Research Strategy

Abbreviations: **PH** (Pulmonary hypertension), **FXN (**frataxin), **Fe-S** (iron-sulfur), **HPAEC** (human pulmonary arterial endothelial cell), **iPSC** (inducible pluripotent stem cell), **ROS** (reactive oxygen species), **RT-qPCR** (Real-time quantitative polymerase chain reaction), **EDN1** (endothelin-1), **RVSP** (right ventricular systolic pressure)

Significance

Pulmonary hypertension is a complex disease that has no cure and remains difficult to treat.

Pulmonary hypertension (PH) is a deleterious disease of the lung vasculature that is clinically defined by increased pulmonary arterial pressure of greater than 25 mmHg at rest during right heart catheterization. Because this condition is caused by multiple disparate triggers, the World Health Organization classifies PH into 5 groups based upon etiology and lesion location. Common to all forms of PH, obstruction in the pulmonary vasculature results in increased resistance to flow, creating a pressure on the right ventricle that eventually results in right heart failure and death[1,12,13]. Due to the insidious onset of nonspecific symptoms, delayed diagnosis occurs in up to 85% of at-risk patients[14]. Current therapies are limited to endothelin-1 antagonists, prostacyclin analogs, and phosphodiesterase inhibitors to manage the symptoms associated with enhanced pulmonary vasoconstriction commonly seen in PH patients[15]. Unfortunately, there are no drugs that reverse the causative pathology of PH, leading to significant morbidity and mortality with patient survival at 5 years is ~61% depending on the etiology[14-16]. Thus, the discovery of new molecular targets for improved treatments is critical.

Deficiencies of iron-sulfur clusters and their assembly proteins are emerging drivers of PH.

Iron-sulfur (Fe-S) clusters are ubiquitous cofactors essential for a diverse number of cellular processes: cytosolic regulation, mitochondrial metabolism and respiration, and nuclear DNA repair. Fe-S clusters also drive oxidation-reduction reactions in the electron transport chain, promoting oxidative phosphorylation[4,17]. The maturation of these multifaceted, bioinorganic compounds depends upon a group of cytosolic and mitochondrial proteins. These assembly proteins ensure Fe-S integrity and are critical for normal physiologic function as evidenced by several complex disease states linked to genetic and acquired deficiencies[18]. My mentor's laboratory recently established how Fe-S cluster deficiency in the pulmonary vasculature causes PH. First, they identified a hypoxia-induced increase in miR-210, which decreased the expression of iron-sulfur cluster assembly proteins 1 and 2 (ISCU1/2) and thus Fe-S-dependent mitochondrial respiration[2]. Next, they demonstrated miR-210 regulation of ISCU1/2 and Fe-S clusters induces changes in mitochondrial function *in vivo* and directs PH development[3]. Importantly, they confirmed these findings via cardiopulmonary exercise testing of a woman with homozygous ISCU1/2 mutations, revealing the first known observation of pulmonary vascular dysfunction in an ISCU-deficient patient. ISCU1/2 are two of over 30 proteins essential for Fe-S cluster biogenesis[17].

Friedreich's ataxia patients experience hypertrophic cardiomyopathy and PH due to FXN deficiency.

Frataxin (FXN) is a separate mitochondrial protein involved in the early steps of Fe-S cluster formation via its interaction with ISCU as well as other scaffolding proteins like NFS1 and ISD11[17]. A trinucleotide repeat mutation in the FXN gene causes Friedreich's ataxia. In this disease, FXN deficiency attenuates Fe-S cluster biogenesis and mitochondrial respiration and results in mitochondrial iron loading[19]. Clinically, Friedreich's ataxia results in neurologic and cardiovascular dysfunction. Mortality is driven by hypertrophic cardiomyopathy which occurs in 60-70% of patients[20,21]. In general, hypertrophic cardiomyopathy is accompanied by PH in greater than one-third of patients and is associated with increased mortality[5]. PH in Friedreich's ataxia patients has historically been considered a result of left ventricular stiffening and hypertrophy. Given that Fe-S cluster deficiency causes PH[2,3], FXN deficiency may promote PH independent of left heart disease in these patients.

In summary, PH patients experience progressive morbidity and mortality[1,14-16]. The mechanisms that drive the development of PH remain largely undefined. Recent discoveries illustrate a link between PH and Fe-S cluster deficiency. Separately, patients with Friedreich's ataxia, a disease with FXN-driven Fe-S cluster deficiency, can present with PH. I propose a direct role for FXN in PH. The identification of FXN as a lynchpin in PH will have significant impact, defining a role of Fe-S biology in human disease, improving management of Friedreich's ataxia patients, and providing alternative drug targets for PH. In this way, this proposal provides insight into the basic biology of Fe-S-driven pathology and, at the same time, has notable clinical ramifications.

Innovation

Conceptual Innovation

1) Define the dynamic control of FXN expression in endothelial cells. FXN deficiency has been described in other diseased tissue types[19,22-25]; however, this proposal will rigorously define the dynamic control of FXN expression in endothelial cells across both genetic and acquired triggers for the first time.

2) Define changes in endothelial cell function resulting from Fe-S cluster deficiency. While the effects of FXN deficiency on Fe-S biogenesis and mitochondrial respiration have been explored[4,19], how this metabolic

shift controls downstream endothelial function is unknown. This proposal is geared toward linking a FXN-dependent and Fe-S-dependent mitochondrial phenotype with the endothelial cell dysfunction observed in PH.

3) Interrogate the dependence of PH on FXN deficiency independent of left heart dysfunction. My mentor's laboratory has generated conditional FXN knockout mice to model the development of PH. If successful, these results would support the idea for the first time that FXN deficiency intrinsic to the endothelium directly drives both a clinical and molecular phenotype of PH.

Technological Innovation

1) Generation of inducible pluripotent stem cell-derived endothelial cells from Friedreich's ataxia patients in the study of PH. Inducible pluripotent stem cells (iPSCs) from Friedreich's ataxia patients were differentiated into endothelial cells to enhance the interrogation of *in vitro* cell-specific changes due to this human mutation-driven loss of FXN.

2) Generation of an inducible Cre/lox mouse model for targeted FXN deletion in the endothelium. FXN flox/flox mice[11] were crossed with VE-Cadherin CreER[10] mice to yield a novel endothelial-specific conditional FXN knockout mice for specific evaluation of FXN deficiency-driven model of PH *in vivo*.

Approach

The foundation for studies proposed in the current application includes published data[2,3,7-9] for my mentor's laboratory combined with my preliminary data.

Preliminary data

1) FXN expression is down-regulated in cultured cells under conditions of hypoxia and in mice and humans with PH. Pilot studies subjected both primary endothelial cells and a HeLa cell line to varying amounts of hypoxic exposure (0,6,24 hours) and showed decreased FXN mRNA (**Fig. 1A**) and protein expression (data not shown) under conditions of hypoxia. A similar down-regulation in FXN was demonstrated in a mouse model of PH and in humans with PH (**Fig. 1B-C**), illustrating consistency between *in vitro* and *in vivo* studies.

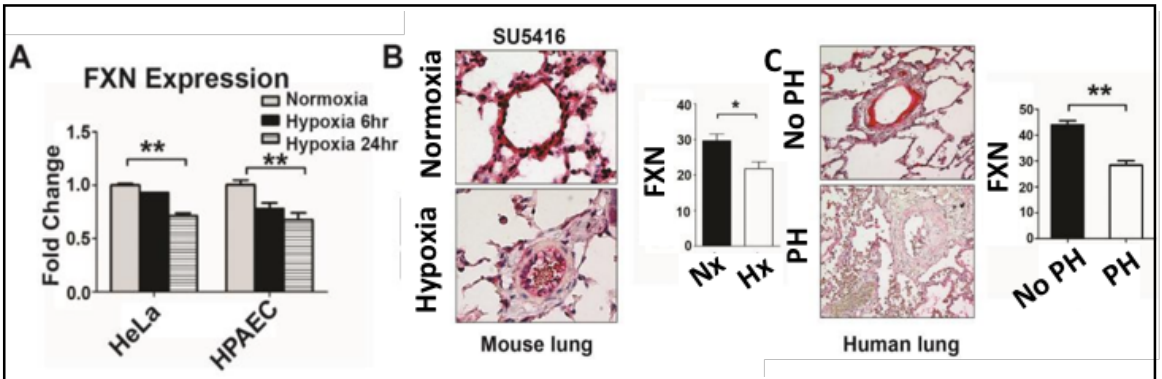
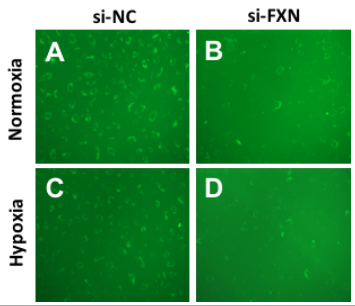


Figure 1. Both HeLa cells and primary HPAECs were cultured under hypoxia (0.2% O_2) or normoxia (~21% O_2). RT-qPCR was performed and demonstrated a decrease of FXN mRNA levels, (relative to β-actin) was dependent upon hypoxic exposure time (**A**). Lungs were collected from a mouse model of PH (SU5416+hypoxia versus SU5416+normoxia control) and from PH patients versus healthy controls. Immunostaining was performed to quantify relative FXN expression (arbitrary units) in the vessel. Both mouse (**B**) and human (**C**) arteries exhibit decreased FXN expression. Error bars reflect SEM, NS $p>0.05$* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

2) Fe-S cluster formation is down-regulated by FXN knockdown and hypoxia in cultured endothelial cells.

Figure 2. Following transduction of lentivirus GRX2 constructs, HPAECs were transfected with siRNA targeting FXN (si-FXN) or negative control (si-NC) and then exposed to normoxic and hypoxic conditions. Hypoxia and/or FXN knockdown decreased fluorescent signal (**B,D**) and thus Fe-S cluster formation. HPAECs containing the GCN4 construct were subjected to the same manipulation and demonstrate no qualitative change in fluorescence between the four groups (data not shown), establishing differences in fluorescence are due to a lack of Fe-S clusters and not an issue with reporter transduction. Quantitative analysis of FXN-dependent Fe-S cluster knockdown will be performed with n=3/group.



My mentor's laboratory has standardized a robust method for quantifying Fe-S clusters *in vitro*[3]. Lentivirus GXR2 (glutathione-dependent oxidoreductase) and GCN4 constructs fused to GFP are transduced into HPAECs. GXR2 homodimerizes only in the presence of Fe-S clusters while GCN4 spontaneously homodimerizes. Fe-S cluster quantification is based on the GXR2 fluorescence normalized to GCN4 control. Following installation of

the reporter system, HPAECs can be further manipulated before quantifying fluorescence intensity with imaging or flow cytometry. Immunoblotting of GXR2 and GCN4 is to be performed as a control (**Fig. 2**).

3) FXN deficiency alters endothelial function by increasing apoptosis and expression of endothelin-1.

To evaluate apoptosis, manipulated cells are treated with a pro-luminescent substrate cleaved by caspase-3 and -7 which yields a luminescent signal correlating to cellular apoptotic activity. Results show an increase in apoptosis in conditions of FXN knockdown and acute hypoxia (**Fig. 3A**). Separately, expression of vasomotor genes including endothelin-1 (EDN1) and nitric oxide synthase 3 (NOS3) were quantified. FXN knockdown, particularly under conditions of hypoxia, increases EDN1 mRNA and extracellular protein expression (**Fig. 3B-C**) while these same conditions decrease NOS3 levels. Further, media from HPAECs under conditions of FXN knockdown or hypoxia causes smooth muscle contraction which is mitigated by the addition of ambrisentan, an endothelin-1 antagonist (data not shown). FXN deficiency increases vasoconstrictive expression and decreases vasodilatory mediators, suggesting contribution to the vasoconstrictive phenotype observed in PH[13,26].

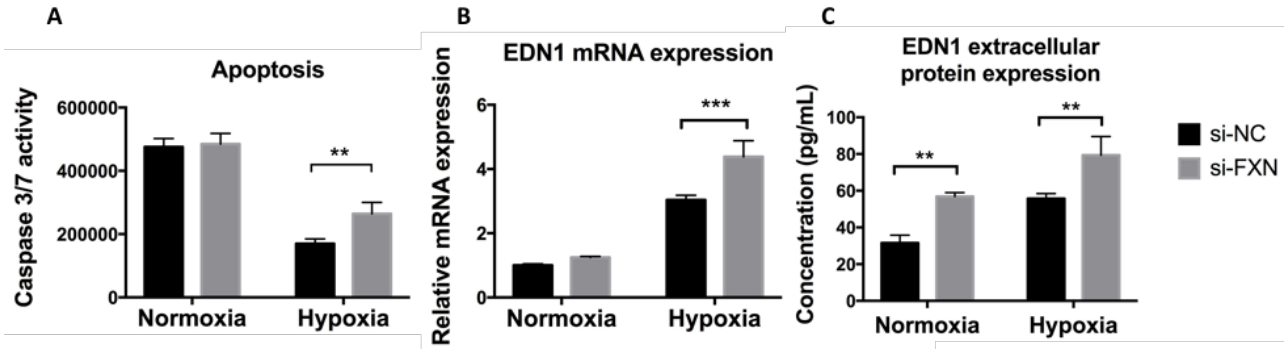


Figure 3. HPAECs were transfected with si-NC or si-FXN and then placed under normoxic and hypoxic conditions. (**A**) To measure apoptosis, luminescence was measured via spectrophotometer. Values were normalized to protein concentration. FXN knockdown under hypoxia increases apoptosis (n=6/group). Under the same conditions, RT-qPCR and ELISA revealed loss of FXN alters vasomotor gene expression; specifically, (**B**) mRNA and (**C**) extracellular protein expression of endothelin-1 (EDN1) are increased with FXN knockdown and hypoxia. (n=3/group; repeated twice). Error bars reflect SEM, NS p>0.05* p<0.05; ** p<0.01; *** p<0.001.

4) FXN knockdown promotes hemodynamic manifestations of PH *in vivo*.

The effects of FXN knockdown were evaluated in C57Bl/6 mice treated with 7C1 nanoparticles containing si-FXN that target the endothelium[3,27]. Increased RVSP, corresponds to mean pulmonary arterial pressure in humans, suggesting intrinsic FXN deficiency drives a PH phenotype. RVSP values were significantly different between groups; however the effect size was modest, perhaps reflective of heterogeneous and thus modestly efficient delivery of the siRNA (**Fig. 4B**). Thus, in this proposal, I plan to use a genetic model of FXN deficiency for a more accurate picture of PH development *in vivo*.

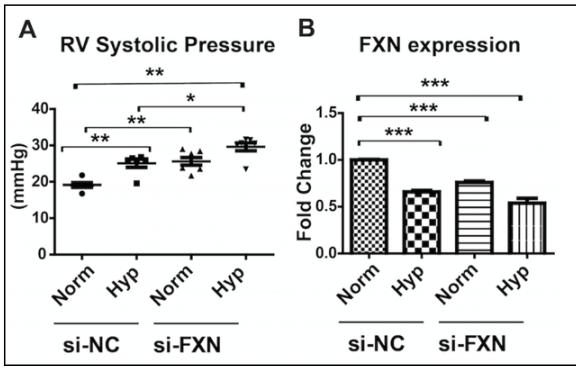


Figure 4. Si-NC or si-FXN were packaged in 7C1 nanoparticle complexes[27] and delivered to the pulmonary vascular endothelium of C57bl/6 mice (n=5/group). Mice were placed in normoxia or hypoxia for 3 weeks. (**A**) Hypoxia and/or FXN knockdown significantly increased right ventricular systolic pressure (RVSP) according to right heart catheterization. (**B**) RT-qPCR of lung endothelial cells showed decreased FXN expression in si-FXN-treated mice and mice exposed to hypoxia,* p<0.05; ** p<0.01; *** p<0.001.

In sum, preliminary data show a hypoxia-dependent decrease in FXN expression in endothelial cells (**Fig. 1A-B**) and consistent *in vivo* evidence for decreased FXN expression in PH (**Fig. 1C-D**). FXN knockdown and hypoxia decrease Fe-S cluster formation (**Fig. 2**) and at the same time, increases endothelial cell apoptosis (**Fig. 3A**) and expression of EDN1 (**Fig. 3B-C**). Pilot *in vivo* data show FXN knockdown, especially in hypoxia, increases RVSP, mirroring hemodynamic changes consistent with PH (**Fig. 4**). Data lend significant support to the feasibility of this proposal and provide the groundwork for interrogating my hypothesis in depth.

Experimental Design

Aim 1: Determine whether hypoxic down-regulation of FXN is controlled by miR-130b.

Rationale & Significance: My mentor's laboratory identified hypoxia-induced miR-210, which decreases the expression of ISCU1/2 and attenuates Fe-S-dependent mitochondrial respiration[2]. As mentioned above, preliminary data show a hypoxia-dependent decrease in FXN expression (**Fig. 1A-B.**) and FXN may be

controlled by a hypoxia-dependent miRNA, similar to ISCU1/2. Nucleotide sequence analysis using TargetScan7.1 has revealed that FXN contains a 3' untranslated region (UTR) binding motif for miR-130b[6] (**Fig. 5**). This microRNA is of particular interest because my mentor's laboratory previously demonstrated the miR-130/301 family, which includes miR-130b, mediates PH development[7-9,28]. Therefore, miR-130b may be a promising mediator of FXN down-regulation in the context of PH. Results will characterize the upstream mechanism that controls FXN loss and expand the role of the miR-130/301 family in PH.

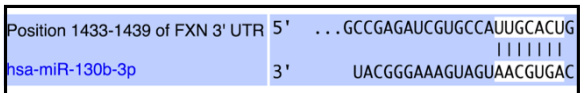


Figure 5. TargetScan7.1 predicted miR-130b target sequence in the FXN 3'UTR.[6]

Aim 1A: Demonstrate decreased FXN expression is dependent upon miR-130b. I have chosen to study primary HPAECs, which provide the most specific and consistent model for studying the endothelium of the lungs *in vitro*. To trigger a PH-like response, HPAECs will be exposed to hypoxic (<0.2% O_2) or normoxic (~21% O_2) conditions to mimic conditions either well below physiologic supply of oxygen in tissues or at normal physiologic levels, respectively. Chronic hypoxia drives disease development in Group III PH patients[1,13] and hypoxia is a well-documented trigger to study PH pathways *in vitro*[2,3,7,29]. For gain- and loss-of-function experiments, HPAECs will be transfected with miR-130b mimic or anti-miR-130b inhibitor vs. proper controls. FXN expression relative to β-actin and miR-130b expression relative to RNU48 will be quantified by RT-qPCR and/or immunoblotting.

Aim 1B: Confirm functional miR-130b binding to the 3'UTR of FXN. To show miR-130b directly binds the predicted site, part of the 3'UTR containing the target sequence will be cloned into a standardized *Renilla* luciferase reporter vector (psicheck-2)[2] to generate psicheck-FXN. Next, cells will be transfected with psicheck-FXN or a control without a miR-130b target sequence (psicheck-cont). Samples will then be co-transfected with miR-130b mimic or control. *Renilla* luciferase activity will be measured and normalized to the firefly luciferase signal, separately encoded in the psicheck-2 parent vector. Next, point mutations will be created in the predicted target site and changes in luciferase activity will be monitored to assess binding specificity of miR-130b.

Anticipated Results: I anticipate knockdown of miR-130b will mitigate the decrease in FXN expression, particularly under hypoxic conditions. Similarly, we predict miR-130b overexpression will further decrease FXN expression from baseline. Using the *Renilla* luciferase reporter system, we anticipate during hypoxia or when treated with miR-130b mimic, luciferase activity will be more robustly repressed cells with psicheck-FXN compared to psicheck-cont. Inhibition of miR-130b during hypoxia will increase luciferase activity encoded by psicheck-FXN. Mutations created in the miR-130b seed sequence will attenuate miR-130b-dependent down-regulation of psicheck-FXN, demonstrating the essential role of miR-130b in this response. Defining miR-130b controls FXN emphasizes the causative importance of the miR-130/301 in PH[7-9,28].

Potential Pitfalls & Alternative Strategies: MiR-130b alone may not sufficiently knockdown FXN given its combinatorial function as a part of the miR-130/301 family. To observe FXN down-regulation, gain- and loss-of-function experiments can be performed using technologies that target the entire miR-130/301 family. Alternatively, there may be more than one miRNA species that controls FXN or miR-130b may not down-regulate FXN under conditions of hypoxia at all. In this case, we will explore alternatively predicted miRNAs also implicated in PH, such as hypoxia-dependent miR-145[30], and other forms of posttranscriptional or epigenetic modification. Lastly, hypoxia is not the only model used to study PH and thus FXN expression can be measured in alternative models such as inflammation[31,32] (IL-6, IL-1β) or variable ECM composition[8-9] (soft versus stiff matrix).

Scientific Rigor: All *in vitro* experiments will have a minimum of n=3/group and each individual experiment will be performed at least three times to ensure consistency in results. The distributions of outcome variables will be analyzed using histograms. If data are normally distributed, they will be presented as mean ± SEM; all paired samples will be analyzed using Student's *t* test, while multiple group comparisons will be performed using ANOVA. If the data are skewed, they will be presented as median (range); all paired samples will be analyzed using Wilcoxon rank-sum tests, while multiple group comparisons will be performed using Mann-Whitney tests.

Aim 2: Establish whether FXN loss attenuates mitochondrial respiration and endothelial function.

Rationale & Significance: The role of FXN as a scaffolding protein required for Fe-S cluster biogenesis and its downstream effects on mitochondrial function and iron handling have been established[4,17,19]. However, these FXN-dependent changes and their effects on endothelial cell function have not yet been explored.

Aim 2A: Confirm FXN deficiency reduces Fe-S cluster integrity and mitochondrial function. Primary HPAECs and induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) from Friedreich's ataxia patients (**Fig. 6**) will be used for *in vitro* modeling of FXN deficiency. I will employ lentiviral transduction of FXN constructs for overexpression or siRNA transfection for knockdown in HPAECs under hypoxic and normoxic conditions to assess necessity and sufficiency of FXN expression. iPSC-ECs will be used simultaneously.

First, Fe-S cluster formation will also be quantified in HPAECs and iPSC-ECs from Friedreich's ataxia patients vs. controls using the laboratory's standardized fluorescent sensor.

Both cell types will then be assessed for measures of mitochondrial metabolic dysfunction previously published by my mentor[2,3,7-9]:

- Complex 1 activity by spectrophotometry[2]
- Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) by Seahorse XF Analyzer[2]
- Reactive oxygen species (ROS) production with DCFDA stain[33]
- Mitochondrial iron concentration by spectrophotometry[34]

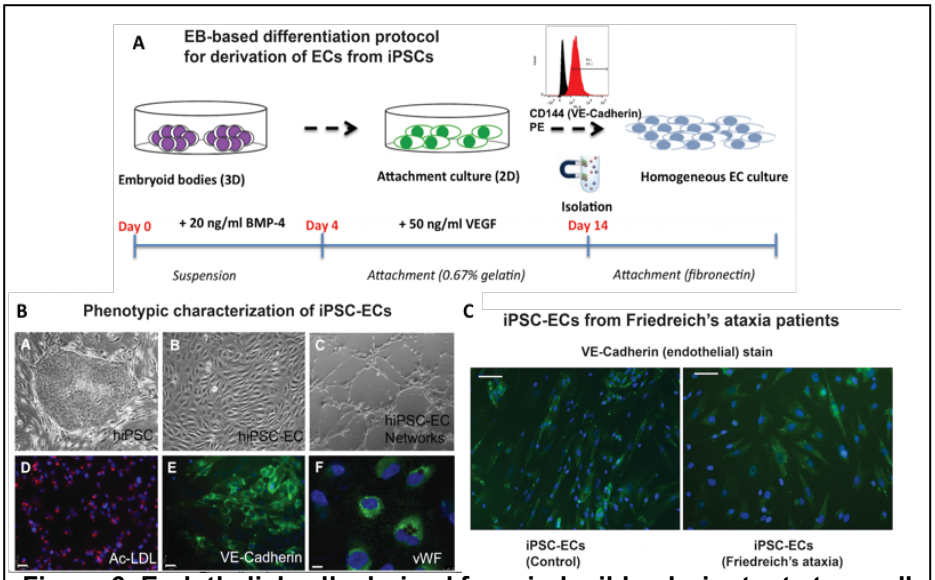


Figure 6. Endothelial cells derived from inducible pluripotent stem cells of patients with Friedreich's ataxia. Lymphocytes from Friedreich's ataxia patients were infected with Sendai virus expressing stem cell factors SOX2, OCT4, KLF4 and c-MYC and grown for ~4 weeks. Candidate iPSC clones were expanded. (**A**) The Qyang lab, a long-time collaborator with my mentor, has optimized an embryoid body (EB) differentiation approach to induce iPSC differentiation toward an EC lineage[39]. (**B**) Isolated CD144+ iPSC-ECs (a) displayed typical cobblestone morphology (b) and formed networks when plated on Matrigel (c). Immunofluorescence revealed that iPSC-ECs took up acetylated-LDL (d) and expressed VE-Cadherin (e) and vWF (f). (**C**) iPSC-ECs (VE-Cadherin immunofluorescent stain, green) from control (left) versus Friedreich's ataxia patients (right) are ready for use.

Aim 2B: Explore endothelial phenotypic changes attributed to FXN loss. Endothelial cells exhibit multiple changes in the context of PH[35-38], some of which remain ill-defined. I will screen several potential phenotypes using assays previously used by the laboratory[2,3,7-9]:

- Apoptosis by caspase-3/7 activity measured via spectrophotometry[2]
- Proliferation by incorporation of bromodeoxyuridine measured via spectrophotometry[7]
- Migration after scratching cell monolayers[2]
- Vasomotor gene expression (EDN1, NOS3, prostaglandinI2 synthase) by RT-qPCR, immunoblot, and ELISA
- Angiogenesis by RT-qPCR and immunoblot of VEGFA expression and by sprouting and tube formation[40]

Anticipated Results: Corresponding with evidence of Fe-S cluster deficiency in HPAECs under conditions of hypoxia and/or FXN knockdown, I anticipate similar loss of Fe-S clusters in iPSC-ECs from Friedreich's ataxia patients. I predict attenuated Complex 1 activity in conditions of FXN knockdown and/or hypoxia. Similarly, cellular oxidative phosphorylation by measures of OCR and ECAR should be decreased while ROS should be increased under the same conditions. I predict endothelial cell proliferation and migration will be attenuated by FXN knockdown and hypoxia. Functional assays of angiogenesis will also be decreased, consistent with disordered angiogenesis in the pulmonary vasculature observed in PH[35]. In parallel, I predict that iPSC-ECs will exhibit phenotypic changes similar to HPAECs after FXN knockdown and hypoxia. Simultaneous gain-of-function experiments should show that overexpression of FXN rescues mitochondrial and whole-cell function. If so, such findings in aggregate would confirm the direct functional link between FXN-dependent mitochondrial changes and endothelial-specific cellular phenotypes after both genetic and hypoxic triggers.

Potential Pitfalls & Alternative Strategies: First, mitochondrial number is relatively low in endothelial cells compared to other cell types[37], potentially making measurement of mitochondrial-specific changes difficult. For aid, the Center for Metabolism and Mitochondrial Medicine here at the University of Pittsburgh provides alternative platform technologies to evaluate bioenergetics of the mitochondria: oxidative phosphorylation, ROS production, electron transport complex activity and expression, apoptotic signaling, and mtDNA/nuclear DNA ratios. Separately, endothelial phenotypes due to short-term hypoxia or FXN knockdown may be reflective of acute, or initial, PH changes while phenotypes in iPSC-ECs from Friedreich's ataxia patients may reflect chronic effects of FXN loss. For instance, there is literature that supports both increased and decreased endothelial cell apoptosis in PH with the former being in acute and the latter being in chronic stages of disease[37,41,42]. Having different induction models, hypoxia and genetic mutation, allows for evaluation of time-dependent responses; both will be important in understanding disease.

Scientific Rigor: Experimental design and statistical analyses are the same as **Aim 1**.

Aim 3: Confirm FXN loss and resulting mitochondrial dysfunction predisposes to PH *in vivo*.

Rationale & Significance: Multiple genetically modified mouse models can be used to study FXN[10]. A cardiac-specific knockout may result in left-sided heart dysfunction that could manifest PH pressures without directly affecting the pulmonary vasculature[11]; instead a conditional knockout is required to isolate the intrinsic effect of FXN deficiency in the endothelium. I will use my mentor's already available endothelial specific conditional knockout mouse model. FXN flox/flox mice[11] were crossed with VE-Cadherin Cre-ER mice[10] to create tamoxifen-induced FXN deletion in the endothelium. To elicit PH, mice will be injected weekly with the vascular endothelial growth factor (VEGF) Flk-1/KDR receptor inhibitor SU5416 in combination with 28 days of continuous exposure to hypoxia versus normoxia[42] (**Fig. 7**).

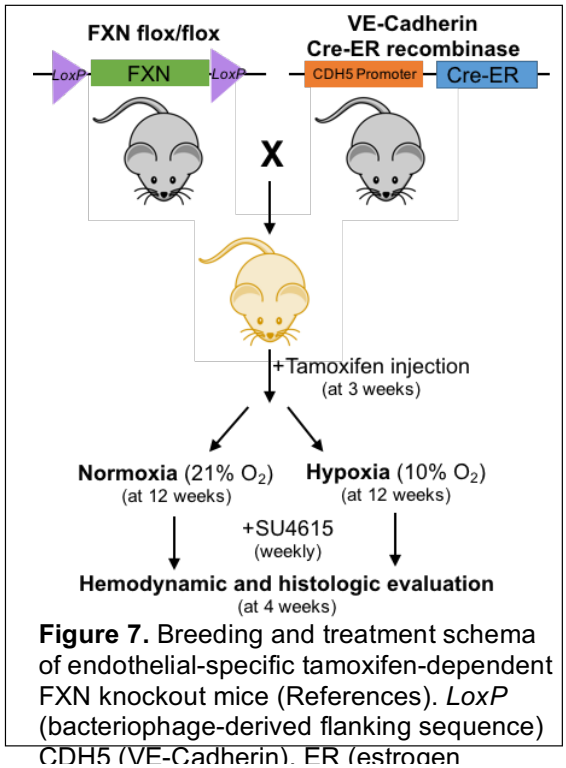


Figure 7. Breeding and treatment schema of endothelial-specific tamoxifen-dependent FXN knockout mice (References). *LoxP* (bacteriophage-derived flanking sequence) CDH5 (VE-Cadherin), ER (estrogen

Mice will then be assessed for mitochondrial, histologic, and hemodynamic measures, as previously published by my mentor[2,3,7-9]:

Mitochondrial indices of PH

- Complex 1 activity in whole lung by spectrophotometry[2]
- OCR and ECAR in endothelial cells isolated via CD31-conjugate beads[3] by Seahorse XF Analyzer[2]
- ROS flux in endothelial cells by flow cytometry[33]

Histologic indices of PH

- *In situ* hybridization and immunofluorescence imaging of FXN in the endothelium of pulmonary arterioles using paraffin-embedded lung samples by confocal microscopy (Nikon A1)
- Vessel remodeling by a ratio of wall thickness to diameter[3]

Hemodynamic indices of PH

- Transthoracic echocardiography by digital ultrasound[43]
- RVSP by right heart catheterization[3,7,44]

Anticipated Results: Whole lung and isolated endothelial cells will exhibit attenuation of Fe-S cluster-dependent mitochondrial function as measured by Complex 1 activity among others. Control experiments measuring FXN mRNA and protein levels will confirm loss of FXN expression in the conditional knockout model. Histologically, the same down-regulation of FXN will be visualized in highly remodeled pulmonary arterioles in lungs of mice exposed to hypoxia. During cardiopulmonary testing, I anticipate an increase in RSVP in mice treated with SU4615+hypoxia, but anticipate a more robust hemodynamic change in conditional FXN knockout mice. Alterations in these indices suggest a loss of FXN in endothelial cells can drive PH independent of other causes.

Potential Pitfalls & Alternative Strategies: No single rodent model recapitulates all aspects of human PH[45]. Thus, an alternative, as my mentor has published in rats and mice[2,3,7-9], could be employed if marginal hemodynamic or histologic changes are observed with the proposed model. Further, the mouse model may not manifest a PH phenotype because endothelial FXN deficiency alone may not drive this disease phenotype. The laboratory has the resources to pursue studies in alternative pulmonary vascular cell types (e.g. smooth muscle cells and fibroblasts) and already has mice to breed smooth muscle cell-specific conditional FXN KO mice[10].

Scientific Rigor and Gender Bias: Mouse numbers will be chosen to achieve 0.80 power for detecting at least a 25% difference among means with a standard deviation of 20% and to balance female and male representation to eliminate sex bias. Statistical analyses are the same as **Aim 1.**

Conclusions: Completion of these aims will rigorously define a unique mechanism of PH development dependent upon FXN. Results could provide an important target for therapeutic intervention and offer a way to identify Friedreich's ataxia patients at risk for PH. This comprehensive approach will ensure I master a breadth of contemporary techniques that build upon my previous training in cardiovascular biology. Studies will take place alongside my mentor's ongoing clinical trial (NCT02594917), which aims to demonstrate pulmonary vascular dysfunction in Friedreich's ataxia patients independent of hypertrophic cardiomyopathy via cardiopulmonary exercise testing. While my proposed project does not rely on the results of this trial, I will greatly benefit from a training environment in which *in vitro* observations are defined in a clinical context, preparing me for my future to bridge the gap between basic molecular discovery and clinical cardiovascular medicine.