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14. ABSTRACT Pulmonary arterial hypertension is characterized by inappropriate proliferation of neointimal cells that occlude the lumen of the microcirculation leading to right ventricular congestive failure and death. The neointimal cells express disorganized fibrils of smooth muscle actin. The origin of the neointimal cells remains unresolved: the neointima may arise from de-differentiation of vascular smooth muscle cells or from microvascular endothelial progenitor cells undergoing endothelial-to-mesenchymal transition. Aim 1 is to determine how endothelial to mesenchymal transition may contribute to neointimal vascular occlusion in pulmonary hypertension using genetic lineage marking in mice. Aim 2 is to characterize how Notch signaling regulates endothelial to mesenchymal transition. During the current funding period, successful Cre-lox genetic labeling of the endothelial lineage was achieved, and specificity of endothelial genetic lineage marking was confirmed by co-immunostaining of endothelial antigens, CD31 and VE-Cadherin. Successful induction of experimental pulmonary hypertension was achieved and demonstrated extensive contribution of endothelial genetic lineage-marked cells to neointimal vascular occlusion.					
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Lineage Analysis in Pulmonary Arterial Hypertension

Final Report 2013

INTRODUCTION:

Human Idiopathic Pulmonary Arterial Hypertension (IPAH) is characterized by neointimal vascular occlusion of the pulmonary microcirculation. Relentless elevations in pulmonary arterial pressures lead to death due to right ventricular failure¹. The pathology of PAH is characterized by abnormal expansions of neointimal cells expressing smooth muscle actin².

There are few data on strategies that suppress neointimal formation being used to treat pulmonary hypertension³. The current medical management of PPH is directed at vasodilatation rather than the prevention of endothelial proliferation and neointimal formation. Prostacyclin may have beneficial effects on vascular remodeling, because some patients who do not demonstrate a vasodilator response to prostacyclin, appear to benefit from its use^{4 5 6}. A number of new agents, including simvastatin, hold the potential to attenuate disease progression^{7 8}.

The pathogenesis of PAH involves 1) pulmonary vasoconstriction, 2) inappropriate proliferation of vascular cells in the intima and media, 3) inflammation and 4) thrombosis *in situ*^{9 10}. All of these mechanisms may contribute to the development of PAH. The hypothesis of pulmonary vasoconstriction leading to medial hypertrophy and pulmonary hypertension was accepted for many years because of its intuitive similarity to the mechanism of development of systemic hypertension. Vasoconstriction associated with increased calcium influx contributes to smooth muscle hypertrophy in response to chronic hypoxia^{11 10}.

Inappropriate hypertrophy and proliferation of cells within small pulmonary arterioles of patients with PAH is evident by analysis of the pathologic plexiform and concentric obliterative lesions that are characteristic of this disease. The lumens of small pulmonary arteries are diffusely narrowed by neointimal proliferation that consists of dedifferentiated vascular smooth muscle cells, myofibroblasts and endothelial cells^{12, 13}. At the level of small pulmonary arteries, the occlusion by neointimal formation significantly exceeds muscularization of the medial component of the vessel wall.

Familial PPH occurs in about 10% of patients, and manifests an identical pathophysiology to sporadic PPH^{14 2}. Recently, Deng et al.¹⁵ and Lane¹⁶ identified Bone Morphogenetic Protein Receptor Type II (BMPR2), located on the chromosome 2q33 as the genetic basis of familial PPH. Nearly 80% of patients with familial PAH have now been demonstrated to carry mutations in the BMPR2 gene. BMP receptors transduce antiproliferative signals to the nucleus through Smad proteins^{17 18}. Thus, familial PAH appears to arise from the loss of an antiproliferative signal or differentiating signal transmitted through the BMP signaling pathway. The important implications from this genetic discovery are that idiopathic PAH and anorexigen-induced PAH, may also arise from loss of antiproliferative signals. BMPR2 expression in the normal human lung is greatest in pulmonary endothelial cells, including microvascular ECs. Notably, lung specimens from patients with PPH and secondary PH showed marked attenuation of expression of BMPR2 in the pulmonary endothelium, with the greatest decreases observed in those patients who carried mutations in BMPR2 predicted to interfere with protein expression¹⁹.

The identity of the neointimal cells that occlude the lumens of small pulmonary arteries causing pulmonary hypertension remains a question of great significance. Based on the expression of smooth muscle actin (SMA), the neointimal cells have been traditionally considered to derive from the medial

wall vascular smooth muscle cells, through a process of dedifferentiation. An alternative explanation was that the neointimal cells represented myofibroblasts that arose from differentiation of migrating adventitial fibroblasts ²⁰. Neointimal cells that derived from the bone marrow were shown to incorporate into the wall of wire-injured systemic arteries, but no bone marrow-derived neointimal cells were observed in the pulmonary vascular lesions in monocrotaline-injected rats ²¹.

Endothelial to mesenchymal transition refers to the process in which a cell releases cell-to-cell contacts, loses polarity and undergoes remodeling of the cytoskeleton. Concurrent with the loss of endothelial antigens such as vWF, VE-Cadherin and PECAM, the cell will increase its expression of SMA and PDGF receptor ²⁰. Arciniegas has been a pioneer in describing EnMT during normal development of the aorta and pulmonary artery in chick. The experiments are technically challenging because they depend on the ability to co-immunostain individual cells that are increasing SMA expression while decreasing expression of vWF or CD31. This lineage transition is a dynamic process and the experimental challenge is to capture the cells undergoing EnMT at the brief moment when there is simultaneous coexpression of different lineage markers.

Voelkel and Tudor described that plexiform lesions in human IPAH showed expression of the endothelial antigen vWF, and this discovery led them to propose that PAH represents a disease of monoclonal expansion of endothelial cells ¹⁴. Other investigators and pathologists did not uniformly embrace this paradigm, because the vast majority of vascular lesions with neointima express SMA but no endothelial antigens. One way to reconcile Voelkel and Tudor's theory of PAH pathogenesis with the absence of endothelial antigens in the majority of neointimal cells, is to consider that neointimal cells may originally have been derived from endothelial progenitor cells that underwent endothelial to mesenchymal transition ²⁰. In this proposal we aim to examine this question by using genetic lineage marking to permanently identify endothelial cells in the pulmonary microcirculation. Mice with endothelial cells permanently marked by expression of green fluorescent protein (GFP) reporter transgene will be subjected to our mouse model of pulmonary hypertension that produces neointimal lesions. If we detect GFP -labeled cells in the neointima, then we will have demonstrated unequivocally, that neointimal vascular occlusion in pulmonary hypertension can involve contributions from resident lung microvascular endothelial cells.

Endothelial to mesenchymal transitions have been shown to be strongly regulated by Notch signaling ²². Transduction of microvascular endothelial cells with activated Notch-1 intracellular domain (Notch-1 ICD) caused a dramatic change in morphology, new expression of SMA, fibronectin, PDGFR and substantial downregulation of expression of VE-cadherin, PECAM-1 and Tie-2. Here we propose to examine whether Notch-1 activation is detected in neointimal cells during the development of pulmonary hypertension. If we demonstrate that Notch-1 activation contributes to neointimal formation, we will test whether inhibitors of Notch activation, gamma secretase inhibitor, may suppress neointimal formation and pulmonary hypertension.

BODY:

Hypothesis: Pulmonary vascular injury triggers proliferation of lung microvascular endothelial progenitor cells capable of restoring the microvascular endothelium or undergoing endothelial to mesenchymal transition into smooth muscle actin (SMA)-expressing neointimal cells that occlude the microcirculation, and regulation of this fate involves Notch-1 signaling.

Aim 1: Determine how endothelial to mesenchymal transition may contribute to neointimal vascular occlusion in pulmonary hypertension using genetic lineage marking in mice. Mice with endothelial-specific expression of Cre recombinase (Tie-2 Cre) will be intercrossed with reporter mice (Rosa26R Floxed Stop lacZ) to permanently label cells of endothelial lineage. Subsequently, mice will undergo pneumectomy followed one week later by intravenous injection of monocrotaline pyrrole. The fate of lacZ-expressing cells will be correlated with immunofluorescent staining of endothelial marker CD31, mesenchymal marker SMA and proliferation marker BrdU. We anticipate demonstrating that lac-Z positive cells of endothelial lineage express SMA during development of pulmonary hypertension.

Aim 2: Characterize how Notch signaling regulates endothelial to mesenchymal transition. Cells actively expressing Notch-1 intracellular domain (Notch1-ICD) will be detected by alpha-VLLS immunostaining. Expression of active Notch1IC will be correlated with cellular expression of endothelial and mesenchymal markers. Gamma secretase inhibitors of Notch activation will be evaluated for efficacy in suppressing EnMT, neointimal vascular occlusion and pulmonary hypertension in mice. We anticipate that inhibition of Notch signaling may represent a novel therapeutic approach to prevent and reverse pulmonary hypertension.

RESULTS:

Experimental pulmonary hypertension in mice: In the pilot study, four wild-type C57Bl/6 male mice underwent left pneumectomy 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**). Mice were sacrificed at Day 42 for organ harvest and histopathology (**Figure 2**). Compared to Control mice (**Figure 2A,B**), pneumonectomized mice injected with MCTP demonstrated substantial 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**).

In the main physiology study, 42 twelve-week old, male C57Bl/6 mice (body weight 24-28g) were studied in five groups: Group C served as a reference control, Group V received DMF vehicle on Day 7 (1µl/g), Group P underwent left pneumectomy on Day 0, Group MCTP received injection of MCTP in DMF (20µg/µl)(1µl/g) on Day 7 and Group P/MCTP received left pneumectomy 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 (**Figure 1C**). Mice that underwent pneumectomy alone, Group P, showed RVSP similar to Group V and Group C (28 ± 1 , 25 ± 2 and 24 ± 2 mmHg). 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 mm Hg). The development of right ventricular hypertrophy correlates with the severity of pulmonary hypertension, and is presented as Fulton's index, $RV/(LV+S)$ (**Figure 1D**). 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).

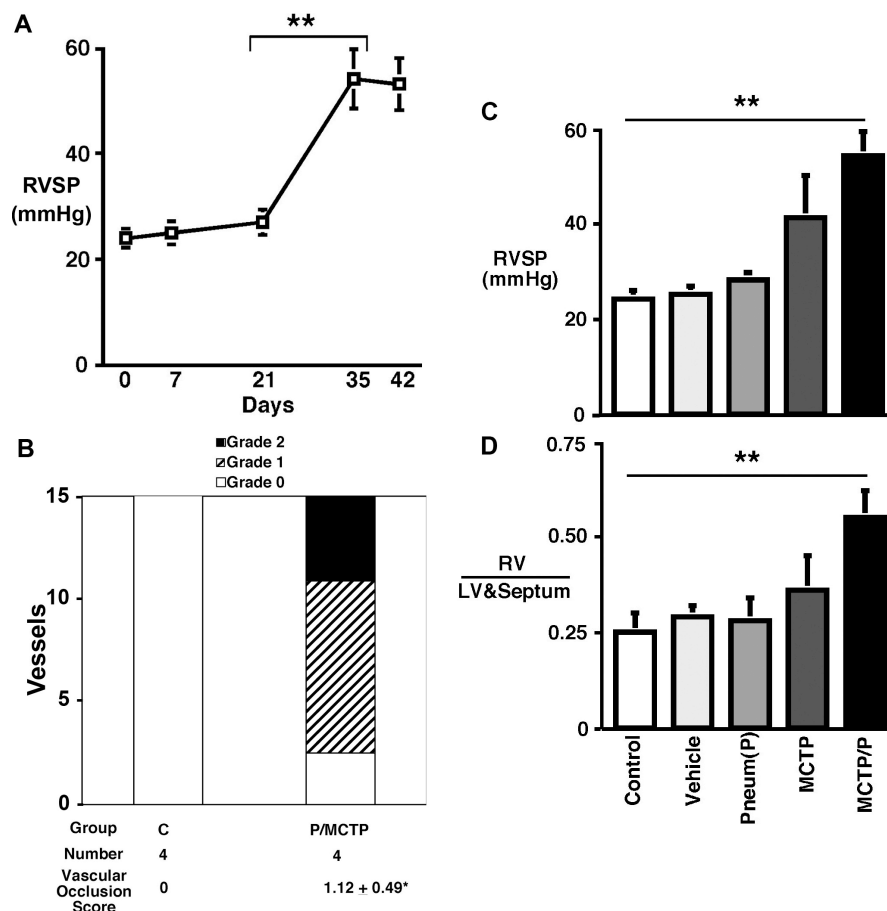


Figure 1. Experimental pulmonary hypertension in mice. A) Pilot study: Time course of development of pulmonary hypertension in pneumonectomized mice injected with monocrotaline pyrrole (P/MCTP, $n = 4$, serial measurements). B) Vascular narrowing analysis of neointima formation in pilot study, Control versus P/MCTP mice sacrificed at Day 42 ($n = 4$). 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, * $p < 0.05$ by t-test.

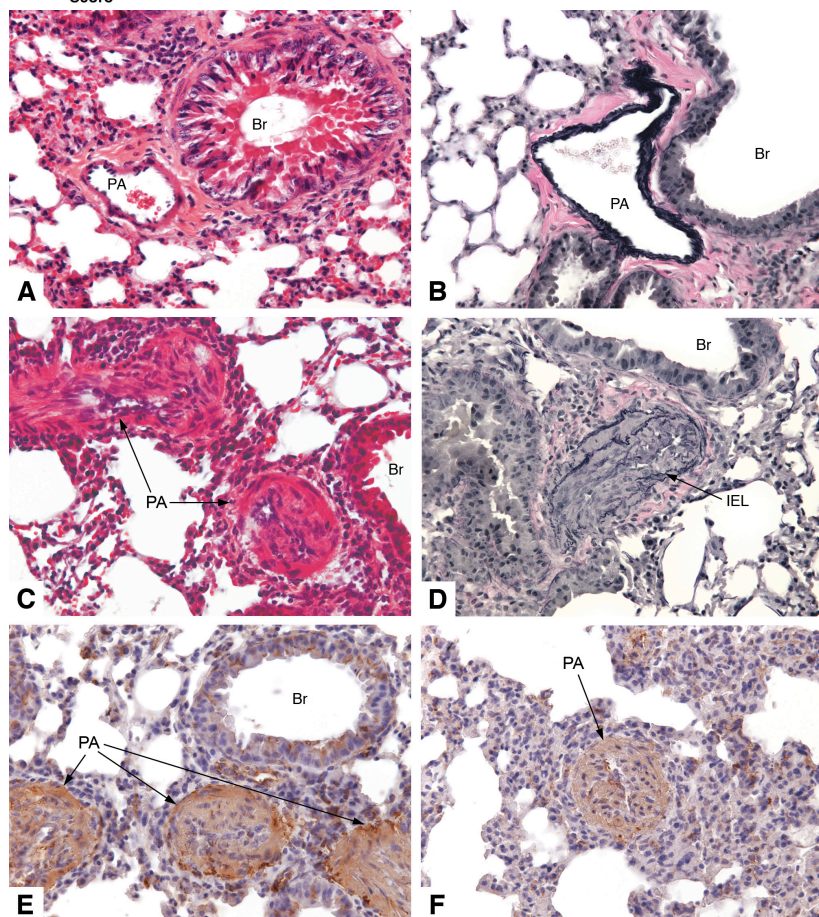


Figure 2. Histopathology of experimental pulmonary hypertension in pneumonectomized mice injected with monocrotaline pyrrole. A, B) Normal muscular pulmonary artery (PA) adjacent to bronchiole (Br) A) hematoxylin and eosin stain (H&E), B) elastin-van Gieson stain (EVG). C, D) Peribronchiolar pulmonary 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 $\times 40$.

Aim 1: Genetic lineage marking and confocal microscopy: Dual fluorescent Cre recombinase reporter mice, ROSA26R *mT/mG*, were intercrossed with transgenic endothelial Cre driver mice, VE-Cadherin Cre²³ or Tie-2 Cre²⁴, 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 use of *mT/mG* reporter mice represented an enhancement over the original proposal to study ROSA26R floxed STOP lacZ mice, and was approved by the IACUC. 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.

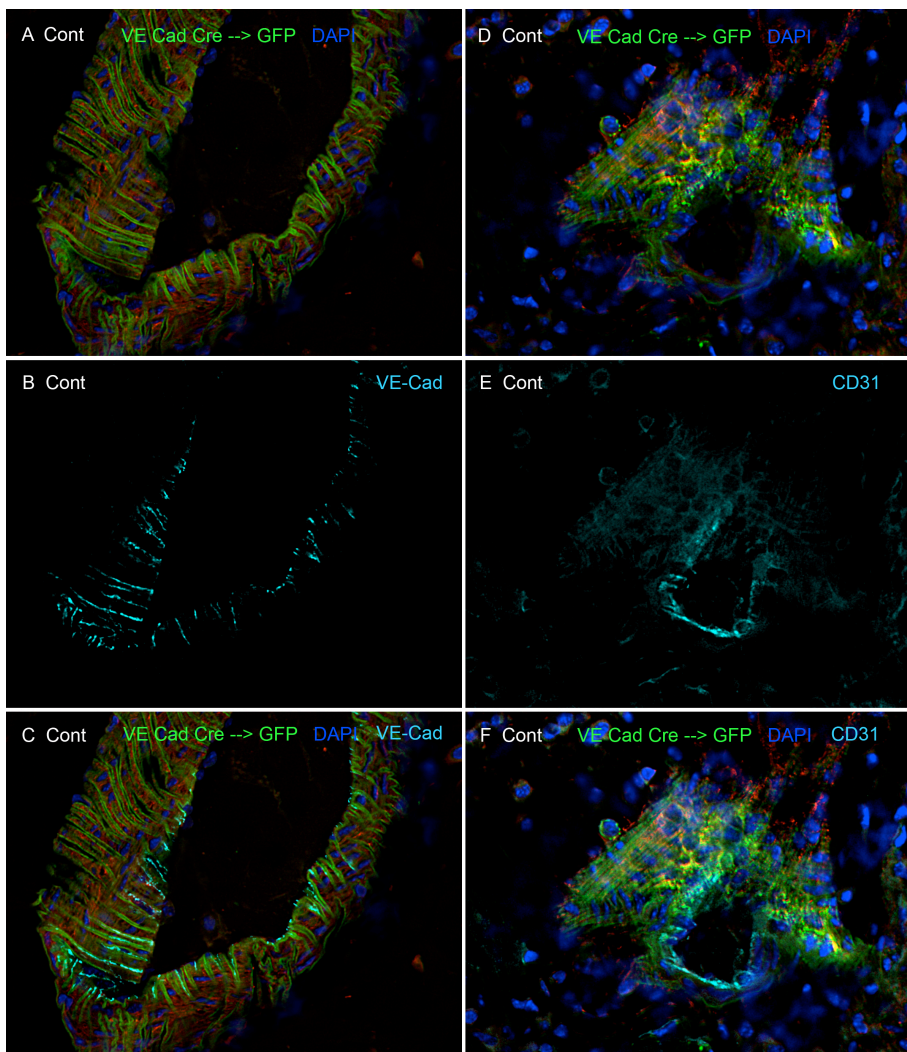


Figure 3. VE-Cad endothelial genetic lineage marking correlates with endothelial antigen expression. (A,C,D,F) Vascular Endothelial Cadherin (VE-Cad) Cre recombinase transgenic mice intercrossed with dual fluorescent mTomato/mGFP Cre reporter mice exhibit endothelial cells with expression of membrane 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 1 μ M confocal

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**).

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**).

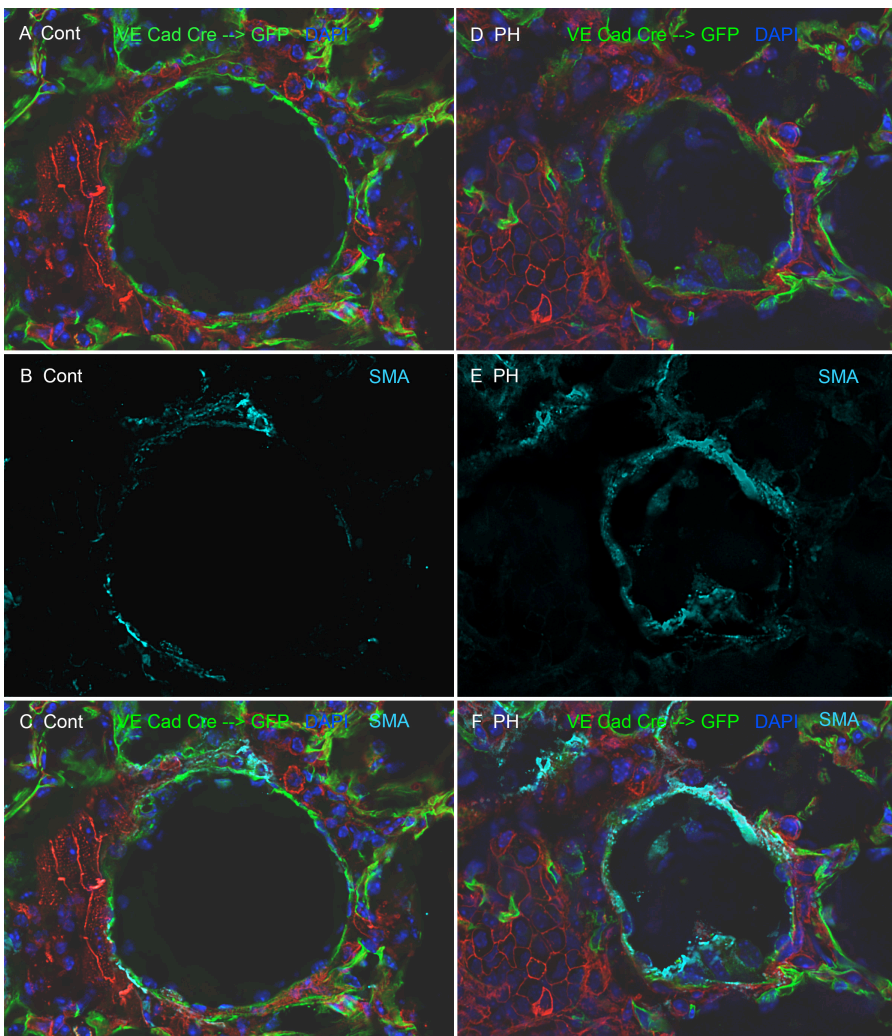


Figure 4. VE-Cad endothelial genetic lineage marking in controls and mice with experimental pulmonary hypertension, and colocalization with α-SMA expression.

(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 1 µM confocal optical sections are presented. Objective magnification x 40.

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**). 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**).

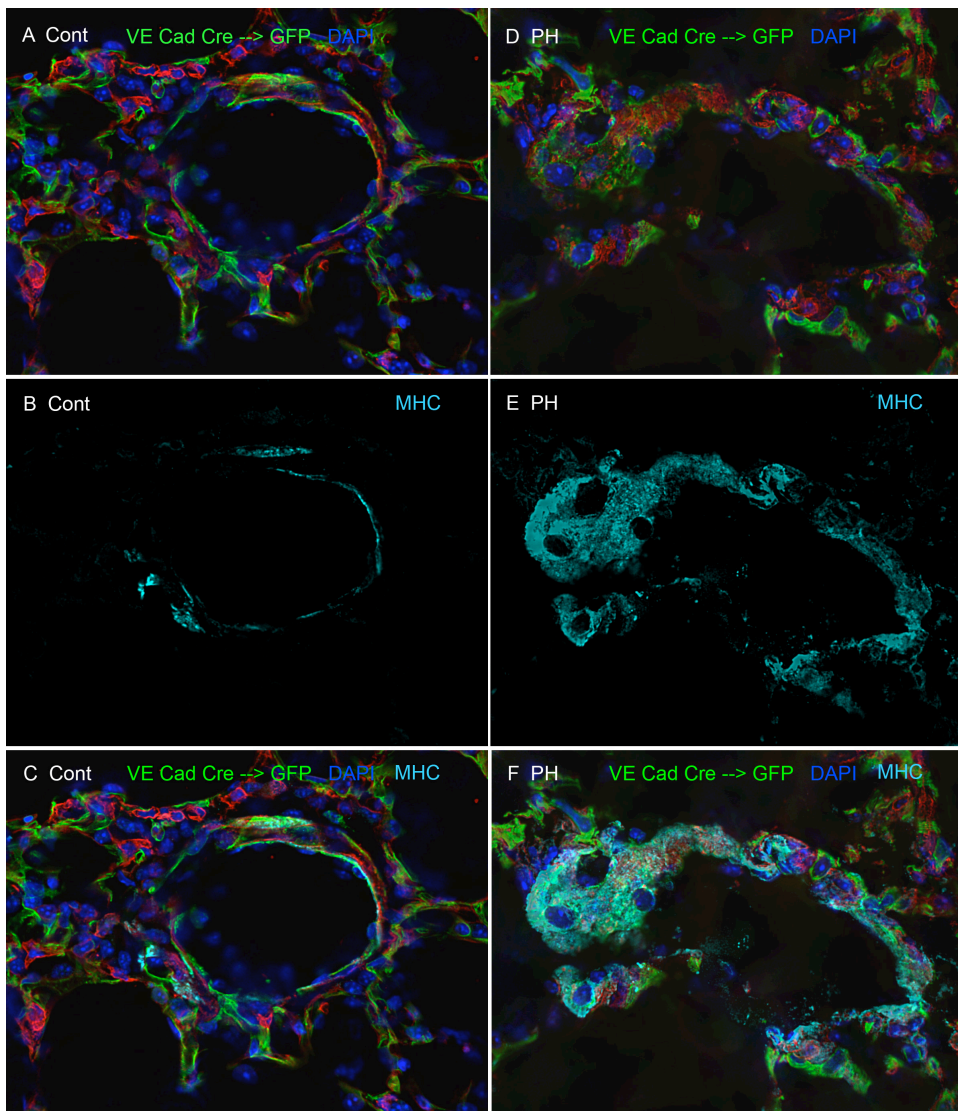


Figure 5. VE-Cad endothelial genetic lineage marking in Controls and mice with experimental pulmonary hypertension, and colocalization with SM-MHC expression. (A,C,D,F) VE-Cad Cre x mT/mG mice marks endothelial cells (green) and non-endothelial cells (red); nuclei are stained with DAPI (blue). (B,C,E,F) 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 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 1 μ M confocal optical sections are presented. Objective magnification x 40.

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. Thus, the partial colocalization of SMA with GFP endothelial lineage-marked cells in Control mice appears restricted to a subset of small pulmonary arteries.

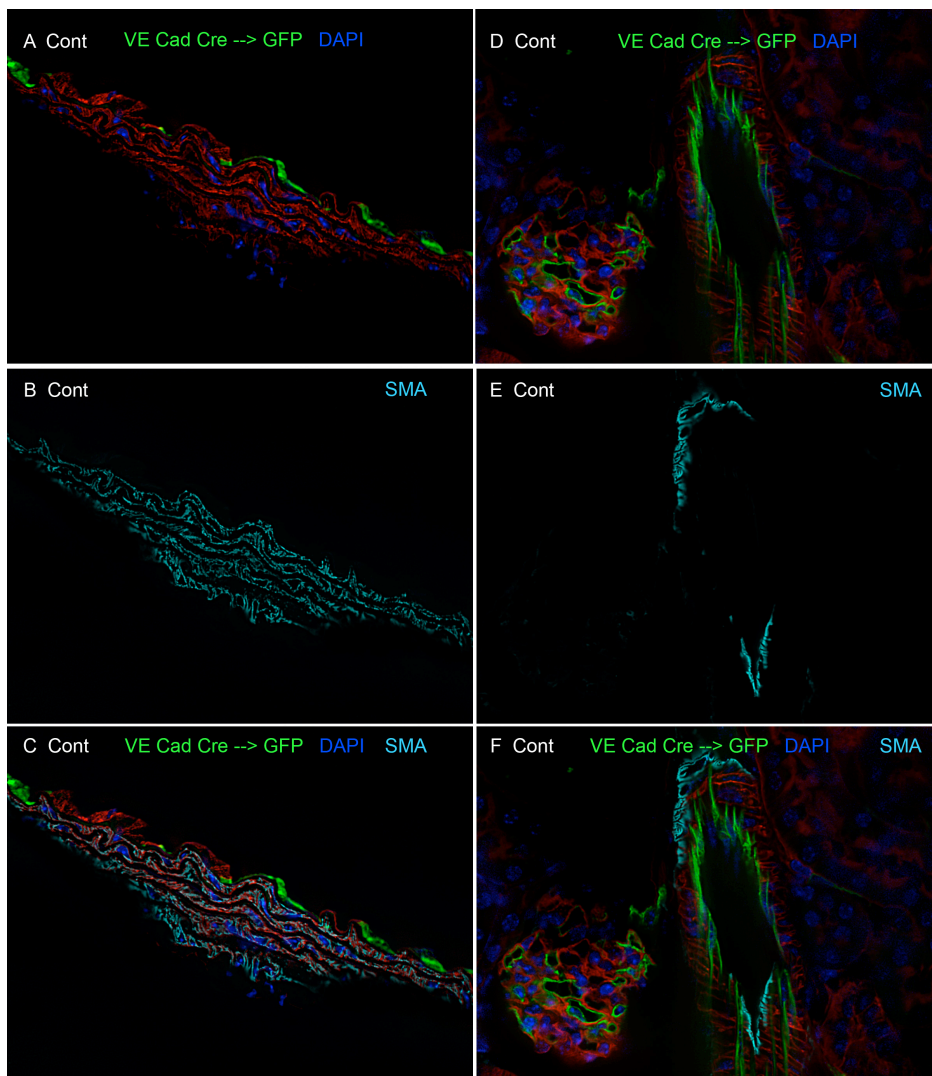


Figure 6. VE-Cad endothelial genetic lineage marking in Control mice and absence of colocalization with α -SMA expression in aorta and kidney. (A,C,D,F) VE-Cad Cre x mT/mG mice marks endothelial cells (green) and non-endothelial cells (red); nuclei are stained with DAPI (blue). (B,C,E,F) Immunostaining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

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**). 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**). Immunostaining for SM-MHC (**Figure 7H, cyan**) demonstrated expression in the neointima with colocalization with GFP endothelial lineage-marked cells (**Figure 7I**).

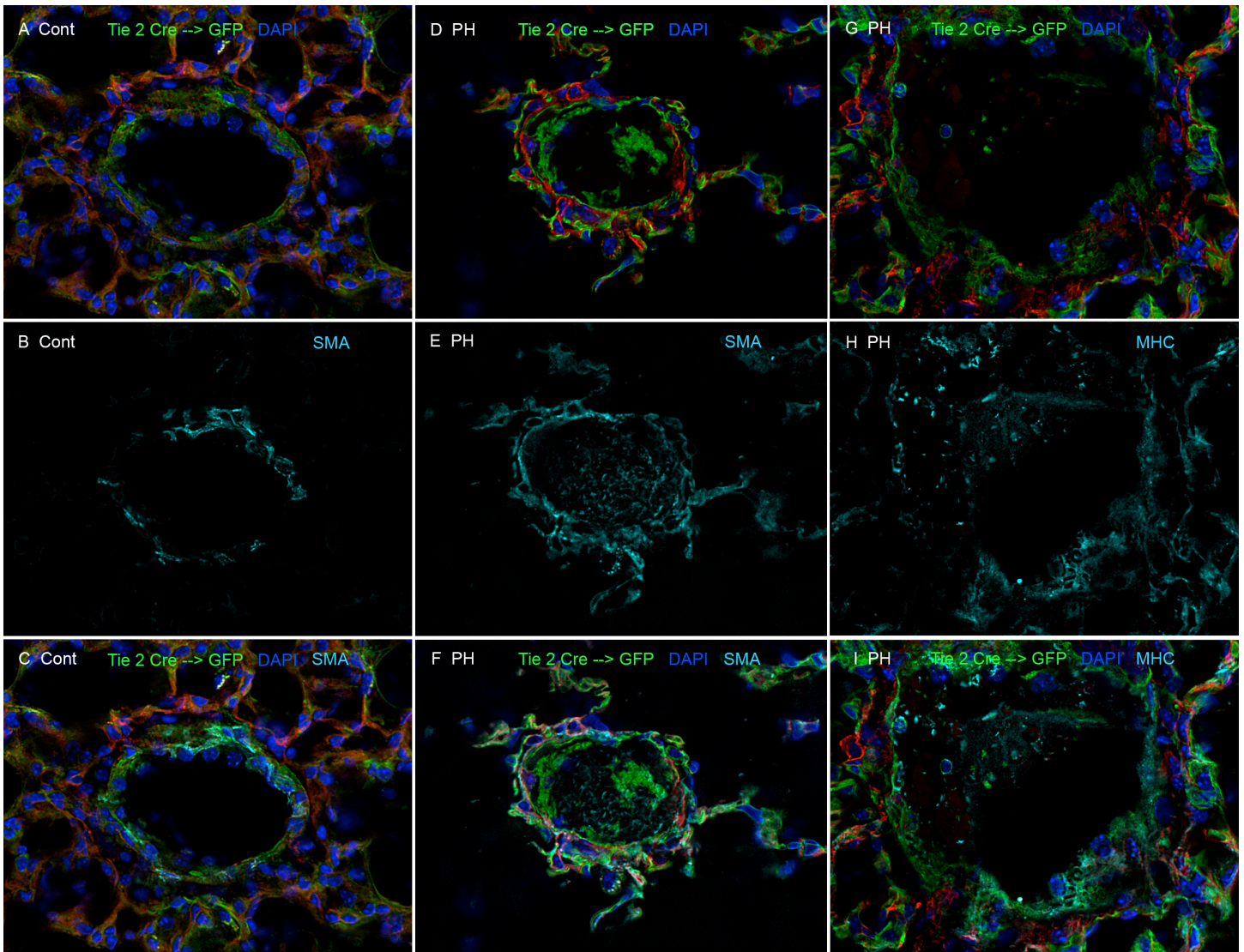


Figure 7. Tie-2 genetic lineage marking in controls and mice with experimental pulmonary hypertension, and colocalization with a-SMA expression. (A,C,D,F,G,I) Tie-2 Cre x mT/mG mice marks endothelial cells (green) and non-endothelial cells (red); nuclei are stained with DAPI (blue). (B,C,E,F) Immunostaining for a-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 a-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 1 μ M confocal optical sections are presented. Objective magnification x 40.

Conclusions: 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. 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 ²³. 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 ²⁵. More recent fate-mapping studies of VE-Cad Cre ²⁶ and Tie-2 Cre ²⁷ 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 ²⁸, concurrent immunostaining of vWF and SMA antigens ²⁹ and clonal analyses of microdissected plexiform lesions expressing Factor VIII antigen ¹⁴.

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 ^{30, 31}, with the exception of one report that bovine endothelial cells express SM-MHC RNA ³². To our knowledge, our discovery that pulmonary artery cells of endothelial genetic lineage activate expression of SM-MHC in neointimal lesions, is novel.

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.

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.

Aim 2: We did not undertake experiments to address this aim due to the challenges we encountered in performing and interpreting the endothelial genetic lineage results.

Continuing Studies:

Our results using endothelial genetic lineage-marking revealed that cells of endothelial genetic lineage activate expression of smooth muscle genes, SMA and SM-MHC, during development of experimental pulmonary hypertension.

An important next step is to determine whether the neointimal cells, which express high amounts of SMA and SM-MHC, include any lineage contribution from differentiated vascular smooth muscle cells. Our preliminary studies using the constitutive SM22 Cre driver mice intercrossed with mT/mG reporter mice, showed that cells with SMA immunostaining generally demonstrated GFP-lineage marking. This result can be interpreted in different ways: 1) that the smooth muscle lineage contributes to the neointima or 2) that neointimal cells activating expression of SMA and SM-MHC also likely activate expression of SM22, and SM22 Cre recombinase, producing genetic recombination and GFP expression.

In order to distinguish between these alternatives, it is necessary to employ conditional, tissue-specific Cre recombinase driver mice. These mice utilize a tissue specific promoter to drive expression of Cre recombinase fused to the estrogen receptor. In the absence of tamoxifen, the CreERT2 fusion protein is retained in the cytoplasm; only when tamoxifen is present does the CreERT2 fusion protein translocate into the nucleus where it produces genetic recombination at loxP flanked sequences (**Figure 8**). We received approval from IACUC and ACURO for new proposed experiments employing conditional smooth muscle Cre driver mice, SMA-CreERT2 and SM-MHC CreERT2 and obtained these mice with permission from the originating labs, facilitated by Dr. Mark Krasnow's lab at Stanford.

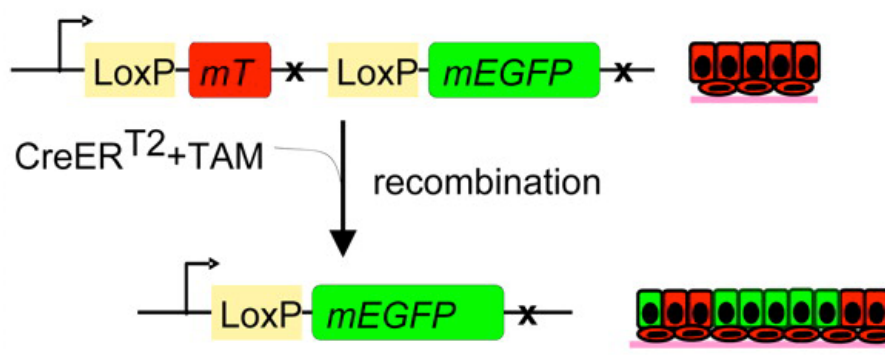


Figure 8. Strategy for conditional genetic lineage marking. Tamoxifen induces cytoplasmic to nuclear translocation of the Cre recombinase-Estrogen Receptor fusion which performs nuclear excision of loxP-flanked mTomato and activates mEGFP expression.

Adult mice that express SMA CreERT2 and mT/mG transgenes will receive daily injection of tamoxifen for 7 days to activate GFP genetic lineage-marking of smooth muscle cells. Subsequently, the mice will undergo pneumonectomy followed one week later by injection of MCTP to produce experimental pulmonary hypertension. Mice will be characterized by RVSP monitoring and sacrificed after 35 days when significant pulmonary hypertension is present. Histopathology with confocal analyses of vascular narrowing and neointima formation will reveal the contribution from the smooth muscle genetic lineage. Extrapolating from our results demonstrating substantial contribution from the endothelial genetic lineage to the neointima (**Figures 4,5,7**), we anticipate that the smooth muscle lineage will not contribute substantially to the neointima (expect red, not green neointima). Such a result would confer support to our hypothesis that the neointima in pulmonary hypertension is derived principally from the endothelial lineage. Furthermore, it would provide additional motivation to search for novel therapies for pulmonary hypertension that suppress the expression of smooth muscle genes in injured pulmonary artery endothelial cells.

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Successful endothelial genetic lineage marking in VE Cadherin Cre x mT/mG mice demonstrates membrane-targeted GFP labeling of pulmonary vascular endothelial cells.
- 2) Novel discovery in Control mice that a subset of endothelial genetic lineage-marked mice demonstrate expression of smooth muscle genes, SMA and SM-MHC.
- 3) Successful induction of experimental pulmonary hypertension in endothelial lineage-marked mice.
- 4) Successful demonstration that neointima lesions in pulmonary hypertension contain GFP endothelial lineage-marked cells.
- 5) Successful demonstration that pulmonary hypertension is associated with induced expression of SMA and SM-MHC in cells of endothelial genetic lineage.
- 6) Approvals granted for acquisition of conditional, smooth muscle lineage directed Cre recombinase mice for planned experiments to characterize the contribution of the smooth muscle lineage to the neointimal lesions in experimental pulmonary hypertension.

REPORTABLE OUTCOMES: Revised manuscript under review.

CONCLUSION: Our results demonstrate that the endothelial genetic lineage contributes to neointimal vascular occlusion in experimental pulmonary hypertension.

Conditional, tissue-specific smooth muscle Cre driver mice are required to perform time-restricted genetic lineage marking prior to the induction of experimental pulmonary hypertension. Approval for these experiments has been obtained, and the experiments are in progress.

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APPENDICES: None