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Award Number: W81XWH-09-1-0383

TITLE: Lineage Analysis in Pulmonary Arterial Hypertension

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REPORT DATE: June 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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# REPORT DOCUMENTATION PAGE

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<b>1. REPORT DATE (DD-MM-YYYY)</b> 01-06-2011		<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED (From - To)</b> 15 MAY 2010 - 14 MAY 2011	
<b>4. TITLE AND SUBTITLE</b> Lineage Analysis in Pulmonary Arterial Hypertension				<b>5a. CONTRACT NUMBER</b>	
				<b>5b. GRANT NUMBER</b> W81XWH-09-1-0383	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Dr. Peter Kao  E-Mail: peterkao@stanford.edu				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> The Leland Stanford Junior University Stanford, CA 94305				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> 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.					
<b>15. SUBJECT TERMS</b> Neointima, vascular occlusion, endothelial cell, Cre-lox recombination					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b>
<b>a. REPORT</b>	<b>b. ABSTRACT</b>	<b>c. THIS PAGE</b>			<b>19b. TELEPHONE NUMBER (include area code)</b>
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# Lineage Analysis in Pulmonary Arterial Hypertension

## Annual Report 2011

### 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 failure (Lilienfeld and Rubin 2000, Rubin 1997). The pathology of PAH is characterized by abnormal expansions of neointimal cells expressing smooth muscle actin (Yi 2000).

There are few data on strategies that suppress neointimal formation being used to treat pulmonary hypertension (Gurubhagavatula and Palevsky 1997). 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 (Higenbottam 1993, Barst, 1996, Rich, 1999). A number of new agents, including simvastatin, hold the potential to attenuate disease progression (Kao and Faul 2003) (Kao 2005).

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* (Fishman 1998, Mandegar 2004). 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 (Yu 1999, Mandegar, 2004).

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 (Tuder 1994, Veyssier-Belot, 1999). 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 (Lee 1998, Yi, 2000). Recently, Deng et al. (Deng 2000) and Lane (Lane 2000) 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 (ten Dijke 2003, Massague, 2000). 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 expression (Atkinson 2002).



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 (Arciniegas 2007). 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 (Sahara 2007).

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 (Arciniegas 2007). 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 Tuder 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 (Lee 1998). 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 Tuder'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 (Arciniegas 2007). 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 (Nosedá 2006). 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-expressing neointimal cells that occlude the microcirculation, and regulation of this fate involves Notch-1 signaling.

**Specific 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, VE-Cadherin Cre) will be intercrossed with reporter mice (mT/mG double fluorescent Cre reporter) 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 GFP-expressing cells of endothelial lineage will be correlated with immunofluorescent staining of endothelial markers CD31 and mesenchymal marker SMA. We demonstrate that GFP-expressing cells of endothelial lineage express SMA during development of pulmonary hypertension. The efficacy of simvastatin will be characterized to suppress EnMT and neointimal formation in experimental pulmonary hypertension.

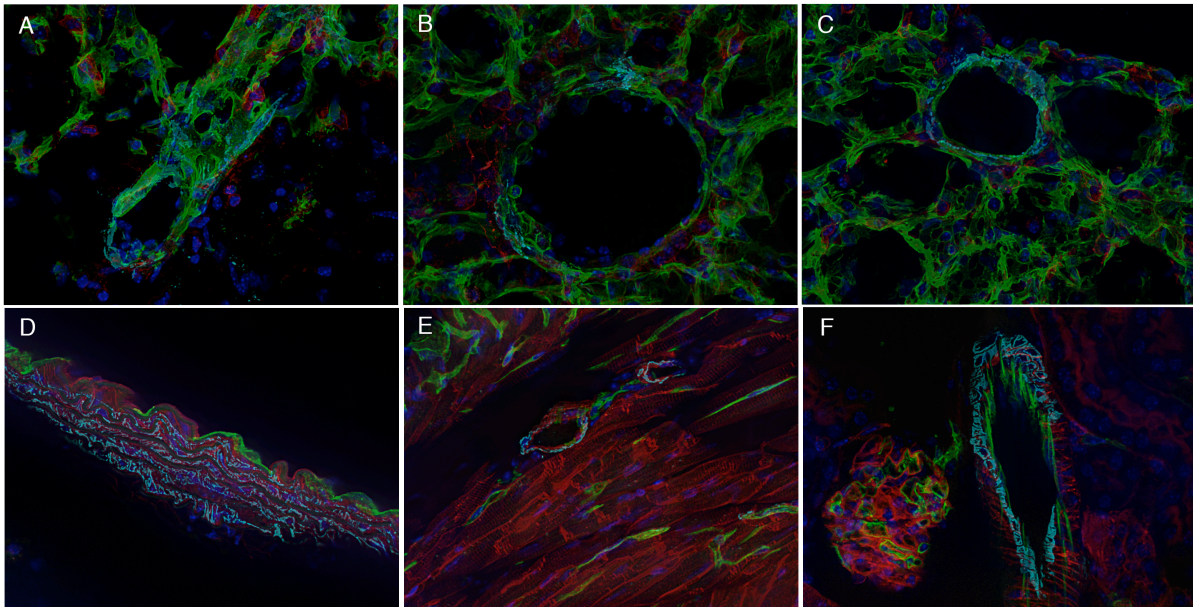
**Specific Aim 2: Characterize how Notch signaling regulates endothelial to mesenchymal transition.** Cells active expressing Notch-1 intracellular domain (Notch1-ICD) will be detected by alpha-VLLS immunostaining. Expression of active Notch1IC will be correlated with cellular expression of endothelial 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:**

**Aim 1:** We achieved endothelial genetic lineage marking by intercrossing VE-Cadherin Cre with mT/mG dual fluorescent Cre reporter mice. The reporter mice (developed by Liquin Luo lab at Stanford) express membrane-targeted tandem dimer Tomato (mT) fluorescent protein in all cells prior to Cre-mediated excision, and membrane-targeted green fluorescent protein (mG) after excision (Muzumdar, 2007). In the hierarchy of endothelial differentiation VE-Cadherin is expressed as a late differentiation antigen of mature endothelial cells.

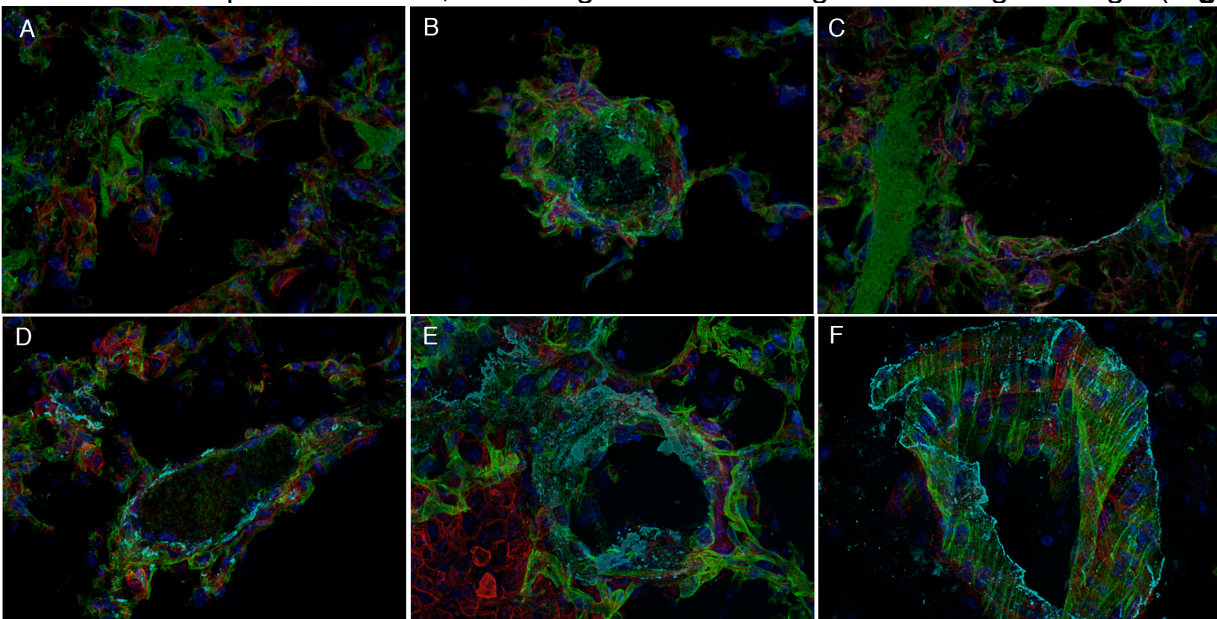
We optimized protocols for mouse lung fixation, cryosectioning, immunostaining and confocal microscopy. We detect endogenous fluorescence of the mTomato and mGFP, plus one channel of immunostaining for endothelial CD31 or VE-Cadherin or smooth muscle alpha actin or myosin heavy chain. Images are acquired using a Leica DMI 6000 inverted fluorescence microscope with 40 and 63x oil immersion apochromatic objective lenses coupled to a BD Carv II white light spinning disk confocal imager. We acquire z-stacks of 1 micron optical sections through physical cryosections of 70 microns, and use deconvolution software for image enhancement followed by 3-D reconstruction through 10-15 microns using NIH Image J. We examining genetic-lineage marked and immunostained thin optical section of mouse lungs with experimental pulmonary hypertension and seek to identify the lineage of origin of the pathological neointimal cells that proliferate within the lumen of small pulmonary arterioles.

During our control experiments characterizing VE-Cadherin Cre x mT/mG mice, we observed good correlation between GFP-expressing endothelial genetic lineage-marked cells and CD31 and VE-Cadherin immunostaining. Notably, we discovered in normal lungs that a fraction of GFP-endothelial lineage-marked cells coexpressed SMA, whereas we observed no expression of SMA by cells of endothelial genetic lineage in the aorta, heart, or kidney (**Figure 1**).



**Figure 1. A subset of endothelial genetic-lineage marked cells express smooth muscle actin in pulmonary arteries but not in aorta, heart or kidney.** VE-Cadherin Cre x mT/mG excises dTomato and induces GFP expression in differentiated endothelial cells. Cyan represents SMA immunostaining. **A-C.** Lung sections showing pulmonary vessels and alveolar capillaries **D.** Aorta **E.** Heart **F.** Kidney

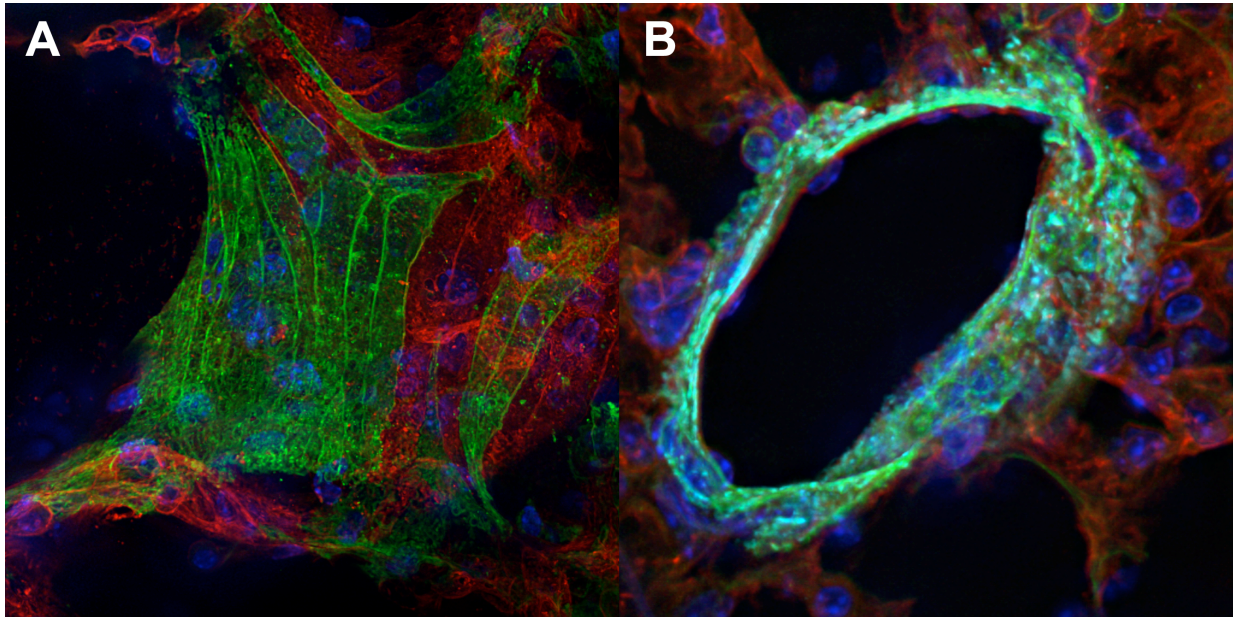
Endothelial genetic lineage-marked mice were treated to develop experimental pulmonary hypertension by left pneumonectomy followed one week later by jugular vein injection of monocrotaline pyrrole in dimethyl formamide. At day 35, mice demonstrated pulmonary hypertension with RVSP increased from  $22 \pm 3$  mmHg to  $54 \pm 5$  mmHg. Histology revealed neointimal vascular occlusion in small pulmonary arteries. Neointimal cells showed prominent and globular expression of SMA, in cells that co-expressed mGFP, indicating an endothelial genetic lineage of origin (**Figure 2**).



**Figure 2. Neointimal vascular occlusion in experimental pulmonary hypertension involves cells of endothelial genetic lineage expressing smooth muscle actin.** **A-C.** Tie2 Cre x mT/mG induces GFP expression in endothelial genetic lineage. Vascular occlusion exclusively by GFP-marked cells with coexpression of SMA (cyan). **D-F.** VE Cad Cre x mT/mG induces GFP expression in endothelial lineage. Vascular occlusion predominantly by GFP-marked cells coexpressing SMA (cyan).

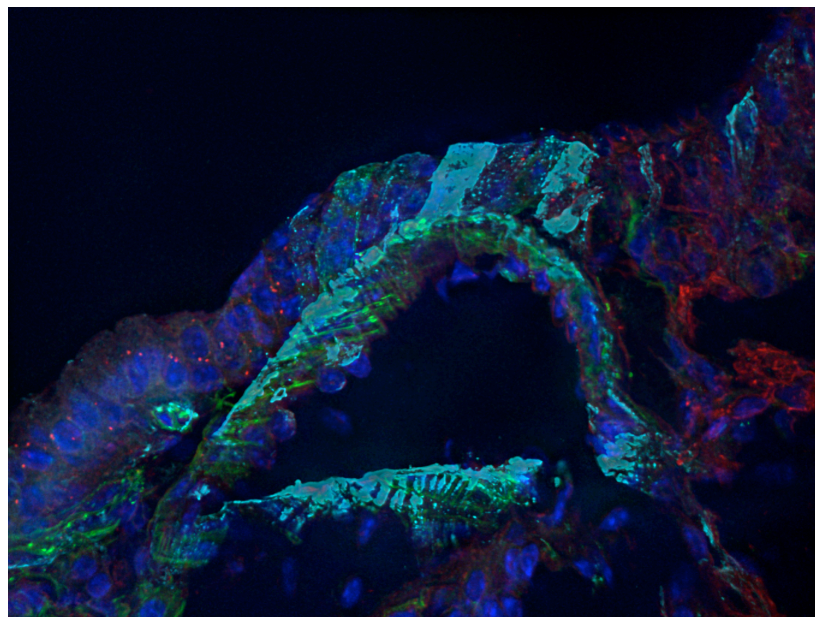


Next, we investigated the contribution of smooth muscle genetic lineage to pathologic neointimal cells. We intercrossed constitutive SM22 Cre driver mice with mT/mG reporter mice and characterized the extent of genetic lineage marking of vascular smooth muscle cells (**Figure 3A**). It is apparent that the borders of single cells are clearly distinguished by the membrane targeted fluorescent protein labeling. Furthermore, the presence of two morphologically identical red cells adjacent to a cluster of green vascular smooth muscle cells indicates that the genetic recombination by SM22 Cre is incomplete. In control mice before induction of experimental pulmonary hypertension, SMA immunostaining is seen predominantly colocalizing with green cells, of smooth muscle genetic lineage (**Figure 3B**).



**Figure 3. SM22 Cre x mT/mG demonstrates GFP labeling of vascular smooth muscle cells in control mice. A.** Interposition of red cells between GFP-labeled vascular smooth muscle cells indicates that genetic lineage recombination is incomplete. **B.** Smooth muscle actin immunostaining (cyan) predominantly overlays green genetic-lineage marked smooth muscle cells.

**Figure 4. Experimental Pulmonary Hypertension in SM22 Cre x mT/mG mice.** SMA immunostaining (cyan) is predominantly colocalized with GFP staining of smooth muscle genetic lineage.



We induced experimental pulmonary hypertension in SM22 Cre x mT/mG mice with smooth muscle genetic lineage marked by GFP. The SMA immunostaining is predominantly associated with green GFP labeling of smooth muscle genetic lineage (**Figure 4**). This result must be reconciled with our previous discovery that SMA immunostaining is associated with GFP labeling of the endothelial genetic lineage in VE-Cadherin Cre and Tie2 Cre x mT/mG mice (**Figure 2**).

**We hypothesize that pathological neointimal cells derive from the endothelial genetic lineage and aberrantly activate a program of smooth muscle gene expression that includes expression of SMA and SM22 and genetic activation of SM22 Cre recombinase transgene.**

Therefore, in order to clarify whether the endothelial or smooth muscle genetic lineage is the origin of the pathologic neointimal cells in experimental pulmonary hypertension, we must use conditional, tissue-specific Cre driver mice that will confer genetic lineage marking prior to the induction of disease.

We have negotiated a Material Transfer Agreement with Dr. Luisa Irula Arispe at UCLA and have obtained through Dr. Mark Krasnow's lab at Stanford the VE Cadherin CreER-T2 mice. Tamoxifen treatment is required for cytoplasmic to nuclear translocation of the Cre recombinase gene in endothelial cells, and genetic recombination. We have characterizing our first intercrosses of conditional VE Cad Cre ER T2 x mT/mG mice, and will assess the degree of recombination of pulmonary endothelial cells (fraction of green labeling to CD31 immunostaining) achievable with one week of daily tamoxifen injections in 2-month old adult mice. Subsequently, these mice with time-stamped genetic labeling of endothelial cells will undergo induction of experimental pulmonary hypertension, and we will characterize the contribution of green, genetically pre-labeled endothelial cells to the pathologic neointima.

We are negotiating a Material Transfer Agreement with Dr. Pierre Chambon at the Institute Pasteur to obtain the SMA Cre ER-T2 mice. These mice will allow us to perform time-stamped labeling of the smooth muscle genetic lineage one week of daily tamoxifen injections in 2-month old adult mice. We will assess the degree of recombination of vascular smooth muscle cells (fraction of green labeled cells to SMA immunostaining). Subsequently, these mice with time-stamped genetic labeling of smooth muscle cells will undergo induction of experimental pulmonary hypertension, and we will characterize the contribution of green, genetically pre-labeled smooth muscle cells to the pathologic neointima.

We anticipate that these experiments using endothelial and smooth muscle tissue-specific conditional Cre driver mice will allow us to determine with new clarity which cell lineage contributes predominantly to pathologic neointima formation in pulmonary hypertension. The results of these studies will help focus future investigations on the cell lineage of origin of the pathologic lesions. Such investigations will include strategies to suppress pathologic responses to injury.

**Aim 2:** We have not yet embarked on experiments addressing this aim. We will perform immunostaining for activated Notch ICD and determine if there is colocalization within neointimal cells.

We are focusing our efforts on using endothelial and smooth muscle conditional Cre driver mice to generate new insights into the cell lineage of origin of the pathologic neointimal lesions that underlie pulmonary hypertension.

**KEY RESEARCH ACCOMPLISHMENTS:**

- 1) Successful Cre-lox labeling of endothelial genetic lineage in mouse lung, by intercrossing Tie-2 Cre and VE-Cadherin Cre endothelial driver mice with mT/mG dual fluorescent switch reporter mice. Microvascular pulmonary endothelial cells express GFP (green) while non-endothelial cells express dTomato (red).
- 2) Successful immunostaining for endothelial antigens CD31 and VE-Cadherin in genetic lineage marked mice confirms endothelial lineage marking is specific and complete.
- 3) Immunostaining for smooth muscle actin in endothelial lineage-marked mice reveals that a subset of endothelial cells coexpress SMA in non-injured lungs. Endothelial cells in heart, kidney and skeletal muscle do not express SMA.
- 4) Neointimal cells contributing to vascular occlusion in experimental pulmonary hypertension originate from endothelial genetic lineage, and coexpress SMA.
- 5) Successful labeling of smooth muscle genetic lineage using constitutive SM22 Cre x mT/mG
- 6) Neointimal lesions in constitutive SM22 Cre x mT/mG mice show colocalization of SMA immunostaining and GFP labeling of smooth muscle genetic lineage
- 7) Constitutive SM22 Cre recombinase may be mediating genetic recombination during aberrant activation of smooth muscle genes in pathologic neointimal lesions
- 8) Acquisition of VE Cadherin CreER-T2 tamoxifen-inducible conditional Cre endothelial driver mice
- 9) Approval to receive SMA CreER-T2 tamoxifen-inducible conditional Cre smooth muscle driver mice

**REPORTABLE OUTCOMES:** Manuscript in preparation

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

Our most recent results indicate that pathologic neointimal cells at some point activate the SM22 smooth muscle gene, which leads to constitutive SM22 Cre-mediated genetic recombination and green labeling of neointimal cells.

Conditional, tissue-specific endothelial and smooth muscle Cre driver mice are required to perform time-restricted genetic lineage marking prior to the induction of disease. These mice have been obtained and are currently breeding.

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**APPENDICES:** None

**SUPPORTING DATA:** None