Endothelial Fate-Mapping in Mice with Pulmonary Hypertension

Running title: Qiao et al.; Endothelial Lineage of Pulmonary Neointima

Lina Qiao, MD, PhD1,4; Toshihiko Nishimura, MD, PhD1; Lingfang Shi, MD1; Dane Sessions1; Ama Thrasher1; James R. Trudell, PhD3; Gerald J. Berry, MD2; Ronald G. Pearl, MD, PhD3; Peter N. Kao, MD, PhD1

1Division of Pulmonary and Critical Care Medicine; 2Dept of Pathology; Dept of Anesthesiology, Stanford University Medical Center, Stanford, CA; 4Dept of Pediatric Cardiology, West China Second University Hospital, Sichuan University, Chengdu, China

Address for Correspondence:
Peter N. Kao, MD, PhD
Pulmonary and Critical Care Medicine
Stanford University Medical Center
300 Pasteur Drive
Stanford, CA 94305-5236
Tel: 650-725-0570
Fax: 650-725-5489
E-mail: peterkao@stanford.edu

Abstract

*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 pathologic plexiform lesions frequently coexpress smooth muscle alpha-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 one week later by jugular vein injection of monocrotaline pyrrole (20µg/µl)(1µl/g) (Group P/MCTP). Compared to Group Vehicle, by Day 35, Group P/MCTP developed higher right ventricular systolic pressure (54 ± 5 vs. 25 ± 2 mmHg, p < 0.01) and right ventricular hypertrophy (0.58 ± 0.16 vs. 0.26 ± 0.05, p < 0.01). Transgenic Vascular Endothelial-Cadherin (VE-Cad) Cre recombinase or Tie-2 Cre mice were intercrossed with mTomato/mGFP double fluorescent Cre reporter mice to achieve endothelial genetic lineage marking with membrane-targeted GFP. In control mice, few endothelial lineage-marked cells lining the lumen of small pulmonary arteries demonstrate expression of smooth muscle alpha-actin (SMA). Concurrent with the development of pulmonary hypertension, endothelial lineage-marked cells are prominent in the neointima and exhibit expression of SMA and smooth muscle myosin heavy chain (SM-MHC). Human pulmonary arterial hypertension neointimal lesions contain cells that coexpress endothelial CD31 or von Willebrand antigen, and SMA.

*Conclusions*—Neointimal cells in pulmonary hypertension include contributions from the endothelial genetic lineage with induced expression of SMA and SM-MHC.

**Key words:** endothelial cell differentiation, genetically altered mice, pulmonary circulation, pulmonary vascular changes, monocrotaline pyrrole, genetic lineage marking, endothelial to mesenchymal transition
Introduction

Idiopathic pulmonary arterial hypertension is characterized by pathological neointima formation within small pulmonary arteries, leading to increased pulmonary vascular resistance and arterial pressure, which in turn causes right ventricle 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.

The pathologic 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 muscularization 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 alpha-actin (SMA) within neointimal cells has long raised questions for pathologists about what cell lineage(s) contribute to the neointima. The most likely cell lineages that may contribute to the pathologic neointima include, vascular smooth muscle cells undergoing dedifferentiation, myofibroblasts derived from migrating adventitial fibroblasts, and endothelial cells transitioning into mesenchymal cells (EnMT) 6, 7. Expression of the endothelial von Willebrand antigen in plexiform lesions and microsatellite analyses were used to infer that individual plexiform lesions represent monoclonal expansions of endothelial cells 8.

Experimental models of pulmonary hypertension that have utilized combinations of chronic hypoxia, pneumonectomy and endothelial injury by monocrotaline or the vascular
endothelial growth factor (VEGF) receptor antagonist, SUGEN 5416, have been reviewed \(^9\). Whereas models based on hypoxia demonstrate predominantly hypertrophy of medial smooth muscles, models based on endothelial injury following exposure to SU5416 or monocrotaline, trigger more local inflammation and proliferative neointimal lesions that exhibit pathologies similar to human idiopathic pulmonary arterial hypertension. Endothelial injury models of experimental pulmonary hypertension are augmented by combination with a second stimulus such as hypoxia exposure following SU5416, and pneumonectomy prior to 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 to monocrotaline alone \(^{15,16}\). Surgical pneumonectomy increases blood flow and also 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 aorto-caval 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 aorto-caval fistula reduced the severity of pulmonary hypertension. We proposed that increased oxygen concentration in the mixed venous blood of animals with an aorto-caval 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, employed rat models of experimental pulmonary hypertension triggered by endothelial injury to characterize novel antiproliferative strategies to reverse established pulmonary vascular disease, such as simvastatin.\textsuperscript{17-20}

In contrast to rats, mice are relatively resistant to induction of pulmonary hypertension by monocrotaline.\textsuperscript{21} Mice administered monocrotaline at 24 mg/kg/day in the drinking water for 6 weeks (ten times higher dose than required in rats to produce 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 (ten times higher dose than used in rats) subcutaneously once per week for 4 to 10 weeks, produced mild increases in right ventricular systolic pressures, right ventricular hypertrophy and vascular narrowing.\textsuperscript{22-25} As in rats, monocrotaline induction of pulmonary hypertension in mice is characterized by perivascular accumulation of inflammatory cells.

Monocrotaline pyrrole is the active metabolite of monocrotaline and is 200-400 times more potent in producing cell injury and triggering the development of pulmonary hypertension\textsuperscript{26,27}. We synthesized monocrotaline pyrrole by chemical dehydration as described by Mattocks \textsuperscript{28}, for direct injection through the jugular vein into the pulmonary circulation to trigger endothelial injury and pulmonary hypertension. Based on our experience in rats\textsuperscript{16}, we elected to combine pneumonectomy with injection of monocrotaline pyrrole into the pulmonary circulation with the intention of producing a two-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 such that a given cell and all its progeny are indelibly labeled.\textsuperscript{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

Monocrotaline pyrrole (MCTP) synthesis

Dehydromonocrotaline, which is monocrotaline pyrrole (MCTP), was synthesized from monocrotaline (MCT, Sigma) by the method of A.R. Mattocks et al. Using mass spectrometry and nuclear magnetic resonance spectroscopy the conversion of MCT 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 aliquoted into vials (500 μL), the solvent was evaporated, and the lyophilized MCTP was stored at -80°C, shielded from light, until just prior to use. Five minutes before injection into mice, MCTP was dissolved into dimethyl formamide (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 five minutes. Endotracheal intubation was performed using a 20g teflon catheter over a fiberoptic light and stylus (Kent Scientific Corporation, Torrington, CT). After intubation, mice were mechanically

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ventilated using a MiniVent Type 845 (stroke volume 200 \( \mu l \), 110 strokes/min, Hugo Sachs Electronik/Harvard Apparatus, Holliston, MA) and anesthesia was maintained with 1.5-2.0% isoflurane. Mice were positioned supine, the forelimbs were gently retracted with tape, and the sternum and left chest were cleaned by aseptic technique. Left thoracotomy was performed in the fourth intercostal space using atraumatic technique and the ribs were retracted using two hooks fashioned from large-size paper clips. The left lung was gently lifted out of the chest, the hilum was clamped with an atraumatic mosquito clamp and ligated using 4-0 silk suture and the left pneumonectomy was completed using 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 (30-35°F, 2 l/m oxygen) for 2 h. Survival one day after pneumonectomy was 90-100%.

**Monocrotaline pyrrole (MCTP) administration**

One week after left pneumonectomy, mice were anesthetized in a plastic box using 2.0% isoflurane, then positioned supine and anesthesia was maintained by hood ventilation using 1% isoflurane. The right internal jugular vein was exposed using aseptic technique. Monocrotaline pyrrole was freshly resuspended in DMF (20\( \mu g/\mu l \)). A Hamilton syringe attached to PE-tubing with a 30g needle was backfilled with 100\( \mu l \) of DMF, then MCTP in DMF (~30 \( \mu l \)) was aspirated into the tubing for injection at a concentration of 20\( \mu g/g \) mouse weight (1 \( \mu l/g \)). A dissecting microscope was used to guide the needle into the right internal jugular vein and the MCTP was injected slowly over approximately one minute. After removing the needle, pressure was applied to the injection site indirectly though a layer of fatty tissue until hemostasis was complete, then the skin was closed using two 4-0 sutures. Survival at 3 days after MCTP
injection was approximately 75%.

**Hemodynamic studies**

For hemodynamic measurements, mice were anesthetized in a plastic box using 2% isoflurane, then placed in a supine position and anesthesia was maintained using 1% isoflurane delivered by hood. Right ventricular systolic pressure (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 phosphate buffered saline (PBS) followed by 10% neutral buffered formalin. The heart was weighed and right ventricular hypertrophy was determined by Fulton's Index: (right ventricle / (left ventricle and septum)). The formalin-fixed lungs were embedded in paraffin and five-micron sections were stained with hematoxylin and eosin (H&E) and with elastin-van Gieson (EVG) to mark internal elastic lamina. Vascular narrowing associated with neointima formation was evaluated as described previously \(^{18,30,31}\): absence of neointima formation was scored 0, neointima formation causing less than 50% luminal narrowing was scored 1, and luminal narrowing greater than 50% was scored 2. The scores of 15 consecutive inter-acinar 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 against \(\alpha\)-smooth muscle actin (Clone 1A4, Dako), and detection was performed using streptavidin-peroxidase and diaminobenzidine substrate.

**Study design**
In the pilot study, four male C57Bl/6 mice (body weight 24-28g) underwent left pneumonectomy on Day 0 and injection of MCTP in DMF (20μg/g) on Day 7. RVSP measurements were made by percutaneous needle puncture of the study mice on Day 0, 7, 21, 35 and 42. Mice were sacrificed on Day 42 for histology.

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

**Statistical analysis**

Data are presented as means ± standard deviations. The pilot study of RVSP was analyzed using a repeated measures analysis of variance (ANOVA) followed by Newman-Keuls tests. The vascular occlusion scores (VOS) of control and P/MCTP mice were compared by t-test. In the main study, Groups V, P, MCTP, and P/MCTP were analyzed by ANOVA and Newman-Keuls tests and also using nonparametric Kruskal-Wallis ANOVA (StatPlus software). Statistical significance was indicated by p < 0.05.

**Genetic Lineage Marking**

Double transgenic mice with endothelial genetic lineage marking were generated by intercrossing VE-Cadherin Cre \(^{29}\) or Tie-2 Cre \(^{32}\) recombinase driver mice with mTomato/mGFP floxed dual fluorescent Cre reporter mice \(^{33}\). Cre-mediated excision of the membrane-targeted dTomato gene is accompanied by expression of membrane-targeted eGFP in endothelial cells.
Immunofluorescence

After sacrifice by exsanguination, the right lung was perfused with PBS followed by 2% paraformaldehyde. Lung lobes were immersed in 2% paraformaldehyde for 2h, followed by dehydration in 30% sucrose overnight and embedding in optimal cutting temperature compound (OCT). Cryosections were cut at 40μM thickness. Immunostaining was performed for CD31 or VE-Cadherin at 1:50 dilution, and for α-SMA and SM-MHC antigens at 1:200 dilution (rat anti-mouse CD31 mAb MEC13.3 conjugated to AlexaFluor 647, BioLegend; rat anti-mouse CD144 VE Cadherin 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 antirat 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 OCT 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; 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 using Alexa Fluor 647 goat anti-mouse IgG1 or IgG2a, demonstrated no specific labeling on the lung sections (data not shown).

Confocal microscopy was performed using a Leica DMI 6000 equipped with BD Carv II confocal imager, Chroma Photofluor metal halide illumination and Leica Imaging Software. Sequential 1μM optical sections were acquired at 350 (DAPI), 488 (GFP), 568 (dTomato) and 647 nm (immunostaining) wavelengths, and contrast was enhanced using deconvolution software.
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, four 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 pulmonary arterial hypertension by Day 35 (Figure 1A). The pilot study of RVSP was analyzed using a repeated measures analysis of variance (ANOVA) followed by Newman-Keuls tests. The RVSP at 35 and 42 days was significantly higher than at 0, 7 and 21 days (p < 0.01). Mice were sacrificed 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 to Control mice (Figure 2A,B), pneumonectomized mice injected with MCTP demonstrated narrowing of peribronchiolar and intraacinar pulmonary arteries associated with both medial hypertrophy and neointima formation (Figure 2C,D). Smooth muscle α-actin (SMA) expression determined by immunohistochemistry was prominent throughout the vascular lesions (Figure 2E,F). A quantitative analysis of vascular narrowing in small pulmonary arteries (< 40μM in diameter, 15 consecutive vessels per mouse) was performed in the control and
P/MCTP groups (n = 4). The average vascular occlusion scores (VOS) on a scale from 0 to 2 are presented (Figure 1B). Compared to 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 five groups: Group C (n = 6) served as a reference control, Group V (n = 6) received DMF vehicle on Day 7 (1μl/g), Group P (n = 6) underwent left pneumonectomy on Day 0, Group MCTP (n = 12) received injection of MCTP in DMF (20μg/μl)(1μl/g) on Day 7 and Group P/MCTP (n = 12) received left pneumonectomy on Day 0 followed by injection of MCTP in DMF (20μg/μl)(1μl/g) on Day 7. The mice underwent hemodynamic measurements and sacrifice on Day 35. Mice that underwent pneumonectomy alone, Group P, showed RVSP similar to Group V and Group C (28 ± 1, 25 ± 2 and 24 ± 2mmHg). Mice that received injection of MCTP alone, Group MCTP, showed higher RVSP (41 ± 9 mmHg). Of the four groups, mice in Group P/MCTP had the highest RVSP (54 ± 5 mmHg). ANOVA demonstrated differences between groups (p < 0.01) with both the monocrotaline pyrrole and the combination of pneumonectomy with monocrotaline pyrrole groups having higher RVSP than the other three groups. The development of right ventricular hypertrophy correlates with the severity of pulmonary hypertension, and is presented as Fulton's index, RV/(LV+S). Mice in Group MCTP showed greater RV hypertrophy than mice in Group V (0.36 ± 0.09 vs. 0.29 ± 0.03). Mice in Group P/MCTP demonstrated the highest RV/(LV+S) 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 five 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, mT/mG, 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 un-recombined 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,C,D,F) was assessed by immunostaining of endothelial antigens, VE–Cadherin (Figure 3B,C, cyan) and CD31 (Figure 3E,F, cyan). Endothelial immunostaining co-localized over green endothelial genetic-lineage marked cells, and did not co-localize over red cells (Figure 3C,F). 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 x mT/mG 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 (Figure 1,2). The fluorescently-labeled pneumonectomized mice injected with MCTP (20μg/g) developed moderate pulmonary hypertension (RVSP ~40 mmHg) over a period of 7-10 weeks. The confocal images (Figure 3-7) are 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; Supplemental Movie 1).

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; Supplemental Movie 2).

Experimental pulmonary hypertension is associated with induction of smooth muscle gene expression, including SM-MHC, in neointima. A distinguishing feature of pulmonary arterial hypertension, and certain experimental models, is the expression of SMA in the neointima. Control and pulmonary hypertensive mice (PH) were analyzed for expression of smooth muscle myosin heavy chain (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; Supplemental movie 3). 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; Supplemental movie 4).
Other organs do not demonstrate colocalization of SMA with endothelial genetic lineage-marked cells

In VE Cad Cre x mT/mG Control mice, careful examination of aorta (Figure 6A-C) and kidney (Figure 6D-F) 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 x mT/mG 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; Supplemental movie 5). Induction of experimental pulmonary hypertension (Figure 7D-I) was associated with neointima formation that partially occluded the lumens with GFP endothelial lineage-marked cells (Figure 7D, G). Immunostaining for SMA (Figure 7E, cyan) demonstrated expression in the neointima with colocalization with GFP endothelial lineage-marked cells (Figure 7F; Supplemental movie 6). Immunostaining for SM-MHC (Figure 7H, cyan) demonstrated expression in the neointima with colocalization with GFP endothelial lineage-marked cells (Figure 7I; Supplemental movie 7).

Human pulmonary arterial hypertension involves neointimal cells with colocalization of endothelial antigens and α-SMA expression

Human lung samples were obtained at lobectomy or autopsy and represented normal histology or pulmonary arterial hypertension (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 to normal lung (Figure
8A,C,E, Supplemental movies 8,9), pulmonary arteries in the PAH lung demonstrated substantial narrowing of the vascular lumen associated with increased number of cells expressing SMA (Figure 8B,D,F, Supplemental movies 10,11). Individual neointimal cells in the PAH lung demonstrate colocalization of SMA and CD31: sometimes in a colinear pattern and at other times globular intracellular SMA staining is enveloped by membrane-directed CD31 staining (Figure 8D, Supplemental movie 10). Compared to normal lung (Figure 8E, Supplemental movie 9), the PAH lung demonstrated substantial narrowing of the vascular lumen associated with neointimal cells that coexpressed SMA and endothelial Von Willebrand Factor (Figure 8F, Supplemental movie 11). Colocalization of SMA and VWF 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 VWF than CD31 (Figure 8F vs. 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 one 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 MCT 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 MCT. Compared to rats, we experienced higher mortality in mice with
experimental pulmonary hypertension that was principally related to respiratory distress that
developed in the first two days after injection of MCTP. The quality of synthetic MCTP is
important for the success of this mouse model. In spite of 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-
Cadherin \(^\text{29}\). 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 \(^\text{35}\). More recent fate-mapping studies of VE-
Cad Cre \(^\text{36}\) and Tie-2 Cre \(^\text{37}\) 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. Following 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 \(^\text{3}\), concurrent immunostaining of vWF and SMA antigens \(^\text{6}\) and
clonal analyses of microdissected plexiform lesions expressing Factor VIII antigen \(^\text{8}\).

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 TGF-β. In contrast, the expression of SM-MHC is believed to be essentially restricted to smooth muscle cells \(^{38,39}\), with the exception of one report that bovine endothelial cells express SM-MHC RNA \(^{40}\). To 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 α-SMA expression in both migrating and adjacent endothelial cells. Upon healing of the wound, SMA-positive myofibroblast cells persisted in the culture, leading Karasak and colleagues to conclude that the myofibroblasts were derived from dermal microvascular endothelial cells \(^{41-43}\). 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 four 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, nor 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 VWF. Neointimal expression of VWF was more extensive than of CD31. These results in human PAH lesions support our conclusions derived from mice, that at least a fraction of the pathologic 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 pulmonary arterial hypertension 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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Conflict of Interest Disclosures: None.

References:


Figure Legends:

**Figure 1.** Experimental pulmonary hypertension in mice. A) Pilot study: Time course of development of pulmonary hypertension in pneumonectomized mice injected with monocrotaline pyrrole (P/MCTP, n = 4, serial measurements). **p < 0.01 by ANOVA. B) Vascular narrowing analysis of neointima formation in pilot study, Control versus P/MCTP mice sacrificed at Day 42 (n = 4). Open bar: VOS = 0, Hatched bar: VOS = 1, Solid bar: VOS = 2. *p < 0.01 by t-test. Main study: Group Control (n = 6), Group Vehicle (DMF, n = 6), Group P (Pneumonectomy, n = 6), Group MCTP (n = 12), Group P/MCTP (n = 12). C) Right Ventricular Systolic Pressure (RVSP) at Day 35, D) Right Ventricular Hypertrophy (RV/LV+S, Fulton's index) at Day 35. **p < 0.01 by ANOVA.

**Figure 2.** Histopathology of experimental pulmonary hypertension in pneumonectomized mice injected with monocrotaline pyrrole. A, B) Normal muscular pulmonary artery (PA) adjacent to bronchiole (Br) A) hematoxylin and eosin stain (H&E), B) elastin-van Gieson stain (EVG). C, D) Peribronchiolar pulmonary arteries in P/MCTP mice demonstrate medial hypertrophy and neointima formation (C, H&E; D, EVG; Internal elastic lamina (IEL) is marked). E, F) Smooth muscle α-actin immunostaining of peribronchiolar pulmonary arteries (E) and intraacinar pulmonary artery (F) in P/MCTP mice. Objective magnification x 40.

**Figure 3.** VE-Cad endothelial genetic lineage marking correlates with endothelial antigen expression. (A,C,D,F) Vascular Endothelial Cadherin (VE-Cad) Cre recombinase transgenic mice intercrossed with dual fluorescent mTomato/mGFP Cre reporter mice exhibit endothelial
cells with expression of membrane targeted GFP (green). Membrane-targeted dTomato (red) marks non-endothelial cells. Nuclei are labeled with DAPI (blue). (B,C) Immunostaining for VE-Cadherin (cyan) and merged with A. (E,F) Immunostaining for CD31 (cyan) and merged with D. Single 1μM confocal optical sections are presented. Objective magnification x 40.

**Figure 4.** VE-Cad endothelial genetic lineage marking in controls and mice with experimental pulmonary hypertension, and colocalization with α-SMA expression. (A,C,D,F) VE-Cad Cre x mT/mG mice marks endothelial cells (green) and non-endothelial cells (red); nuclei are stained with DAPI (blue). (B,C,E,F) Immunostaining for α-SMA expression. (A-C) Control mouse (D-F) Pneumonectomized mouse injected with monocrotaline pyrrole to induce development of experimental pulmonary hypertension with neointima, PH. C) Merge of A and B demonstrates limited colocalization of endothelial genetic lineage marking with α-SMA expression in Control mouse. F) Merge of D and E demonstrates increase in colocalization of endothelial lineage marking with α-SMA expression in P/MCTP mouse. Single 1μM confocal optical sections are presented. Objective magnification x 40.

**Figure 5.** VE-Cad endothelial genetic lineage marking in Controls and mice with experimental pulmonary hypertension, and colocalization with SM-MHC expression. (A,C,D,F) VE-Cad Cre x mT/mG mice marks endothelial cells (green) and non-endothelial cells (red); nuclei are stained with DAPI (blue). (B,C,E,F) Immunostaining for SM-MHC expression. (A-C) Control mice (D-F) Pneumonectomized mice injected with monocrotaline pyrrole to induce development of experimental pulmonary hypertension with neointima, PH. C) Merge of A and B demonstrates limited colocalization of endothelial genetic lineage marking with SM-MHC expression in Controls and mice with experimental pulmonary hypertension.
Control mouse. F) Merge of D and E demonstrates increase in colocalization of endothelial lineage marking with SM-MHC expression in P/MCTP mouse. Single 1μM confocal optical sections are presented. Objective magnification x 40.

**Figure 6.** VE-Cad endothelial genetic lineage marking in Control mice and absence of colocalization with α-SMA expression in aorta and kidney. (A,C,D,F) VE-Cad Cre x mT/mG mice marks endothelial cells (green) and non-endothelial cells (red); nuclei are stained with DAPI (blue). (B,C,E,F) Immunostaining for α-SMA expression. (A-C) Aorta in transverse section. (D-F) kidney showing glomerulus and tubule. C) Merge of A and B demonstrates absence of colocalization of endothelial lineage marking and α-SMA expression. F) Merge of D and E demonstrates absence of colocalization of endothelial lineage marking and α-SMA expression. Single 1μM confocal optical sections are presented. Objective magnification x 40.

**Figure 7.** Tie-2 genetic lineage marking in controls and mice with experimental pulmonary hypertension, and colocalization with α-SMA expression. (A,C,D,F,G,I) Tie-2 Cre x mT/mG mice marks endothelial cells (green) and non-endothelial cells (red); nuclei are stained with DAPI (blue). (B,C,E,F) Immunostaining for α-SMA expression. (H,I) Immunostaining for SM-MHC expression. (A-C) Control mouse. (D-I) P/MCTP mouse with neointima, PH. C) Merge of A and B demonstrates limited colocalization of endothelial genetic lineage marking with α-SMA expression in Control mouse. F) Merge of D and E demonstrates increase SMA expression with colocalization with endothelial lineage-marked cells in P/MCTP mouse. I) Merge of G and H demonstrates colocalization of endothelial lineage marking with SM-MHC expression in P/MCTP mouse. Single 1μM confocal optical sections are presented. Objective magnification x 40.
Figure 8. Human pulmonary arterial hypertension involves neointimal cells with colocalization of endothelial antigens and α-SMA expression. (A,C,E) Human normal lung. (B,D,F) Human PAH lung. (A-D). Lung sections are double-immunostained for endothelial CD31 (green) and α-SMA (red). (E,F) Lung sections are double-immunostained for von Willebrand's factor (VWF, green) and α-SMA (red). Nuclei are labeled with DAPI (blue). Single 1μM confocal optical sections are presented. Objective magnification: x 20 (A,B); x 40 (C-F).
Figure 3
Figure 5
Figure 6
Figure 8
Endothelial Fate-Mapping in Mice with Pulmonary Hypertension
Lina Qiao, Toshihiko Nishimura, Lingfang Shi, Dane Sessions, Ama Thrasher, James R. Trudell, Gerald J. Berry, Ronald G. Pearl and Peter N. Kao

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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 Pulmonary Hypertensive 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.