Porter Physiology Development Fellowship in Part Sample Application

This is a sample of a previous successful application. The information included within this sample is being shared with the permission of the applicant. In an effort to maintain the applicant's privacy, the entire application will not be shared, only the parts listed below.

Included within this sample application are the following:

- Statement regarding specific interest in studying the physiological sciences
- Dissertation Research Proposal
- Training Potential

Please note: This application was submitted in 2015 and is an example of a successful application at that time. Please ensure that your application is up to date, meets the current criteria, and is aligned with the best way to communicate your research. We strongly encourage you to read the requirements carefully to avoid an illegible application due to a missing part.

c. Statement regarding my specific interest in studying the physiological sciences and career aspirations

Upon completion of my Ph.D., my primary career goal is to become an independent and productive research investigator with a focus on translational research. My long-term research interests are to understand the physiology behind human disease at the level of the cell, the organ and the whole organism. In pursuing these interests, it is my intent to dedicate myself to creating opportunities for students from diverse backgrounds to gain access to the resources, mentorship and opportunities that are together required to launch a career in science.

My interest in physiological sciences was initiated through multiple research rotations at [redacted]. As a doctoral student in Cellular and Molecular Physiology I have received direct training and guidance from two faculty members who are experts in electrophysiology. This has opened a new avenue for me to lead my team in our study of renal channels and has advanced my dissertation research to new levels. In my career I aim to pursue research in renal and cardiovascular physiology utilizing these technical skills and knowledge base. My interest in continuing studies in this field and my ability to conduct physiological research have also been strengthened by my programs graduate coursework and teaching opportunities in physiology. The department's thorough and nurturing training program has exposed me to a variety of focuses and original approaches in the field and has expanded my network by enabling me to personally host physiologists from different universities.

Moreover, the research career that I am pursuing will be one dedicated to mentoring, leadership and service. My participation in a variety of research programs for minority science researchers has been formative in my academic success and I will continue to support these programs throughout my career. The opportunities that are afforded through the Porter Physiology Development Fellowship would be enormously valuable in helping me to prepare myself for this career. If I am fortunate enough to receive this Fellowship I will dedicate myself to ensuring that I make the best possible use of every opportunity that is offered to me and will do my best to recreate them for my future trainees.

Specific Aims The polycystin proteins generate and interpret signals that regulate renal tubular epithelial cell growth and morphogenesis. New studies indicate that cells that lack polycystin-1 expression exhibit profound perturbations in their energy metabolism. Even in the presence of oxygen, these cells substantially reduce their reliance on mitochondrial oxidative phosphorylation and instead produce the bulk of their energy through glycolysis. This fascinating behavior indicates that the polycystin proteins participate, directly or indirectly, in the signaling pathways that govern cellular energy generation, although the mechanisms responsible for this participation have yet to be elucidated. We have recently identified that the polycystin complex is regulated by components of the cellular oxygen-sensing machinery. We **hypothesize** that the polycystin complex channel is oxygen sensitive and its activity could directly govern mitochondrial energy production by contributing to the calcium-dependent control of pyruvate dehydrogenase (PDH) a key regulator of oxidative phosphorylation (OXPHOS). In order to test this hypothesis we aim to:

1. Investigate the role of oxygen in polycystin-dependent calcium signaling. We find that polycystin-1 interacts with and is modified by the cellular oxygen sensor EGLN3 and that the ER polycystin channel-complex activity is regulated by oxygen levels. In this aim we will investigate the potential role of oxygen in polycystin-dependent cellular and mitochondrial calcium signaling, with the intent of apply our findings to ongoing studies on Ca^{2+} sensitive regulators of oxidative metabolism. We will explore whether polycystins modulate agonist induced intracellular calcium elevation, alter basal mitochondrial Ca^{2+} concentration or modulate mitochondrial calcium uptake and determine if the modulation is oxygen dependent. In addition, we will elucidate whether PC2 channel activity is required for the polycystin effects on energy production.

2. Determine how the polycystins alter mitochondrial function. We find that polycystins play important roles in regulating cellular energy production. Our preliminary data suggest that the polycystins regulate the activity of pyruvate dehydrogenase, an enzyme whose activity plays a critical role in determining the level of mitochondrial oxygen consumption. In this aim we will investigate how polycystins affect function and levels of mitochondrial enzymes essential for oxidative metabolism and mitochondrial membrane potential. We also assess whether any such potential regulation works through calcium or oxygen dependent mechanisms.

Background

Autosomal dominant polycystic kidney disease (ADPKD) is a hereditary disease characterized by massive enlargement of the kidney and replacement of the normal kidney parenchyma with fluid-filled cysts [1]. Mutations in the *Pkd1* and *Pkd2* genes encoding polycystin-1 (PC1) and polycystin-2 (PC2) cause ADPKD [2]. Intracellular calcium signaling is important in kidney development, and a defect in this signaling is likely the basis of cyst formation in polycystic kidney disease [3]. Polycystin-1 and 2 interact to form a functional channel complex found in the plasma membrane and endoplasmic reticulum (ER) that when active enables calcium flux into the cell from the extracellular space or calcium release from the ER.

ER calcium can flow directly from the ER to the mitochondria at points of close contact termed Mitochondria Associated ER membrane (MAMs)[4]. Mitochondrial Ca^{2+} enhances the activities of several key enzymes for oxidative metabolism including pyruvate dehydrogenase (PDH), TCA enzymes, and the F0F1 ATPase [5]. The variety of roles for Ca^{2+} in cellular metabolism highlights the importance of maintaining regulated cytosolic and mitochondrial calcium signaling pathways.

A recent study has implicated a role for polycystins in cellular energy metabolism demonstrating that cells homozygous for ADPKD-causing mutations have considerably perturbed energy production, with unexpectedly high levels of aerobic glycolysis and low levels of oxidative metabolism [6]. The mechanism causing this metabolic perturbation has not been fully described, but recently our group has identified a novel pathway involving the cellular oxygen-sensing machinery that we believe is responsible for the excessive glycolytic activity seen in ADPKD cells (see Preliminary Studies).

Purpose

Recent data from our lab showed that the polycystins interact with cellular oxygen-sensing machinery, specifically the oxygen sensor EGLN3, and affect mitochondrial function. Absence of polycystin-1 in these roles could lead to progression of ADPKD. The nature of the link between the polycystin channel's oxygen sensitive modulation by EGLN3 and the reduced activation of PDH seen in the absence of PC1 remains unknown and will be a major focus of this proposal. We will address this in our first aim by investigating whether and how polycystins modulate cellular and mitochondrial calcium in response to oxygen levels and in our second aim by determining the effect of polycystins activity on mitochondrial enzyme function and membrane potential.

Significance

The proposed studies serve to explore the molecular basis of the ADPKD by identifying a role for polycystins in the modulation of Ca^{2+} flux from the ER into the mitochondria and establishing a direct connection between polycystin channel activity and energy metabolism. Our efforts will also provide significant basic knowledge on intracellular calcium signaling in renal cells and perhaps suggest new therapeutic targets for ADPKD.

Preliminary Data

These data, obtained by lab members and collaborators, form the foundation for aspects of the work proposed in the present proposal.

1. **Polycystin-1 interacts with and is modified by EGLN3**

Polycystin-1 interacts with the oxygen sensor and prolyl hydroxylase EGLN3 through its C-term, which is demonstrated by co-immunoprecipitation followed by western blotting **(Fig. 1)** and this interaction does not depend on EGLN3 catalytic proyl hydroxylase activity. We find that EGLN3 acts on polycystin-1 as a substrate, specifically that polycystin-1 C-term is hydroxylated on proline residues and that the EGLN3 inhibitor DMOG reduces this proline hydroxylation (data not shown).

2. **Oxygen levels regulate polycystin complex channel activity**

To test whether hydroxylation of polycystin-1 by EGLN3 affects polycystin-2 channel activity, we performed single channel recordings using bilayer membranes into which were fused ER microsomes prepared from LLC-PK_1 cells over-expressing polycystin-1 and polycystin-2. Interestingly, inhibition of EGLN3 function by low oxygen treatments altered the polycystin channel properties **(Fig. 2)**. This work suggests that the polycystin complex has the potential to regulate mitochondrial function in response to changes in O_2 levels through the modulation of its channel properties by the oxygen sensor EGLN3.

3. **Lack of Pkd1 expression affects mitochondrial function**

To understand how the absence of functional PC1 causes metabolic perturbations, we measured oxygen consumption rate (OCR). Utilizing previously characterized $Pkd1^{-/-}$ and $Pkd1^{flox/-}$ mouse renal proximal tubule cell lines that completely lack or are heterozygous for polycystin-1 expression, respectively, we found that $Pkd1^{-/-}$ cells have significantly reduced OCR compared to

Pkd1$^{flox/-}$ cells **(Fig. 3)** in line with the decreased OXPHOS by these cells. Lastly, we find that polycystin-1's regulation of OXPHOS involves the activities of both EGLN3 and Pyruvate dehydrogenase (PDH), a calcium activated mitochondrial enzyme that links glycolysis to the TCA cycle (data not shown). Therefore, we **hypothesize** that the polycystin complex channel activity could directly govern mitochondrial energy production by contributing to the calcium-dependent control of PDH **(Fig. 4)**. Specifically we propose that ER to mitochondrion Ca^{2+} leak, facilitated by the polycystin complex, activates PDH phosphatase and thus increases the activity of PDH and OXPHOS. We expect that decreased oxygen-dependent prolyl hydroxylation of the polycystin complex decreases ER to mitochondrion calcium leak, resulting in increased inactive pPDH and decreased OXPHOS.

In summary, our group finds that polycystins interact with the cellular oxygen sensing machinery, that O_2 levels modulate the polycystins channel activity and most relevant to this proposal, we find that the polycystin complex serves as an O_2-sensitive regulator of the level of mitochondrial oxidative metabolism.

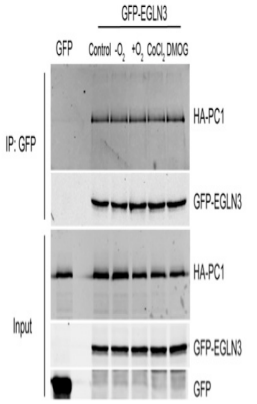

Fig.1 Co-IP of GFP-EGLN3 and PC1 in HEK cells. Cells were incubated in hypoxia (-O_2) or hyperoxia (+O_2) for 2hrs or with 1mM DMOG or 200 µM $CoCl_2$ for 24 hrs.

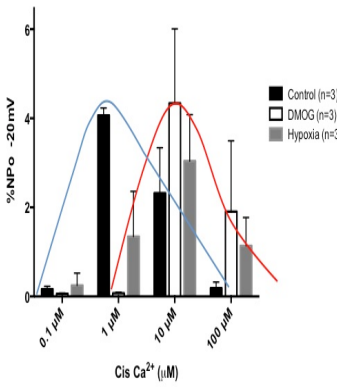

Fig. 2 PC1 proline hydroxylation regulates polycystin complex channel activity. Single channel recording on microsomes from LLC-PK cells over-expressing Pkd1 and Pkd2 incubated under control conditions or in hypoxia for 2 hrs or with 1mM DMOG 24 hrs; the blue line outlines the channel activity in control conditions, while the red line outlines the shift in calcium sensitivity induced by DMOG and hypoxia treatments. The oxygen effect on channel activity is statistically significant, P=0.0018 as assessed by 2way ANOVA.

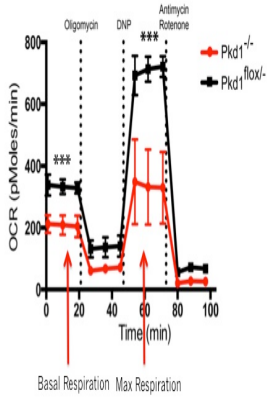

Fig. 3 Lack of Pkd1 expression reduces mitochondrial oxygen consumption rate. Oxygen consumption rate (OCR; in pMoles/min) analysis in Pkd1$^{-/-}$ and Pkd1$^{-/+}$ proximal tubule cells.

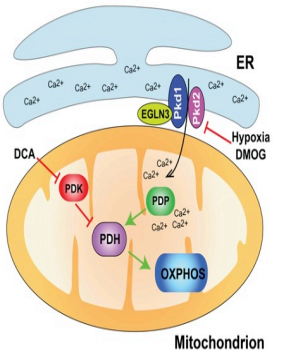

Fig. 4 Model of the regulation of PDH and OXPHOS by the polycystin complex. ER to mitochondrion Ca^{2+} leak, facilitated by the polycystin complex, activates PDH phosphatase and thus increases the activity of PDH and OXPHOS. Decreased oxygen-dependent prolyl hydroxylation of the polycystin complex decreases ER to mitochondrion calcium leak, resulting in increased pPDH and decreased OXPHOS. PDK= PDH kinase, PDP = PDH phosphatase.

Figures 1-4

Research Design and Methods

Aim 1: Investigate the role of oxygen in polycystin-dependent calcium signaling.

1.1: Do the polycystins modulate agonist induced intracellular calcium transients and is the modulation oxygen dependent? We want to test the hypothesis that the polycystins can modulate cytosolic and nuclear calcium signaling in response to oxygen levels. We ask whether expression of the polycystins leads to increased agonist induced cellular calcium signaling and if hypoxic conditions can induce or modify these calcium transients. To answer these questions we will perform Fura-2 ratiometric calcium imaging to measure $[Ca+2]_I$ levels in PC1 and 2-expressing LLC-PK1 cells mounted in a perfusion chamber. Cells will be subjected to continuous flow with solutions that 1) contain agonists such as ATP or vasopressin to induce a Ca^{2+} signal or 2) that have been bubbled with gases to render them either hyperoxic or hypoxic, or 3) perfusate containing the well established PHD-inhibitors $CoCl_2$ and DMOG to inhibit

EGLN3 thus mimicking hypoxic conditions. To confirm that the effects observed are attributable to the polycystins' channel activity we will employ a LLC-PK PC1 cell line expressing a channel dead mutant of PC2 (D511V) and expect observed Ca^{2+} transients to be abolished. **1.2: Do the polycystins alter basal mitochondrial Ca^{2+} concentration?** The concentration of calcium in the mitochondria regulates mitochondrial function. We hypothesize that in basal conditions polycystin-1 modulates Ca^{2+} uptake into the mitochondria, enabling basal activation of PDH. To determine if polycystins have an effect on mitochondrial calcium concentration we will perform Rhod-2/AM imaging to measure the concentration of free mitochondrial matrix Ca^{2+} in cells that do or do not express Pkd1 or Pkd2 using a well-established protocol [42]. If these experiments show that polycystin null cells have higher basal levels or that there is no difference in mitochondrial calcium concentrations, then we will conclude that polycystin-1's enhancement of PDH activity at baseline either does not act through mitochondrial calcium or that it acts by altering protein levels of regulators of PDH activity, specifically PDP and PDK, a possibility that will be explored in aim 2.1. **1.3: Does polycystin-1 modulate mitochondrial calcium uptake and is it oxygen dependent?** We want to test the hypothesis that polycystins regulate shuttling of calcium into the mitochondria directly from the ER. We have found that subcellular polycystin-1 can localize near mitochondria and that without PC1 the mitochondrial enzyme PDH is less active. These findings suggest a dysregulation of PDH phosphatase (PDP), a Ca^{2+} activated enzyme essential to PDH activation, and further suggest that polycystin-1 can alter mitochondrial Ca^{2+} levels. To assess a role for PC1 in modulating mitochondrial Ca^{2+} and determine if it is oxygen-dependent we will measure mitochondrial Ca^{2+} uptake through live cell confocal imaging of $Pkd1^{flox/-}$ and $Pkd1^{-/-}$ cells that have been transfected with the genetically encoded calcium indicator MTS-RCaMP that is targeted to the inner mitochondrial membrane facing the matrix. Elevating cytosolic Ca^{2+} levels, for example by activating cell surface purinergic receptors with extracellular ATP, induces mitochondrial Ca^{2+} uptake that stimulates PDH phosphatase (PDP), resulting in increased mitochondrial PDH activity [39]. To determine if the observed Ca^{2+} transients are oxygen sensitive we will treat cells with DMOG. **1.4: Is the channel activity of PC2 required for polycystins' effects on energy production?** To elucidate the role of polycystin-2 and it's channel activity we will repeat the experiments described in **1.3** using well-characterized $Pkd2^{flox/-}$ and $Pkd2^{-/-}$ cells that do or do not express PC2, respectively. If PC2 participates in mediating Ca^{2+} shuttling from the ER to mitochondria (and in regulating pPDH levels) then we expect that extracellular ATP-induced mitochondrial Ca^{2+} (and PDH activation) will be blunted in the $Pkd2^{-/-}$ cells.

<u>Aim 2: Determine how the polycystins alter mitochondrial function.</u>

We find that polycystins play critical roles in regulating energy production. Our preliminary data suggest that the polycystins regulate the activity of pyruvate dehydrogenase, an enzyme whose activity plays a critical role in determining the level of mitochondrial oxygen consumption. In this aim we will investigate how polycystins affect function of mitochondrial enzymes and mitochondrial membrane potential. We also ask if regulation of potential works through a Ca^{2+} or O_2 dependent mechanism and will answer these questions by performing the following experiments.

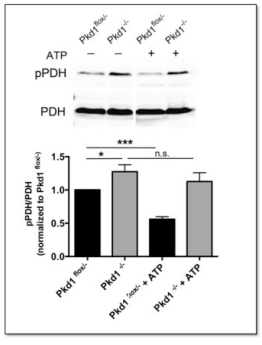

Fig. 5 Western blot analysis of pPDH/PDH ratio normalized to $Pkd1^{flox/-}$ treated with .1mM ATP for 10 min. Data from three independent experiments are shown as mean +- s.e.m; *<0.05, *** P<0.001

2.1: **Do polycystins influence activation or protein levels of mitochondrial enzymes?** To answer this question we utilize biochemical and functional studies. We will use western blotting to determine whether the presence of polycystins increases PDH activation and/or protein levels.

We have already used western blotting to determine that $Pkd1^{-/-}$ cells have less of the active form of PDH and that purinergic receptor activation (and subsequent Ca^{2+} signaling) leads to a reduction in the size of the pPDH pool (pPDH normalized to total PDH) in $Pkd^{flox/-}$ cells but not in $Pkd1^{-/-}$ cells **(Fig. 5)**. These findings are consistent with our working model **(Fig. 4).** Next, we will determine if induction of PDH activity in polycystin-expressing cells is associated with downregulation of pyruvate dehydrogenase kinase (PDK), which inhibits PDH via phosphorylation, and/or with elevated expression of calcium-activated pyruvate dehydrogenase phosphatase 2 (PDP2), which stimulates PDH via dephosphorylation. PDH activity will be confirmed by a commercial assay. Activation of PDH will be measured at baseline, or after treatment with either ATP to increase mitochondrial Ca^{2+}, DMOG and $CoCl_2$ to mimic hypoxia or with DCA to inhibit PDH kinase. By elucidating exactly how polycystins regulate PDH activity we will define how aberrant signaling by the polycystin complex can have broad repercussions on cellular metabolism. **2.2: Do polycystins influence mitochondrial membrane potential?** We hypothesize that the membrane potential, which is vital for energy production, will be sensitive to oxygen levels in polycystin-expressing cells. We will use live cell imaging through confocal microscopy to measure mitochondrial membrane potential using the fluorescent probe TMRM (tetramethylrhodamine methyl ester) in intact $Pkd1^{flox/-}$ vs $Pkd1^{-/-}$ cells. These studies will provide quantitative data on mitochondrial polarization or depolarization at a single-cell resolution by measuring the total fluorescence signal from a region of interest that encompassed the cell. Cells will be exposed to $CoCl_2$ and DMOG and increases or decreases in fluorescence will be measured, representing hyperpolarization or depolarization of the mitochondrial membrane potential, respectively. In conclusion, in Aim 2 we will elucidate how the polycystins regulate metabolic enzymes and the proton gradient harvested for ATP synthesis. Together with the proposed calcium imaging studies in Aim 1 we will determine if the polycystin channel's oxygen sensitive modulation by EGLN3 can directly affect PDH activation and cause negative repercussions on mitochondrial function when PC1 is absent. These analyses will not only explore a novel role for the polycystin complex, but will also enable us to decipher the mechanism that, when disturbed, is a major factor in determining the dramatic reduction in oxidative phosphorylation that characterizes polycystin-1 null cells.

Expected Outcomes & Limitations

Aim 1: All of the tools and techniques required to perform these experiments are in routine use in the laboratory. We anticipate that if the channel activity of the surface and ER polycystin complex is modulated by O_2 through the activity of EGLN3, then hypoxic treatments should result in variations in the level of intracellular calcium in polycystin expressing cells. We propose that polycystins are important transducers that will communicate information about oxygen levels to metabolic pathways through Ca^{2+} signaling. If, however, our work does not confirm this, alternatively we will explore the effects of metabolic products on polycystin-dependent Ca^{2+} transients. We expect $Pkd1^{flox/-}$ to have higher basal concentrations of mitochondrial matrix Ca^{2+} compared to $Pkd1^{-/-}$ as this would explain the increased activation of PDH in the $Pkd1^{flox/-}$ cells. We expect that hypoxia will enhance ATP-induced mitochondrial Ca^{2+} uptake in the $Pkd1^{flox/-}$ cells without altering uptake in the $Pkd1^{-/-}$ cells. **Aim 2:** We expect that $Pkd1^{flox/-}$ will have higher protein levels of PDP or lower protein levels of PDK. Either result will shed light on the observed increase in PDH activity at baseline and in the presence of ATP. We believe DMOG and $CoCl_2$ treatment will reduce polycystin-dependent basal mitochondrial Ca^{2+} and thus expect treatment to also reduce basal activation of PDH. Lastly, we expect that polycystin expressing cells can alter mitochondrial membrane potential in response to O_2 levels.

References

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b. Training Potential

The studies outlined in this application will provide a wealth of opportunities to acquire training in the design and execution of a variety of experimental approaches. The overall goal for my graduate training is to gain a set of cellular and molecular skills that will complement my previous research training in order to study renal pathophysiology. Thus, during the fellowship I will: 1) learn how to design and conduct experiments using biochemical techniques and microscopy for the study of renal pathophysiology, 2) learn how to use live cell imaging and electrophysiological techniques to understand the physiological role of renal channels of interest, 3) learn how to use the techniques of cell culture to study mechanisms of renal pathophysiology. As a whole, this research fellowship will enable me to pursue novel research ideas and apply unique physiological approaches to the study of Polycystic Kidney Disease.

Another important goal for my doctoral training is to enhance my career development. Thus, I will improve my skills in the areas of: 1) written and oral communication, 2) critical review of manuscripts and grants, and 3) research ethics. The doctoral program in Physiology at [redacted] has given me the opportunity to present my research findings to leading physiologists and specialists in my field during departmental and interdepartmental Polycystic Kidney Disease seminars. During my graduate training I have written and reviewed manuscripts and grants, resulting in authorship in a published book chapter and in primary literature in press, and in a NSF Graduate Research Fellowship. These early experiences with academic writing and workshops hosted by the Graduate Writing Center provide me with the skills needed for preparing my dissertation research work for publication. Moreover, I have received training on ethical conduct in scientific research, which has subsequently allowed me to lead formal discussions on research ethics for new graduate students.

Towards the later part of my doctoral training, I will also focus on training in the area of: 1) the postdoctoral application process, 2) teaching, and 3) job experience and networking. The APS provides numerous resources for preparing and identifying postdoctoral positions. I have significant teaching experience from instructing physiology courses at the college and graduate level, and teaching physiology to elementary school students during the annual Physiology Understanding Week that myself and other APS members recently implemented at [redacted]. During my research training I will participate in comprehensive teaching workshops and earn the [redacted] Graduate Teaching Certificate in order to become an effective faculty lecturer. To acquire job experience, I have participated in an industry research opportunity and this semester I will build an international network of collaborators during brief research collaborations at [redacted]

The goals outlined here align with those of my sponsor. Therefore, the training provided for this fellowship will strengthen my ability to successfully transition into an independent and productive research investigator.