AWARD NUMBER: W81XWH-17-1-0158

TITLE: Pharmacology of Polycystic Kidney Disease Proteins

PRINCIPAL INVESTIGATOR: Erhu Cao

CONTRACTING ORGANIZATION: University of Utah
Salt Lake City UT 84112

REPORT DATE: MARCH 2019

TYPE OF REPORT: Final Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MARCH 2019	Final	1 Jun 2017 – 30 Nov 2018

4. TITLE AND SUBTITLE

5a. CONTRACT NUMBER
W81XWH-17-1-0158

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Erhu Cao

E-Mail: Erhu.Cao@biochem.utah.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Utah
201 S President Circle Rm 408
Salt Lake City, UT 84112-9023

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

The primary goal of this proposal is to identify regulators, in particular activators, of PKD1 & PKD2 proteins for future pharmacological dissection of their structures & functions that will shed light on pathogenesis of ADPKD. Aim 1: Identify small molecule activators of PKD proteins by liposome flux assay. Aim 2: Identify cone snail peptide regulators of PKD proteins. Aim 3: Generate conformation-sensitive, functional antibodies for PKD proteins.

15. SUBJECT TERMS
Pharmacology, Polycystic Kidney Disease,

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	9	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. Introduction:
The major aim of this project is to develop pharmacological tools (e.g., small chemical compounds, peptide toxins from venomous animals, and conformation-sensitive, functional antibodies) for dissecting structures and physiology of polycystic kidney disease proteins. Such pharmacological studies of PKD proteins will also help establish therapeutic principles for treating autosomal dominant polycystic kidney disease (ADPKD).

2. Keywords:
PKD1, PKD2, ADPKD, Pharmacology, Receptors, animal venoms, and Ion channels.

3. Accomplishments:

	Proposed Timeline	**Completion Date**
Specific Aim 1: Identify small molecule activators of PKD proteins by liposome flux assay		
Subtask 1: Purify and reconstitute PKD2 and PKD2 mutants into liposomes of optimal lipid composition	Months 1-6	We have purified and reconstitute the PKD2 channel and a gain-of-function mutant into liposomes. These goals have been accomplished.
Subtask 2: Purify and reconstitute PKD1 and the PKD1/PKD2 complex into liposomes of optimal lipid composition	Month 1-6	The full-length human PKD1 turned out to be extremely challenging for purification than we initially expected. We have overcome this obstacle by focusing on purifying several human PKD1 variants that retain major functional components of the receptor. We have also cloned >20 PKD1 homologs and orthologs from various species for identification of the best candidate for reconstitution.
Milestone(s) Achieved: Reconstitution of PKD proteins into liposomes	Month 6	We have accomplished reconstitution of the PKD2 channel and a gain-of-function mutant into nanodiscs and liposomes.
Subtask 3: Optimize liposome flux assay for PKD2	Months 7-12	We have successfully purified a gain-of-function PKD2 mutant and are exploiting this mutant to establish a robust liposome flux assay for high-throughput screening of small molecule compounds. We determined the structure of this mutant, which was published on *Nature Communication* recently.
Subtask 4: Optimize liposome flux assay for PKD1 and the PKD1/PKD2 complex	Months 7-12	We were able to purify several PKD1 variants. and are reconstituting them into liposomes.
Milestones(s) Achieved: Optimize liposome flux assay for PKD proteins	Month 12	In conclusion, we have successfully purified PKD2 and a PKD2 gain-of-function mutant, as well as several PKD1 variants. We are optimizing liposome flux assay for high-

		throughput screening. We published a *Nature Communication* article that reports the structure of the PKD2 gain-of-function mutant.
Specific Aim 2: Identify cone snail peptide regulators of PKD proteins		
Subtask 1: Reconstitute PKD2 channels into nanodiscs	Months 1-2	We reconstituted PKD2 and PKD2 mutant into nanodiscs. This has been accomplished.
Subtask 2: Use biochemical approach to pull down binding peptides of the PKD2 channel from crude cone snail venoms for identification by mass spectrometry	Months 3-4	We tried this approach with several cone snail venoms, which has yet to yield positive results. However, we established an alternative oocyte-based assay that is robust for medium-throughput screening of venoms.
Subtask 3: Reconstitute PKD1 and the PKD1/PKD2 complex into nanodiscs	Months 1-6	We have purified several PKD1 variants and are exploring different lipids for nanodisc formation.
Milestone(s) Achieved: Reconstitute PKD proteins into nanodiscs	Month 6	We have successfully reconstituted PKD2 and mutant into nanodiscs.
Subtask 4: Screen cone snail venoms against PKD2 liposomes by liposomal flux assay Cone snail venoms will be provided by the lab of Dr. Baldomero Olivera at the U of U	Months 7-8	We used an alternative oocyte based assay, which we found is more robust and sensitive. We have used this approach and finished screening of > 50 cone snail venoms on the PKD2 channel.
Subtask 5: Screen cone snail venoms against PKD1 and the PKD1/PKD2 complex liposomes by liposomal flux assay Cone snail venoms will be provided by the lab of Dr. Baldomero Olivera at the U of U	Months 7-8	It turned out that the human PKD1 full-length protein is challenging for biochemical purification. We were successful in purifying several human PKD1 variants. We have also cloned more than >20 PKD1 orthologs and homologs from variant species (e.g., chick, pig, etc.), which will be screened for expression and purification during the remaining months of this grant.
Subtask 6: Use biochemical approach to pull down binding peptides of PKD1 and the PKD1/PKD2 complex from crude cone snail venoms for identification by mass spectrometry	Months 7-8	As described above, we have attempted this approach on PKD2. We will continue to explore this method, but we are also using a more robust oocyte based assay for accomplishing this goal.
Subtask 7: Characterizing identified cone snail regulators of PKD proteins by electrophysiological recording	Months 8-9	We are using our newly established oocyte recording to accomplish this goal.
Milestone(s) Achieved: Identify cone snail peptide regulators of PKD proteins	Month 9	We have screened more than 50 cone snail venoms on the PKD2 channel. Although a positive candidate has yet to emerge from the

		ongoing screen, we have a robust system in place to screen more cone snail venoms. We have also established collaboration with another animal venom lab (Glenn King) and we plan to continue to screen their collection of > 700 venoms.
Specific Aim 3: Generate conformation-sensitive, functional antibodies for PKD proteins		
Subtask 1: Immunize mice with liposomes or nanodiscs containing PKD proteins using results obtained in Aim 1 and Aim 2	Months 7-12	This goal has been accomplished for the PKD2 channel.
Subtask 2: Screen hybridoma clones with nanodiscs containing PKD Proteins	Months 13-15	Ongoing for the PKD1 receptor; we have already accomplished the task for the PKD2 channel.
Subtask 3: Determine the effects of antibodies on PKD proteins by electrophysiological recordings	Month 16-18	Ongoing
Milestone(s) Achieved: Raise and identify conformationsensitive, functional antibodies for PKD proteins	Month 18	Ongoing

Accomplishments:

The overall goal of this proposal is to develop pharmacological tools (e.g., antibodies and peptide toxins from animal venoms) for dissecting structures and functions of the polycystic kidney disease (PKD) proteins that are implicated in the polycystic kidney disease. Thanks to the DoD discovery grant, we have made significant progress toward this ambition goal, which are detailed below. On a related note, I was recently named a Pew scholar with $300,000 award to support any research I am enthusiastic to pursue. When the DoD funding ends within few months, I plan to use part of this award to build on and expand the PKD pharmacology research initiated by the DoD discovery grant. I am fully committed to achieve our long-term goal of developing molecular pharmacology for the PKD proteins, which I believe will eventually benefit discovering novel therapeutic strategies for treating PKD patients.

a. Feng Zhang and Xiaoyong Yang, two postdoctoral fellows in my group, have successfully reconstituted PKD2 and a gain-of-function PKD2 mutant (F604P) into lipid bilayer

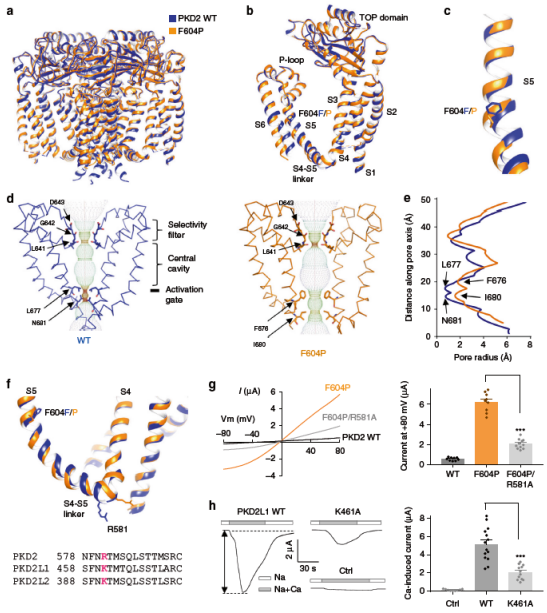

Figure 1 The structure of PKD2 F604P reveals a mechanism of channel activation. (a - f) comparison of F604P with the wild type PKD2 channel, revealing the expansion of the lower gate in the mutant. (**g-h)** the F604P is constitutively open as confirmed by electrophysiological recordings in oocytes.

systems (*in vitro* nanodiscs and liposomes), which are actively being optimized and adapted for high-throughput fluorescence-based screen in lipid vesicles. Supported by our R01 grant, we further determined and published the structure of the PKD2 gain-of-function mutant on *Nature Communication* recently (Figure 1).

b. Feng Zhang has established a medium-throughput, conventional two-electrode oocyte recording for assaying animal venoms on the PKD2 channel. Using this assay, he has finished screening of more than 50 different cone snail venoms. Although a positive hit has yet to emerge from such screen, this methodology is robust to screen other venoms (i.e., more cone snail venoms and other animal venoms). We are optimistic that we will identify a novel peptide toxin that can modulate PKD2 function when more crude venoms are analyzed.

c. We have obtained antibodies by employing two parallel strategies: First, in collaboration with the Tony Kossiakoff group at the University of Chicago, we have identified 4 Fabs that all bind to the extracellular domain of the PKD2 channel as confirmed by size-exclusion assay and other methods (Figure 2). Second, we have obtained 3 monoclonal antibodies for PKD2 via conventional hybridoma method. We suspect that these monoclonal antibodies bind to the cytosolic domains of the PKD2 channel. We are extremely optimistic that these antibodies will allow us to pinpoint and visualize essential structural elements of the PKD2 channel that have not been discovered in our previous structures. We are actively determining structures of PKD2 in complex with these antibodies by single-particle cryo-EM. We expect that structures of PKD2/antibodies are forthcoming by the end of this year. Since antibodies were developed via the DoD grant support, any future publication of these structures will acknowledge the DoD grant. We are also testing whether some of these antibodies can modulate PKD2 channel function. More broadly, these antibodies will also be invaluable reagents for other researchers in the field because they can be used to locate endogenous PKD2 channel in native tissues. We plan to make these antibodies available to academic labs in the field.

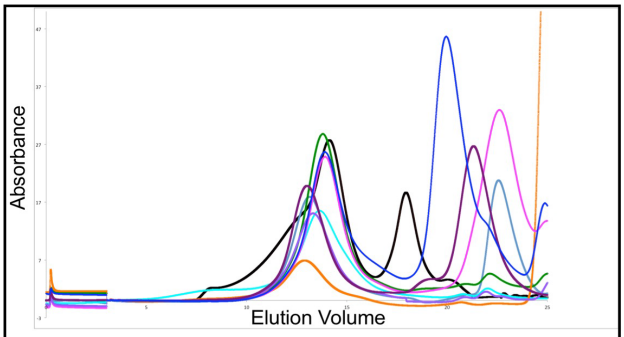

Figure 2. Anti-PKD2 Fabs obtained from a Fab phage display library. In size exclusion chromatography analyses, binding of 4 Fabs to PKD2 increases channel size, resulting in earlier elution (the leftmost peak in each curve) from the column as compared to PKD2 alone (black curve). 4 other Fabs are likely weaker binders, so their binding only leads to subtle shift of the elution profile.

d. We have established collaboration with the Glenn King group at Australia. Dr. King group has a collection of > 700 animal venoms (spiders, centipede, snakes, etc), we plan to continue screening these venoms in oocytes first, and in lipid vesicles as well in the future.

Training Opportunities:
Xiaoyong Yang and Feng Zhang are being trained in ion channel pharmacology and biochemistry.

Result Dissemination:
The antibodies will be shared with the academic community.

Plans for Next Reporting Period
We will finish screening the >20 PKD1 constructs we recently cloned in order to identify the best candidate for reconstitution and screening for pharmacological agents.

4. Impact:
Nothing to Report.

5. Changes/Problems:

- Changes in approach and reasons for change. No Major changes.

- Actual or anticipated problems or delays and actions or plans to resolve them. The human PKD1 purification is more challenging than expected. However, we have successfully cloned >20 PKD1 orthologs and homologs. We are confident that some of these PKD1 proteins will be more amenable to biochemical purification and reconstitution.

- Changes that had a significant impact on expenditures. No

- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. No

6. Products:
- Publications, conference papers, and presentations.
    - Wang Zheng[#], Xiaoyong Yang[#], Ruikun Hu, Ruiqi Cai, Laura Hofmann, Zhifei Wang, Qiaolin Hu, Xiong Liu, David Bulkey, Yong Yu, Jingfeng Tang*, Veit Flockerzi, Ying Cao, **Erhu Cao**[*], and Xing-Zhen Chen[*]. Hydrophobic pore gates regulate ion permeation in polycystic kidney disease 2 and 2L1 channels. Nature Communication. [#] Co-first authors * Corresponding authors. *The DoD grant was not acknowledged in this publication because the major structural determination effort was supported by our R01 grant.*

7. Participants & Other Collaborating Organizations
- What individuals have worked on the project?

Name:	**Erhu Cao**
Project Role:	Principle Investigator
Researcher Identifier:	0000-0003-3102-5692
Nearest person months worked:	6.26CM (over 18 months)
Contribution to Project:	Dr. Cao has provided guidance and oversight over this project as well as training Xiaoyong Yang and Feng Zhang.
Funding Support:	NIH: R01DK110575; 2017 Pew Biomedical Scholars Program

Name:	**Xiaoyong Yang**
Project Role:	Postdoctoral Fellow
Researcher Identifier:	0000-0001-7263-3913
Nearest person months worked:	9.74CM (over 18 months)
Contribution to Project:	Dr. Yang has purified and reconstituted all proteins into nanodiscs and liposomes.
Funding Support:	No direct other funding support
Name:	**Feng Zhang**
Project Role:	Postdoctoral Fellow
Researcher Identifier:	0000-0002-7659-8470
Nearest person months worked:	6.65CM (over 18 months)
Contribution to Project:	Dr. Zhang has established oocyte-based screening system for assaying cone snail venoms and other venoms.
Funding Support:	No direct other funding support

- Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period?
  Nothing to Report

8. Special Reporting Requirements:

Bibliography for Publications Supported by this Grant:

Thuy N. Vien, Jinliang Wang, **Erhu Cao**,and Paul G. DeCaen.Top domain variants cause a loss of polycystin-2 ion channel function in the primary cilia. *Manuscript in preparation.*

Erhu Cao. Structural mechanisms of the transient receptor ion channels.*Journal of general physiology* (invited review; submitted).

Qinzhe Wang and**Erhu Cao.** Structural determination of the polycystin-2 channel by electron cryo-microscopy.*Invited book chapter; submitted*

Conference Presentation Abstracts:

9. Appendices: N/A