2 Cre enabled the characterization of endothelial-to-mesenchymal transition in the atrioventricular canal of the developing mouse heart. More recent fate-mapping studies of VE-Cad Cre and Tie-2 Cre recombination revealed lineage contributions of endothelial cells to hematopoietic stem cells. In separate experiments, we used VE-Cad Cre and Tie-2 Cre driver mice, intercrossed with mTomato/mGFP double-fluorescent Cre reporter mice, to achieve permanent labeling of the endothelial lineage with membrane-targeted GFP. The lungs were perfused free of blood, and we did not observe significant evidence of GFP-labeled hematopoietic cells in our microscopy sections.
After induction of experimental pulmonary hypertension, we observed that the neointimal cells were predominantly green, consistent with an endothelial lineage of origin. Our results using genetic recombination for endothelial fate mapping in pulmonary hypertension support earlier inferences of endothelial contribution to the neointima based on morphology, concurrent immunostaining of von Willebrand factor and SMA antigens and clonal analyses of microdissected plexiform lesions expressing factor VIII antigen.

The neointimal cells, which we interpret to be derived substantially from the endothelial genetic lineage, demonstrated expression of smooth muscle genes, SMA, and SM-MHC. Endothelial cells are known to activate expression of SMA during vascular remodeling or in response to treatment with transforming growth factor-β. In contrast, the expression of SM-MHC is believed to be essentially restricted to smooth muscle cells, with the exception of 1 report that bovine endothelial cells express SM-MHC RNA. To the best of our knowledge, our discovery that pulmonary artery cells of endothelial genetic lineage activate expression of SM-MHC in neointimal lesions is novel.

Wounding by scratch of cultured human dermal microvascular endothelial cells induces a transition from epithelioid to spindle-shaped morphology; migration into the wound was accompanied by induction of SMA expression in both migrating and adjacent endothelial cells. On healing of the wound, SMA-positive myofibroblast cells persisted in the culture, leading Karasek and colleagues to conclude that the myofibroblasts were derived from dermal microvascular endothelial cells. In this mouse model of experimental pulmonary hypertension, injury to pulmonary endothelial cells by MCTP eventually leads to activation of a program of smooth muscle gene expression. Our studies involved confocal microscopy using 4 distinct color channels, one of which was available for immunostaining. We therefore were unable to perform simultaneous immunostaining for endothelial and smooth muscle antigens within single cells in the neointima. We do not yet know at what rate an injured pulmonary artery endothelial cell activates expression of smooth muscle genes or the rate at which it may lose expression of endothelial genes. We do not know whether the neointima arises from a small population of apoptosis-resistant pulmonary artery endothelial cells that proliferate after injury to produce vascular narrowing or whether many pulmonary artery endothelial cells are permissive for activation of smooth muscle gene expression after injury.

Human PAH lungs demonstrated SMA-expressing neointimal cells with coexpression of endothelial antigens, CD31, or von Willebrand factor. Neointimal expression of von Willebrand factor was more extensive than that of CD31. These results in human PAH lesions support our conclusions derived from mice that at least a fraction of the pathological neointima originates from the endothelial genetic lineage.

Whether activation of smooth muscle gene expression in cells of endothelial lineage may be suppressed or reversed is a question of critical importance. How the cellular milieu, including modulation by perivascular inflammatory cells, affects neointima formation and induction of smooth muscle gene expression in endothelial cells represent important areas for investigation. Novel therapies for PAH might include agents that promote the differentiated endothelial phenotype and suppress activation of smooth muscle gene expression in small pulmonary arteries after injury.

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Disclosures
None.

References
smooth muscle gene expression in small pulmonary arteries after injury. Arterial hypertension might include agents that promote the differentiated endothelial phenotype and suppress activation with induced expression of smooth muscle -actin and smooth muscle myosin heavy chain. Novel therapies for pulmonary hypertension may include contributions from the endothelial genetic lineage with induced expression of smooth muscle -actin and smooth muscle myosin heavy chain.
Endothelial Fate Mapping in Mice With Pulmonary Hypertension
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SUPPLEMENTAL MATERIAL
Movie Legend:

Supplemental Movie 1: VECadContSMA. VE Cad Cre x mT/mG Control mice with immunostaining for SMA, z-stack of 1 µM confocal images. Green: GFP-endothelial lineage marked cells; red: dTomato labeled non-endothelial lineage marked cells; blue: DAPI nuclear staining; cyan: SMA immunostaining. There is limited colocalization of SMA immunostaining over GFP endothelial lineage-marked cells along the lumen of a small pulmonary artery.

Supplemental Movie 2: VECadPHSMA. VE Cad Cre x mT/mG Pulmonary Hypertensive mice with immunostaining for SMA, z-stack of 1 µM confocal images. Green: GFP-endothelial lineage marked cells; red: dTomato labeled non-endothelial lineage marked cells; blue: DAPI nuclear staining; cyan: SMA immunostaining. There is substantial colocalization of SMA immunostaining over GFP endothelial lineage-marked cells in the neointima within the lumen of a small pulmonary artery.

Supplemental Movie 3: VECadContMHC. VE Cad Cre x mT/mG Control mice with immunostaining for SM-MHC, z-stack of 1 µM confocal images. Green: GFP-endothelial lineage marked cells; red: dTomato labeled non-endothelial lineage marked cells; blue: DAPI nuclear staining; cyan: SM-MHC immunostaining. There is limited colocalization of SM-MHC immunostaining over GFP endothelial lineage marked cells along the lumen of a small pulmonary artery.

Supplemental Movie 4: VECadPHMHC. VE Cad Cre x mT/mG Control mice with immunostaining for SM-MHC, z-stack of 1 µM confocal images. Green: GFP-endothelial lineage marked cells; red: dTomato labeled non-endothelial lineage marked cells; blue: DAPI nuclear staining; cyan: SM-MHC immunostaining. There is substantial colocalization of SM-MHC immunostaining over GFP endothelial lineage-marked cells in the neointima within the lumen of a small pulmonary artery.

Supplemental Movie 5: Tie2ContSMA. Tie-2 Cre x mT/mG Control mice with immunostaining for SMA, z-stack of 1 µM confocal images. Green: GFP-endothelial lineage marked cells; red: dTomato labeled non-endothelial lineage marked cells; blue: DAPI nuclear staining; cyan: SMA immunostaining. There is limited colocalization of SMA immunostaining over GFP endothelial lineage-marked cells along the lumen of a small pulmonary artery.

Supplemental Movie 6: Tie2PHSMA. Tie-2 Cre x mT/mG Pulmonary Hypertensive mice with immunostaining for SMA, z-stack of 1 µM confocal images. Green: GFP-endothelial lineage marked cells; red: dTomato labeled non-endothelial lineage marked cells; blue: DAPI nuclear staining; cyan: SMA immunostaining. There is substantial colocalization of SMA immunostaining over GFP endothelial lineage-marked cells in the neointima within the lumen of a small pulmonary artery.

Supplemental Movie 7: Tie2PHMHC. Tie-2 Cre x mT/mG Control mice with immunostaining for SM-MHC, z-stack of 1 µM confocal images. Green: GFP-endothelial lineage marked cells;
red: dTomato labeled non-endothelial lineage marked cells; blue: DAPI nuclear staining; cyan: SM-MHC immunostaining. There is substantial colocalization of SM-MHC immunostaining over GFP endothelial lineage-marked cells in the neointima within the lumen of a small pulmonary artery.

Supplemental Movie 8: HumanNormalCD31SMA. Human Normal Lung double-immunostained with CD31 (green) and SMA (red); nuclei are stained with DAPI (blue). Z-stack of 1µM confocal images.

Supplemental Movie 9: HumanPAHCD31SMA. Human Pulmonary Arterial Hypertension Lung double-immunostained with CD31 (green) and SMA (red); nuclei are stained with DAPI (blue). Z-stack of 1µM confocal images. There is colocalization of CD31 and SMA in individual cells.

Supplemental Movie 10: HumanNormalVWFSMA. Human Normal Lung double-immunostained with anti-Von Willebrand Factor (green) and SMA (red); nuclei are stained with DAPI (blue). Z-stack of 1µM confocal images.

Supplemental Movie 11: HumanPAHVWFSMA. Human Pulmonary Arterial Hypertension Lung double-immunostained with anti-Von Willebrand Factor (green) and SMA (red); nuclei are stained with DAPI (blue). Z-stack of 1µM confocal images. There is substantial colocalization of VWF and SMA immunostaining in the neointima.