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TITLE: Dissecting the Cystogenic Pathway Driving Polycystic Kidney Disease

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14. ABSTRACT *Research Problem:* Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common life threatening, inherited disease, affecting ~1 in 1000 Americans. It and other related Polycystic Kidney Diseases (PKD) are characterized by growth of fluid filled cysts that destroy healthy kidney tissue. Treatment options for ADPKD are currently limited to a drug called tolvaptan. Tolvaptan delays renal failure with an average increase in life expectancy of 2.6yrs. However, it may cause side effects of chronic thirst, frequent urination, and liver toxicity and is often discontinued for these reasons. There is no cure for PKD and in ADPKD, more than half of patients develop kidney failure requiring kidney transplant and/or dialysis, by their 60's. The high incidence of ADPKD means that patients constitute 10% of individuals in the US with kidney failure. This places a large burden on the health care system and greatly impacts patient quality of life. As such there is a pressing need for new PKD treatments.

Innovation: Through comparative analysis of mice engineered to model two distinct forms of human PKD, we discovered a shared factor that causes kidney cysts. Expression of this factor is negligible in healthy kidneys but switches on in PKD. By removing this factor prior to disease development, we prevented cysts from forming. Importantly, removal of this factor in healthy kidneys had no ill effect. Significantly, we discovered this key factor turns on a process often active in cancer, which reinforces the disease. This project explores this process and examines additional candidate members of the pathway.

Progress: Due to delays caused by COVID19 disruptions and slower than expected establishment of required mouse colonies, this project has only made progress with initial optimization aims. With such obstacles now overcome, significant progress into major experiment aims can be achieved in the upcoming year.

Applicability and Impact: Our unique approach has led to discovery of a new pathway that causes cyst growth. In this project we will examine additional candidate members of this pathway that could lead to new treatments with potential to increase the quality of life for PKD patients and reduce the burden on the health care system.

15. SUBJECT TERMS
Kidney, Polycystic Kidney Disease (PKD), Autosomal Dominant Polycystic Kidney Disease (ADPKD), Renal Cyst, Cyst, Cystogenesis

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1. **INTRODUCTION:** Kidney Disease (PKD) is characterised by the formation of multiple fluid-filled cysts. Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common form of PKD but also the most common life-threatening, inherited monoallelic conditions, affecting ~1 in 1000 Americans. The expense of medical treatments with kidney dialysis and renal transplant is great, while the single therapy, tolvaptan, only modestly slows kidney enlargement. Furthermore, tolvaptan causes side effects of chronic thirst and frequent urination, with potential for liver toxicity. As such there is a need for better therapies. We recently discovered that removal of AURKA, effectively cured ADPKD and other forms of PKD. When AURKA is present it drives cystogenesis, in part via promoting AKT activity, but surprisingly does so independent of its kinase activity. We identified a series of candidate factors which may be effectors in this AURKA-AKT cystogenic pathway and this project seeks to systemically test their influence using a new CRISPR based screening process known as CROPseq, both in live ADPKD mice and human organoid cultures.

2. **KEYWORDS:** Kidney, Polycystic Kidney Disease (PKD), Autosomal Dominant Polycystic Kidney Disease (ADPKD), Renal Cyst, Cyst, Cystogenesis, AURKA, AKT, CROPseq

3. **ACCOMPLISHMENTS:**
    - **What were the major goals of the project?**

Specific Aim 1: Determine AURKA-AKT cytogenic pathway candidates in mouse model	**Months from start March 31st 2021**	**Status**
Internal Animal Breeding Ethics, Internal Animal Experimental Ethics, Internal Human Ethics, Internal Gene Technology Regulator Approval (Animal), Internal Gene Technology Regulator Approval (Human), U.S. Army ACURO approval and other DoD negotiations.	-5	Complete
Major Task 1: To perform CRISPR library enrichment screen		
Subtask 1: Proof of principle and optimization.	1-5	Partial-Delayed
Subtask 2: Screen for cyst repressors downstream of *Aurka* deletion.	5-11	Not started-Delayed
Subtask 3 (concurrent with subtask 2): Screen for cyst exaggerators.	5-11	Not started-Delayed
Subtask 4: Bioinformatic analysis of subtask 2,3.	12	Not started-Delayed
Subtask 5: CROPseq screen.	12-17	Not started-2nd year Program
Subtask 6: Bioinformatic analysis of subtask 5.	18	Not started-2nd year Program

Milestone(s) Achieved: identification of specific gene targets in AURKA-AKT cytogenic pathway which promote or prevent cyst formation in mice.	18	Incomplete
Specific Aim 2: Further validate AURKA-AKT cytogenic pathway candidates refined from Aim 1 in human model		
Major Task 2: To perform CROPseq screen in patient tubuloids		
Subtask 1: Optimize tubuloid culture	12-18	Partial-Brought forward due to Major Task 1 delays
Subtask 2: CROPseq tubuloid screen.	18-21	Not started-2nd year Program
Subtask 3: Bioinformatic analysis of subtask 2.	21-24	Not started-2nd year Program
Milestone(s) Achieved: confirmation of specific gene targets in AURKA-AKT cytogenic pathway which promote or prevent cyst formation in human cells.	24	Incomplete

- **What was accomplished under these goals?**
  - A major activity was in obtaining the Cas9-GFP mouse strain and commencing the cross-breeding program required to generate the strains required for Major Task 1. The cross-breeding program has unfortunately been slower than expected meaning the stated goals have not been met in the original time frame. The setback was due to an animal technician mispairing two sets of animals in error. This mistake was not identified until their progeny pups were found to have impossible genotypes. Upon identification of the mistake, the mating pairs were corrected, however this setback the breeding program a mouse generation (~12 weeks). In addition, the progeny mice with the required multiple transgenic genotypes to advance the program, have been harder to obtain also slowing progress. After almost a year of inbreeding, we are at a point where the final intended strains are almost ready.
  - A second major activity was in starting Major Task 1, Subtask 1: The proof of principle and optimisation of hydrodynamic tail vein (HTV) injection of lentivirus into live mice. Our animal welfare officer insisted upon two supervised training sessions for staff members. The first was to attempt HTV injection of 1ml saline into unconscious mice without recovery. The second was to attempt HTV injection of 1ml saline into unconscious mice with recovery and animal monitoring for 4 days. No long-term adverse effects were observed in the training mice. A report was then required to our animal ethics committee, and then conditional approval has been given to try two more small cohorts after which another report is due. We have since HTV injected 1ml of Lentivirus (once) into 6 unconscious mice with recovery and euthanasia after two

weeks and observed successful kidney transduction. The next cohort of mice will repeat this work but trialling whether 1, 2 or 3 rounds of HTV injection of lentivirus leads to the highest transduction efficiency. This next optimisation phase was unfortunately delayed due to COVID19 lockdown, vaccine mandates impacting staff availability and other restrictions imposed by the governments of Australia. Now that restrictions have eased, we are resuming the last phase of this optimisation as I write this report. Following completion, we will submit the report to our animal ethics committee and then obtain full approval to proceed towards Major Task 1: Subtasks 2 to 6.

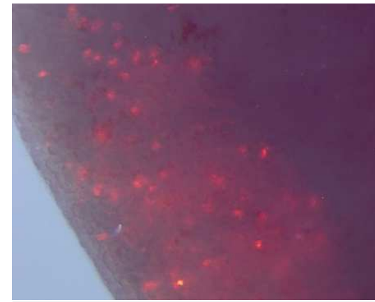

Fig 1: Dissected murine kidney, 2 weeks after HTV injection of live mouse with lentivirus, demonstrating RFP (overlaid on Brightfield).

- A third major activity has been in starting Major Task 2, subtask 1: The optimisation of tubuloid cultures. Key personal Dr Alex Combes and his lab, began to work up the isolation of kidney cells excreted in human urine samples. Work to date has shown it is feasible to grow organoids in culture from normal kidney epithelial cells collected from urine, however the technique required modification from the reference protocol published by the Clevers laboratory. This goal has been achieved earlier than expected and the next phase will be to apply the approach to cells isolated from ADPKD patient urine. Urine derived organoids (from healthy donors) are also currently being characterised to establish their tubule identity.

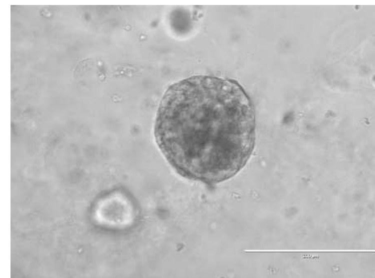

Fig 2: Image of kidney organoid cultures from cells derived from human urine (healthy volunteer).

- **What opportunities for training and professional development has the project provided?**
  - Three training activities were provided to Dr Cottle and several animal technician staff in the optimization of the hydrodynamic tail vein injection technique to date.
- **How were the results disseminated to communities of interest?**
  - Yes, the training and optimisation results were disseminated to our Animal Ethics Committee as per conditional approval conditions. Our animal welfare officer has also used this information to inform the design of HTV injection experiments in other laboratories.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - The delays due to establishment of the required mouse colonies has now been overcome, so the intended experiments can commence.
  - The delays due to COVID19 restrictions and government mandated lockdowns have eased, allowing the intended experiments to commence.
  - We are intending to catch up on the goals which have suffered delays. We are investigating the possibility of subtasks 2/3/4 to run concurrently with subtasks 5/6 to regain time. Failing that an extension without additional funds may be required.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Nothing to Report.
- **What was the impact on other disciplines?**
  - Nothing to Report.
- **What was the impact on technology transfer?**
  - Nothing to Report.
- **What was the impact on society beyond science and technology?**
  - Nothing to Report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - Originally, we proposed importing strain 26175 from Jax labs but with the slowdown in the frequency of international shipping due to COVID-19 this would likely be a long and risky process. We however found another Cas9 strain (28551 Jax) had cryopreserved sperm available locally. These animals are functionally equivalent for our purposes also providing Cre-induced expression of Cas9 and GFP from a CAG promoter, with the gene cassette knocked into the Rosa26 locus. With our Grant Officer's permission (email received 18th February 2021) we substituted this strain.
  - Our Monash Functional Genomic Platform (MFGP), which performs CROPseq services including lentiviral vector cloning and lentivirus production, revised down the recommended library size in order to boost the chances of success. We are planning on following these recommendations and use a more focussed library with our Grant Officer's permission (email received 18th February 2021).
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - Unfortunately, our animal facility mispaired two sets of mice during the breeding process to bring together the Cas9-IRES-GFP allele into our ADPKD disease model. This wasn't identified until pups of "impossible"

genotypes were detected. The mistake has now been remedied but it has set back the breeding program by approximately one mouse generation (12 weeks). Mice of the desired (rarer) genotypes to start the next generation in our inbreeding strategy have also proven more elusive than hoped, further slowing down progress. To overcome this issue, we have had to breed more litters to find the rare mice of the right genotypes. After a year of breeding, we are now producing mice of suitable genotypes to enter the experimentation program and the problem is resolved.

- A second delay has also been incurred due to Covid19. As you may be aware Melbourne was forced into lockdown on August 5th, with stay-at-home orders until October 22nd. After a brief reopening, restrictions returned with Delta and now Omicron waves at the end of 2021 and start of 2022. There are still currently restrictions in place with the unvaccinated not permitted on campus and a mandate to work from home (where possible) only lifting on February 28th. For much of last year's lockdown, schools were closed with it being parental responsibility to care for and manage remote learning (home school) for children during this time. While some exceptions were given to universities for research purposes, this still has had a knock-on effect with the availability of animal technician staff to both train or directly perform specialised techniques such as hydrodynamic tail vein injection. This has slowed progress towards optimising the technique but we have now demonstrated we can infect kidney cells with lentivirus via hydrodynamic tail vein injection as a proof of concept. The next phase of optimisation is about to be undertaken in the next few weeks. This coupled with the mouse strains being ready means we will be able to soon proceed with the tasks outlined.

- Our Monash Functional Genomics Platform has also warned the creation of a dual sgRNA lentiviral while possible, might have destabilising effects on the lentivirus due to repeated sequences. In our original proposal, we had planned to treat a cohort of *Pkd1 f/+; HoxB7-Cre; Cas9-GFP* animals with a dual sgRNA lentivirus, where one of the sgRNAs was against the remaining *Pkd1* allele in order to trigger adult onset ADPKD, while the other sgRNA was against a member of our candidate library. This cohort would thereby identify factors which prevent cyst formation (and also those which accelerate it). In light of the potential for dual sgRNA lentivirus issues, a solution to this anticipated problem would be to use a cohort of *Pkd1 f/f; Pax8-rtTA; TetO-Cre; Cas9-GFP* animals. These animals could alternatively have adult onset ADPKD induced by Doxycycline administration in their drinking water and then be injected with the same single sgRNA lentivirus as used for other cohorts. As the *Pax8-rtTA; TetO-Cre* system has a broad kidney epithelial expression range, only collecting duct cells would be analysed to restore matched comparisons with other cohorts using the *HoxB7-Cre* system. In this regard, FACS would still collect GFP+RFP cells but also then select those additionally labelled with DBA-biotin-Streptavidin Alexafluor647, where the DBA-lectin marks collecting duct cells. A stock line of *Pkd1 f/f; Pax8-rtTA; TetO-Cre;*

Cas9-GFP animals is available in the laboratory having been bred for another project, and the Doxycycline induced system is also routine in our hands. We hope this cohort substitution solution will be acceptable.

- **Changes that had a significant impact on expenditures**
    - The cost of IVF and establishment of the living 28551 Jax strain from sperm, was comparable to the importation cost of live mice from Jax, so no significant cost chance occurred.
    - Due to the pooled nature of the library cloning and viral production, the production cost is unchanged. The smaller library merely has more of any one construct.
    - The delays in our breeding program have had significant impact on expenditure. It has resulted in the colonies being needed to be maintained longer and in larger numbers to find the desired multitransgenic animals.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - Nothing to Report.
- **Significant changes in use or care of human subjects**
    - Nothing to Report.
- **Significant changes in use or care of vertebrate animals.**
    - Nothing to Report.
- **Significant changes in use of biohazards and/or select agents**
    - Nothing to Report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**
    - **Journal publications.** Nothing to Report.
    - **Books or other non-periodical, one-time publications.** Nothing to Report.
    - **Other publications, conference papers, and presentations.** Nothing to Report.
    - **Website(s) or other Internet site(s)**
      Nothing to Report.
    - **Technologies or techniques**
      Nothing to Report.
    - **Inventions, patent applications, and/or licenses** Nothing to Report.
    - **Other Products**
      Nothing to Report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	Dr Denny Cottle
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-5047-6515
Nearest person month worked:	6
Contribution to Project:	Dr Denny Cottle is the Principal Investigator and has worked to coordinate and manage the project, including animal and human ethics and DoD reporting requirements. He is responsible for managing the mouse breeding program, developing the HTV injection of lentivirus protocol (in conjunction with animal technicians) and performing the subsequent analysis.
Funding Support:	This award (50%) and other grants (50%)
Name:	Prof Ian Smyth
Project Role:	Key Personnel
Researcher Identifier (e.g. ORCID ID):	0000-0002-1727-7829
Nearest person month worked:	1
Contribution to Project:	Prof Ian Smyth hosts PI Dr Denny Cottle and provides access to the laboratory infrastructure required to perform the work.
Funding Support:	Other sources 100%
Name:	Prof Sefi Rosenbluh (and MFGP lab)
Project Role:	Key Personnel
Researcher Identifier (e.g. ORCID ID):	0000-0001-9815-8049
Nearest person month worked:	1
Contribution to Project:	Prof Sefi Rosenbluh is the head of the MFGP laboratory. The MFGP is responsible for the construction of the required lentiviral vectors and production of lentivirus.
Funding Support:	Other sources 100%

Name:	Dr Alex Combes (and lab)
Project Role:	Key Personnel
Researcher Identifier (e.g. ORCID ID):	0000-0001-6008-8786
Nearest person month worked:	3
Contribution to Project:	The Dr Alex Combes laboratory is responsible for the optimisation of kidney epithelia organoid growth from cells extracted from human urine.
Funding Support:	Other sources 100%
Name:	Prof David Nikolic-Paterson
Project Role:	Key Personnel
Researcher Identifier (e.g. ORCID ID):	0000-0001-5734-2931
Nearest person month worked:	1
Contribution to Project:	Prof David Nikolic-Paterson is responsible for the human ethics approval and coordination of the collection of clinical urine specimens, required for kidney organoid growth.
Funding Support:	Other sources 100%

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to Report
- **What other organizations were involved as partners?**
  - Nothing to Report
    - **Organization Name:** Nothing to Report
    - **Location of Organization:** Nothing to Report
    - **Partner's contribution to the project** Nothing to Report
      - **Financial support** Nothing to Report
      - **In-kind support** Nothing to Report
      - **Facilities** Nothing to Report
      - **Collaboration** Nothing to Report
      - **Personnel exchanges** Nothing to Report
      - **Other** Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** Nothing to Report
- **QUAD CHARTS:** Nothing to report

9. **APPENDICES:** Nothing to Report