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TITLE: Lineage Analysis in Pulmonary Arterial Hypertension

PRINCIPAL INVESTIGATOR: Peter N. Kao, M.D., Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, CA 94305-6203

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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, a subset of pulmonary arterial vascular lining cells was discovered with endothelial genetic lineage and coexpression of smooth muscle antigens, SMA, SM-MHC, and SM22. Induction of experimental pulmonary hypertension with neointima vascular occlusion is associated with augmented expression of smooth muscle antigens in cells of endothelial genetic lineage. Conditional endothelial and smooth muscle lineage marking will be used to determine the principal lineage contributing to the pathologic neointima and suggest novel therapies.
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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 pathphysiology 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 (Noseda 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 pneumonectomy 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 neoointimal 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 (Figure 1).
Endothelial genetic lineage marked-cells in pulmonary arterioles also express smooth muscle genes. 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 2). Therefore, we propose that a subset of pulmonary arteriolar cells exhibit dual endothelial and smooth muscle lineage and gene expression.

Figure 2. 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
Experimental pulmonary hypertension augments smooth muscle actin and myosin heavy chain expression in pulmonary arteriolar cells of endothelial genetic lineage: 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. Expression of smooth muscle alpha actin in neointimal lesions is a distinguishing feature of pulmonary hypertension. Immunostaining of pulmonary hypertensive mice demonstrated smooth muscle actin in both Tie-2 Cre (Figure 3A-C) and VE-Cad Cre (Figure 3D-F) genetic-lineage marked endothelial cells. Some of the vascular lesions demonstrated SMA expression in endothelial genetic lineage marked cells in splotches (Figure 3E) or along edges potentially involved in migration (Figure 3F).

![Figure 3A-C](image)

**Figure 3. 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).

We observed that smooth muscle myosin heavy chain expression increased in endothelial genetic-lineage marked cells surrounding pulmonary arteries (Figure 4D-F). In Tie-2 Cre-marked pulmonary hypertensive mice, GFP-labeled cells endothelial cells comprised the neointimal lesions and demonstrated widespread SM-MHC expression (Figure 4D). In VE-Cad Cre-marked pulmonary hypertensive mice there was substantial augmentation of SM-MHC expression associated with GFP-labeled endothelial cells (Figure 4E,F).
A subset of vascular lining cells demonstrates a smooth muscle genetic lineage. SM22alpha (transgelin) is a marker of contractile smooth muscle cells, structurally related to actin-binding protein, calponin. We intercrossed SM22alpha Cre driver mice with mT/mG reporter mice and characterized the extent of smooth muscle genetic lineage marking (Figure 5). At low magnification we observed circumferential GFP-genetic lineage marking of smooth muscle cells surrounding bronchi and large pulmonary arteries (not shown). At higher magnification (x400) we observed single cells clearly outlined by the membrane-targeted GFP (green) labeling. The cells appeared thin, rectangular and tightly joined with morphology similar to pulmonary arteriolar endothelial cells. Interestingly, we observed the smooth muscle genetic lineage marking of some but not all, vascular lining cells (see red unrecombined cells adjacent to green cells in Figure 5A). Furthermore, the smooth muscle genetic lineage marking in small arterioles was evident at branch points (Figure 5B). SMA expression was predominantly colocalized with green cells of smooth muscle genetic lineage (Figure 5B and C).

Figure 4. Smooth muscle myosin heavy chain expression in cells of endothelial genetic lineage is augmented in experimental pulmonary hypertension (PH). A-C. VE-Cad Cre x mT/mG mice demonstrate SM-MHC expression (cyan) in a subset of pulmonary artery endothelial cells (green). D. Tie-2 Cre x mT/mG mice with PH demonstrate extensive SM-MHC expression (cyan) in neointimal cells of endothelial lineage (green). E-F. VE-Cad Cre x mT/mG mice with PH demonstrate extensive SM-MHC expression (cyan) in neointimal cells of endothelial lineage (green).

Figure 5. SM22 Cre x mT/mG results in GFP-lineage marking of cells that express smooth muscle genes. Smooth muscle actin (SMA) immunostaining (cyan) colocalizes with GFP smooth muscle lineage marking. Control mice before induction of experimental pulmonary hypertension.
Experimental pulmonary hypertension is associated with activation of smooth muscle gene expression in vascular lining cells. We induced experimental pulmonary hypertension in SM22 Cre x mT/mG mice, by injecting monocrotaline pyrrole into the pulmonary circulation of pneumonectomized mice. Marked enhancement of SMA expression in vascular lining cells is revealed by immunostaining (Figure 6, SMA immunostaining in cyan). We appreciate that injured vascular lining cells activate a program of smooth muscle gene expression, and this is demonstrated in complementary ways by SMA immunostaining, and by genetic recombination driven by SM22 Cre recombinase, inducing a dTomato red to GFP green switch (Figure 6). This explains why pulmonary artery vascular lining cells in control SM22 Cre mice initially demonstrate partial GFP labeling, reflecting a subset of presumed endothelial cells that coexpress SM22, whereas after vascular injury essentially all of the endothelial cells activate SMA expression and simultaneously undergo SM22-Cre mediated recombination and switch from red to GFP expression (Figure 5 vs. 6B).

We hypothesize that pathological neointimal cells derive from the endothelial genetic lineage and activate a program of smooth muscle gene expression demonstrated through expression of SMA and activation of SM22 Cre recombinase resulting in genetic recombination and activation of GFP expression.

In order to determine with precision whether the endothelial or smooth muscle cells represent the principal lineage of origin of the pathologic neointimal cells in experimental pulmonary hypertension, we need to study tissue-specific, conditionally-activated Cre driver mice that will confer conditional 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 will assess the degree of recombination of pulmonary endothelial cells (fraction of GFP labeling to CD31 immunostaining) achieved with 5 sequential days of tamoxifen injections in 2-month old adult mice. These mice will then undergo our experimental pulmonary hypertension model and we will characterize the contribution of GFP-endothelial lineage marked cells to the pathologic neointima.
We have negotiated Material Transfer Agreements with Dr. Pierre Chambon at the Institute Pasteur to obtain the SMA Cre ER-T2 mice and with Dr. Stefan Offermanns at the Max Planck Institute for Heart and Lung Research to obtain SM-MHC CreER-T2 mice. These mice will allow us to perform conditional labeling of the smooth muscle genetic lineage with 5 sequential days of tamoxifen injections in 2-month old adult mice (Figure 7). We will assess the degree of recombination of vascular smooth muscle cells (fraction of GFP-labeled cells to SMA immunostaining). Subsequently, these mice with conditional genetic labeling of smooth muscle cells will undergo induction of experimental pulmonary hypertension, and we will characterize the contribution of GFP-smooth muscle lineage marked cells to the pathologic neointima.

We anticipate that during the progression of experimental pulmonary hypertension, the pulmonary artery luminal endothelial cells will decrease expression of endothelial antigens, VE-Cadherin and CD31, and the developing neointimal cells will demonstrate increasing expression of smooth muscle genes, SMA and SM-MHC (Figure 7A).

**Conditional, endothelial genetic lineage marking:**

*Early injection of tamoxifen (Days -5 to 0):* We anticipate that VE-Cad CreER^{T2}; mT/mG mice injected with tamoxifen on Days -5 to 0 before pneumonectomy will demonstrate 75-80% GFP-labeling of pulmonary arteries and alveolar capillaries, and that ~80% of neointimal cells at Day 35 will demonstrate GFP-labeling, supporting an endothelial lineage of origin (Figure 7B). Control mice will be injected with tamoxifen on Days -5 to 0 and sacrificed on Day 35, without pneumonectomy or MCTP injection, to assess penetrance of endothelial labeling and persistence of GFP-marked endothelial cells in uninjured lungs.

*Late injection of tamoxifen (Days 28-33):* VE-Cad CreER^{T2}; mT/mG mice will be injected with tamoxifen on Days 28-33 following pneumonectomy. We anticipate that neointimal cells will no longer express VE-Cadherin at this time point (Figure 7A), and therefore will not undergo recombination, and that the majority of neointimal cells at Day 35 will be red (Figure 7B, lower line). This result will
provide strong support for endothelial-to-mesenchymal transition, specifically loss of endothelial phenotype, contributing to disease pathogenesis.

**Conditional, smooth muscle genetic lineage marking:**

*Early injection of tamoxifen (Days -5 to 0):* We anticipate that SM-MHC CreERT² and SMA CreERT² x mT/mG mice injected with tamoxifen on Day -5 to 0 before pneumonectomy will exhibit 75-80% GFP-labeling of airway and vascular smooth muscle cells and limited GFP-labeling of endothelial cells of pulmonary arteries (~10%), consistent with dual endothelial/smooth muscle lineage expression in a subset of pulmonary artery endothelial cells (*Figure 2A-C, Figure 4A-C, Figure 7C*). Schema C1: We anticipate that the neointima on Day 35 will consist of a mixture of GFP- and dTomato-labeled cells, in the same proportion as prior to the induction of disease (10%), and this will imply that pathogenesis involves activation of smooth muscle gene expression in injured, unrecombined pulmonary artery endothelial cells. Scheme C2. It is possible that a limited pool of endothelial progenitor cells (the 10% that is identified by SM-MHC recombination before disease) possesses high proliferative potential and will expand dramatically during disease and eventually contribute to 100% of the neointima.

*Late injection of tamoxifen (Days 28-33):* SM-MHC CreERT² and SMA CreERT² x mT/mG mice will be injected with tamoxifen on Days 28-33 following pneumonectomy and MCTP injection. We anticipate that all neointimal cells express smooth muscle genes at this time point (*Figure 7A*) and therefore expect 90-100% recombination manifesting as GFP-marked neointimal cells at Day 35 (*Figure 7C.3, lower line*).

These studies hold the potential for providing important new insights into the molecular events and potential endothelial lineage transitions that contribute to the pathogenesis of pulmonary hypertension.

**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. Pulmonary arterial and microvascular endothelial cells express GFP (green) while non-endothelial cells express dTomato (red). Successful immunostaining for endothelial antigens CD31 and VE-Cadherin in genetic lineage marked mice confirms endothelial lineage marking is specific and complete.
2) Discovery and characterization of a novel subset of pulmonary arterial vascular lining cells of endothelial genetic lineage that coexpress smooth muscle genes, SMA and SM-MHC in lungs of control mice. No such dual endothelial and smooth muscle lineage cells are observed in heart, kidney or skeletal muscles.
3) Experimental pulmonary hypertension is associated with augmented expression of smooth muscle genes, SMA and SM-MHC in cells of endothelial genetic lineage.
4) Constitutive SM-22 Cre; mT/mG mice demonstrate recombination in bronchial and vascular smooth muscles and also mosaic recombination in a subset of pulmonary artery vascular lining cells with morphology of endothelial cells in lungs of control mice.
5) Experimental pulmonary hypertension in constitutive SM22 Cre x mT/mG mice demonstrates genetic recombination (expression of GFP) in neointimal lesions that express SMA and SM-MHC antigens.
6) Appreciation of the need for conditional endothelial and smooth muscle genetic lineage marking to achieve genetic recombination at defined time windows relative to the induction of experimental pulmonary hypertension:
   a) Acquisition and breeding of VE Cadherin CreER-T2 tamoxifen-inducible conditional Cre endothelial driver mice.
   b) Acquisition and breeding of SMA CreER-T2 tamoxifen-inducible conditional Cre smooth muscle driver mice; Acquisition and breeding of SM-MHC CreER-T2 tamoxifen-inducible Cre smooth muscle driver mice.

REPORTABLE OUTCOMES: Manuscript in revision for Circulation Research

CONCLUSION: We discovered and characterized a subset of pulmonary arterial vascular lining cells with dual endothelial and smooth muscle lineages. Experimental pulmonary hypertension is associated with augmented expression of smooth muscle genes in cells of endothelial genetic lineage. Studies in progress utilizing conditional, endothelial and smooth muscle Cre driver mice will allow conditional genetic lineage marking prior to the induction of experimental pulmonary hypertension. These experiments will determine with precision the predominant lineage of origin of the pathologic neointima in pulmonary hypertension, and suggest novel therapeutic approaches.
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**APPENDICES:** None

**SUPPORTING DATA:** None