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# Cellular Models of PAH-Associated Molecular Defects as a Tool for Identifying New Therapeutic Targets

Elinoff

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## Sub-Project 1

Rare Genetic Defect in Glucose Metabolism as a Model for Investigating Mechanisms Underlying Vascular Remodeling in PAH Glucose-6-phosphatase catalytic subunit 3 (G6PC3) is a ubiquitously expressed enzyme that maintains intracellular glucose homeostasis by catalyzing the hydrolysis of glucose-6-phosphate to glucose in the endoplasmic reticulum. Loss-of-function mutations in G6PC3 lead to an autosomal recessive, multi-system syndrome of severe congenital neutropenia with a broad phenotypic spectrum that includes a high incidence of congenital heart defects. A subset of affected patients exhibits Dursun syndrome, a triad of congenital neutropenia, atrial septal defect and PAH. While the effect of G6PC3 deficiency on neutrophil function has been thoroughly studied, little is known about its impact on the vasculature. We hypothesize that investigation of a rare but well-characterized genetic cause of disrupted cellular energy homeostasis will provide valuable insight into how metabolic reprogramming contributes to PAH pathobiology.

### Aim 1

Determine the phenotypic consequences of G6PC3-silencing in human pulmonary artery and human pulmonary microvascular endothelial cells (ECs). In FY23, based on the results of hemodynamic studies in G6pc3 knockout (KO) mice, we examined the effects of shear stress and glucose concentration on NOS3, END1, ACE2, ACTA2, and VWF gene expression in G6PC3-deficient primary

human pulmonary artery ECs.

## Aim 2

Investigate the impact of G6pc3 deficiency on pulmonary vascular function in vivo using KO mice under both normoxic and chronic hypoxic conditions. These murine studies were done under an ACUC approved Animal Study Proposal (CCM 20-03). In FY23, G6pc3 KO mice and wild-type (WT) littermate controls were exposed to either normoxia or hypoxia for 5 weeks and then underwent cardiac catheterization. Compared to WT littermate control animals, male G6pc3 KO mice have significantly higher baseline right ventricular systolic pressure (RVSP) under normoxic conditions whereas female KO mice do not. Exposure to chronic hypoxia further increases RVSP in male KO mice compared to male WT controls. Aim 3: Develop and characterize patient-specific in vitro models of endothelial dysfunction using induced pluripotent stem cell (iPSC)-derived endothelial cells. In FY23, in collaboration with Dr. David McDermott, peripheral blood mononuclear cells were collected from two new subjects with loss-of-function mutations in G6PC3 (homozygous for c.246 G>A). These cells will be used to develop patient-specific iPSCs.

## Sub-Project 2

Mechanisms Leading to Interferon Activation in Caveolin-1 (CAV1) deficient primary human pulmonary artery ECs Recently, comprehensive in vitro characterization of CAV1 deficiency in human lung endothelium revealed a proliferative, interferon (IFN)-biased inflammatory phenotype driven by constitutively activated STAT and AKT signaling. PAH patients with CAV1 mutations also had elevated serum CXCL10 levels and their fibroblasts mirrored phenotypic and molecular features of CAV1-deficient PAECs. Moreover, immunofluorescence staining revealed endothelial CAV1 loss and STAT1 activation in the pulmonary arterioles of patients with idiopathic PAH, suggesting that this paradigm might not be limited to rare CAV1 mutations. Finally, inhibiting JAK/STAT and/or PI3K/AKT reversed this aberrant cell phenotype and may ameliorate vascular remodeling in PAH (Gairhe et al. PNAS 2021).

In FY23, we continued investigations into the mechanisms underlying STAT1 activation following CAV1 loss in human PAECs. In addition to constitutively activated STAT1, we also previously observed that NOS3 phosphorylation at serine 1177, a post-translational modification associated with NOS3 uncoupling and increased oxidant stress, is also increased in CAV1-deficient PAECs (Gairhe et, PNAS 2021). Consistent with this observation, we observed both increased reactive oxygen species (ROS) and evidence of nitrosative stress in CAV1-silenced PAECs. Importantly, co-silencing NOS3 in CAV1-deficient cells not only attenuated STAT1 phosphorylation and ROS production but also reversed the proliferative phenotype. Thus, NOS3 dysfunction appears linked to STAT1 activation and the abnormal phenotype of CAV1-silenced PAECs. Importantly, both PKA and AKT can phosphorylate NOS3 at serine 1177 and therefore represent possible signaling mediators in the context of CAV1 deficiency. Increased phosphorylation of nuclear CREB, a known target of PKA and AKT, further implicated a role for either or both of these kinases in shaping the phenotype of CAV1 deficient PAECs.

Interestingly, while PKA inhibition blocked phosphorylation of STAT1 and NOS3 in CAV1-deficient PAECs, inhibiting transmembrane adenylyl cyclase (AC), a major regulator of PKA, did not. In addition to transmembrane AC, a soluble isoform of AC also exists and its activation is associated with impaired endothelial function. Using two different small molecule inhibitors, we demonstrated that blocking soluble adenylyl cyclase (AC10) attenuated ROS production, STAT1 and NOS3 phosphorylation and cell proliferation in CAV1-silenced PAECs. We plan to further investigate the role of soluble adenylyl cyclase and PKA in STAT1 activation with the goal of identifying therapeutic

targets that can ameliorate the inflammatory remodeling in PAH.

### Sub-Project 3

Efficacy of PI3K/AKT Pathway Inhibition on Pulmonary Vascular Remodeling in Rat Models of Pulmonary Arterial Hypertension Activation of the PI3K/AKT pathway is a prominent, shared feature across our models of PAH-associated molecular defects. Leniolisib is a PI3K-delta inhibitor that has been very well tolerated in children with activated PI3K-delta syndrome and reversed the hyperproliferative, apoptosis-resistant cellular phenotype in our in vitro PAH cellular models.

In collaboration with Novartis/Pharming, we first tested RB-50-LV29 (abbreviated RB), a tool compound for leniolisib, in our rat SU5416-hypoxia PAH model. More recently we have obtained leniolisib for additional in vivo studies. These animal studies are conducted under ACUC approved Animal Study Proposals (CCM 19-03, CCM 19-07 and CCM 23-01). In FY23, in vivo testing of RB was completed. Male and female Sprague-Dawley rats (N=54) were subcutaneously injected with SU5416 (20 mg/kg), exposed to hypoxia (10% FiO<sub>2</sub>) for 3 weeks and then returned to normoxia for 5 weeks (referred to as SuHx rats). SuHx rats were randomly assigned to receive either RB (n=14, 60 mg/kg/d; n=12, 120 mg/kg/d) or placebo (n=28) from weeks 5 to 8. Age-matched male and female normoxic controls were also randomly assigned to receive either RB (n=8, 60 mg/kg/d) or placebo (n=8) from weeks 5 to 8. Compared to placebo-treated SuHx rats, mean right ventricular (RV) systolic pressure was lower in SuHx rats that received high dose RB (120 mg/kg/d) but not low dose RB (60 mg/kg/d). Treatment with high dose, but not low dose RB also reduced the number of completely occluded pulmonary arterioles but did not reduce pulmonary arteriolar muscularization or RV hypertrophy in SuHx rats. Left heart catheterization revealed no significant difference in mean systemic blood pressure or left ventricular end diastolic pressure between normoxic controls and SuHx rats with or without RB treatment.

## Proteasomal Degradation of XPB as a Novel Mechanism for Treating Inflammation

Elinoff

[NIH link](#)

In collaboration with the NCATS Functional Genomics Lab, we have created and validated a high-throughput luminescent reporter assay suitable for identifying small molecule degraders of XPB and genome-wide RNAi screening.

Using this state-of-the-art high-throughput assay we will:

1. Identify novel, small-molecule XPB degraders with potent anti-inflammatory effects that can be accelerated into clinical trials;
2. Characterize molecular partners necessary for drug-induced XPB degradation in order to construct a more generalizable pharmaceutical paradigm.

This innovative assay platform will enable discovery of clinically relevant XPB degraders that modulate lung vascular inflammation in PAH patients, and the NIH Clinical Center is ideally suited for spearheading trials testing these first-in-class anti-inflammatory drugs.

## Project Milestones

1. Screen the NCATS Small Molecule Library (up to 150,000 compounds) for drugs that induce XPB-HiBiT degradation. Candidate drugs will be selected through bio/chemi-informatic analyses, validated at 11 concentrations, and undergo counter screens to exclude cytotoxic effects and/or assay interference. Top candidates will be resynthesized and their activity confirmed.
2. Perform a second, high-throughput screen of candidate XPB degraders for potent anti-inflammatory effects across heterogeneous signaling pathways. Novel drugs identified in both screens will be further examined for their effects on inflammatory gene transcription and cytokine production in cellular models of PAH-associated endothelial cell dysfunction.
3. Perform a genome-wide RNAi screen to define the molecular mechanism underlying drug-induced XPB degradation. Target deconvolution of candidate drugs that induce proteasome-dependent XPB degradation and the NCATS pharmacologically annotated chemical toolbox will be used to substantiate gene silencing effects. Candidate XPB degradation genes will be confirmed in primary human pulmonary vascular endothelium.
4. Apply state-of-the-art medicinal chemistry techniques to optimize pharmacokinetics, potency, and efficacy of lead anti-inflammatory drugs for translation into the clinic.

## Personalized protein-protein interactomes and precision medicine in pulmonary arterial hypertension

[NIH link](#)

Maron

### Project Summary/Abstract

Pulmonary arterial hypertension (PAH) is a highly morbid cardiopulmonary disease characterized by an obliterative vasculopathy involving distal pulmonary arterials that promotes right heart failure. Complex and integrated pathobiological signaling pathways drive vascular remodeling in PAH: the arteriopathy includes numerous endophenotypes (i.e., specific features, such as fibrosis, cellular proliferation, others) that occur to differing extent across patients. Wide variability in the proteomic and genetic profile is also observed in PAH, which accounts for phenotypic heterogeneity and inconsistent clinical response to drug therapies reported in clinical trials and at point-of-care.

Overall, these observations suggest that opportunity exists to improve clinical outcome by individualizing the pathobiology-clinical phenotype relationship. Nonetheless, precision medicine in PAH remains unrealized, which we postulate is due to limitations inherent in conventional analytical methods that average biological data and overlook functionally important signaling pathways. Work from our laboratory and others has demonstrated the importance of studying functionally significant signaling pathways using network medicine to discover novel and modifiable therapeutic targets in PAH. This approach differs from classic reductionist methods that infer functionality based on transcript or protein quantity alone, which may erroneously implicate molecular bystanders in the pathogenesis of disease. However we have innovated a precision-based network medicine strategy that generates patient-specific protein-protein interaction (PPI) networks (e.g., patient-level molecular wiring map).

Our approach unmask molecular interactions that distinguish (and group together) individual patients with the same clinical phenotype. We present novel preliminary data in the accompanying

application to support the central hypothesis: Developing patient-specific PPI networks will personalize clinical phenotyping and optimize prognosis in PAH. Our findings will also clarify the relationship between PAH genetic risk and pathobiology on an individual-patient level, and inform rationale and personalized drug selection using the PPI networks. To test our hypothesis, we will leverage a rich dataset from the United Kingdom PAH Phenome Biobank.

This dataset includes comprehensive proteomic, genomic, clinical, and outcome data across two timepoints (1 yr apart) for idiopathic PAH, hereditary PAH, asymptomatic family members of patients with PAH, including three patients that developed PAH during the study, and healthy volunteer controls (N=500 total).

The Aims are:

1. Profile patient-specific PPI networks using proteomic and genetic data, and analyze temporal differences in network features by patient group
2. Develop, test, and validate a network score that informs phenotype and outcome of individual PAH patients. As an exploratory aim, we will use the PPI networks to predict patient-specific drug therapies.

Overall, findings from this project will advance precision medicine in PAH with direct relevance to the clinical management of patients.

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