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TITLE: Inducing Somatic Pkd1 Mutations in Vivo in a Mouse Model of Autosomal-Dominant Polycystic Kidney Disease

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14. ABSTRACT

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is one of the world's most common life-threatening genetic diseases. Over 95% of diagnosed cases of ADPKD are caused by mutations in *PKD1* or *PKD2* genes. The overall goal of this project is to identify, at the single cell level, the mechanisms that drive the progression of a homozygous *Pkd2* null renal cell towards a pathogenic clonal cyst in a mouse model of ADPKD. We have established several genetic models to induce mutations: two during embryogenesis (with Six2-cre and CVM-cre) and one in the adult (Villin-cre). One of the embryonic models has generated clones of wildtype and mutant cells that persist in the adult. The adult model has failed to induce sufficient recombination. In this report we summarize the results obtained with the embryonic model.

15. SUBJECT TERMS

None listed.

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1. **INTRODUCTION:** Autosomal Dominant Polycystic Kidney Disease (ADPKD) is one of the world's most common life-threatening genetic diseases. Over 95% of diagnosed cases of ADPKD are caused by mutations in PKD1 or PKD2 genes. The disease is characterized by numerous renal cysts that grow over time, ultimately causing renal failure. A better understanding of the cellular and molecular mechanisms underlying cyst formation could lead to new strategies to prevent the progression of ADPKD. The overall goal of this project is to identify, at the single cell level, the mechanisms that drive the progression of a homozygous Pkd1 or Pkd2 null renal cell towards a pathogenic clonal cyst in a mouse model of ADPKD. Ultimately, we aim at identifying the footprint of the pre-cystic cells in order to better target them for directed therapies.
2. **KEYWORDS:** Kidney, renal cystic disease, mouse model, Pkd2, proliferation, primary cilia.
3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**
- Major Task 1.1: Assess the level of recombination achieved with the Six2–cre and Villin-cre lines using the MADM system (weeks 1-20). 100% completed
- Major Task 1.2: Histological characterization of the embryonic two-hit model. Do cysts appear? And when? (Weeks 1-70). 100% completed
- Major Task 1.3: To identify defects in Planar Cell Polarity (PCP) associated with Pkd2 somatic mutations and how they correlate with cyst formation (weeks 18-70). 100% completed
- Major Task 1.4: To identify changes in proliferation associated with Pkd2 somatic mutations and how they correlate with cyst formation (weeks 18-70). 100% completed
- Major Task 2.1: RNAseq of control vs mutant cells (weeks 36-65). See section 5 changes/problems.
- Major Task 3.1: Identify the fate of Ddx11-/- cells in our model of ADPKD (weeks 1-50). See section 5 changes/problems.
- **What was accomplished under these goals?**

Major activities:

Generation of the desired animal models:

We have generated 3 different animal models to study the behavior of Pkd2 mutant cells in the mouse kidney. All three models are based on the MADM system. In Fig.1 we present a diagram of how the MADM system works. In short, we have developed a system to generate mutant and wildtype cells in an otherwise heterozygous background. Importantly, these cells are labeled with a fluorescent protein (wildtype cells with tdTomato red fluorescent protein, mutant cells with the

green fluorescent protein GFP) so we can follow these cells and their progeny. Of note, these cells are generated at very low frequency, so we can easily follow the progeny of each clone.

The generation of these **wildtype**/**mutant** clones is driven by cre-mediated recombination. We originally proposed the use of Six2-cre mice (Tg(Six2-EGFP/cre1Amc)) to induce recombination during embryonic development and Villin-cre mice (Tg(Vil-cre)997Gum)) to induce recombination in the adult. As stated below in the results and conclusions section, the very low level of recombination in the Villin-cre mice completely precluded their use. Hence, in our 1-year report we proposed to use a third model using the Cmv-cre mice (Tg(CMV-cre)1Cgn/J).

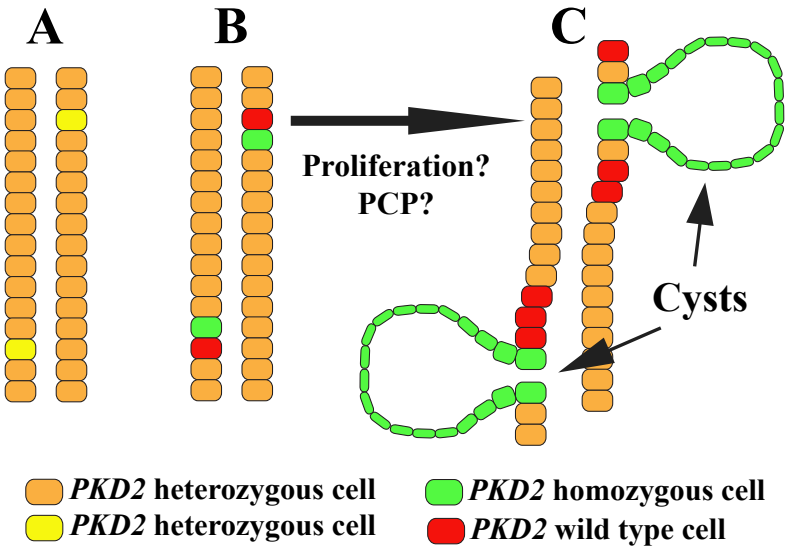

Fig.1. The MADM mice recapitulate the two-hit model, in which Pkd2+/- cells are normal but when affected by a second mutation they become homozygous and undergo cystogenesis. In the MADM system the second hit is cre-mediated and generates two sister cells, one homozygous mutant (green) and one wild type (red).

In all cases the cre lines were crossed into the MADM-TG line while the Pkd2-null allele was crossed into the MADM-GT line to generate animals that were cre +/-; MADM TG/TG or Pkd2+/-; MADM GT/GT. When these animals were intercrossed, we generated control samples that were cre+/-; MADM TG/GT, Pkd2+/+ in which both green and red cells were wildtype for Pkd2 and experimental littermate samples that were cre+/-; MADM TG/GT; Pkd2+/- in which green cells were mutant and red cells were wildtype. As an internal control in these last experimental samples, yellow (green and red) cells are also generated but these are all heterozygous for Pkd2.

Characterization of the mutant and wildtype phenotype:

Control and experimental littermates from the genotypes specified above were analyzed at birth (P0) and postnatally at 2, 4, 12 and 52 weeks of age. The kidneys from these mice were collected and processed for FACS analysis and further purification or fixed in 4%PFA and processed for thick sectioning (200um) using a vibrating microtome. **Wildtype**/**mutant** clones were identified and classified according to their phenotype.

Specific objectives:

Establishment of a mouse model to recapitulate the two-hit model in ADPKD:

ADPKD patients are heterozygous for either PKD1 or PKD2 deleterious mutations; homozygous mutations are considered incompatible with life. An intriguing feature of ADPKD is the relatively late onset of the disease. An affected individual may present only a few cysts for the first two decades of life, but by the age of 50, hundreds or thousands of renal cysts can be detected. Interestingly, loss-of-heterozygosity studies have documented the monoclonal nature of the epithelial cysts in ADPKD patients and, more importantly, a second mutation in the wild type allele of PKD1 or PKD2 has been detected in up to 80% of the cysts analyzed. Taken together, these findings support the two-hit model in which one mutation in either PKD1 or PKD2 is inherited, but a second (somatic) mutation is required for the clonal expansion of the epithelial cells and progression of cystic disease. Our first objective was to generate a mouse genetic model in which only a few cells would become homozygous for Pkd2 in an otherwise heterozygous background. The most relevant aspect of this approach relies on the ability to track these mutant cells due to their expression of a fluorescent protein.

Cellular and molecular characterization of the earliest events leading to cyst formation in ADPKD:

Once our model was generated we aimed at identifying the differential behavior of the mutant cells. Do they become cystic? If they do, are all the cells in the cysts labelled? Do the cysts incorporate non-mutant cells? Is proliferation increased? Does proliferation increase before cyst formation? Which genes are up or down-regulated in the mutant cells before cyst formation? And after? Answering these questions would significantly increase our understanding of cyst growth, a first step in developing novel therapies to slow ADPKD progression.

Analysis of the deleterious effect of ablating mutant cells from the diseased kidney:

Our last objective was to use our genetic model to introduce a gene mutation that would kill the mutant cells before they would become cystic. As stated bellow, the change in approach prevented us from implementing these experiments.

Significant results and conclusions:

By generating Six2cre; MADM5 tg/gt; Pkd2+/- mice we aimed at inducing homozygous mutations of Pkd2 during embryonic kidney development. Specifically, the Six2cre enhancer drives the expression of cre in the nephron progenitors throughout nephrogenesis. The activity of cre-recombinase will induce rare inter-chromosomal recombination of the MADM5 allele that would

result in two sister cells, one eGFP-labeled Pkd2-/- and the other tdTomato-labeled Pkd2+/+. When we analyzed kidneys from these mice at birth we observed the presence of green (mutant) and red (wildtype) clones. We also observed yellow (green and red) clones that are composed of heterozygous cells. While the results obtained were consistent with our expectations, we also observed the following: At birth, mutant clones were morphologically indistinguishable from wildtype clones, with no evidence of cyst development at that stage. Also, the frequency of clones was very low indicating that the cre expression was enough to induce inter-chromosomal recombination but at a relatively low rate, Fig.2.

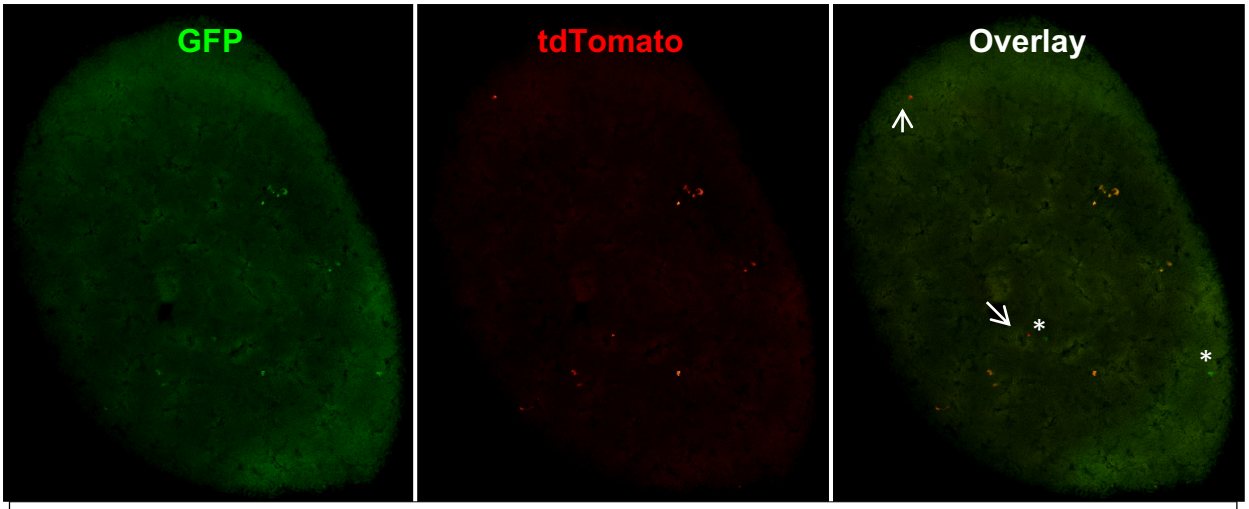

Fig.2. Kidney from 2-week-old mutant (Six2cre; MADM5 tg/gt; Pkd2+/-). Kidneys were isolated, fixed in 4%PFA overnight and processed for vibratome sectioning at 100um thickness. Several clusters of recombined cells are visible. Asterisks point to green-only cells, arrows point to red-only cells.

By generating Villincre; MADM5 tg/gt; Pkd2+/- mice we aimed at inducing homozygous mutations of Pkd2 during adult proximal tubule growth. The rationale for this approached was to circumvent a possible severe phenotype with our embryonic kidney model. Unfortunately, as we mentioned in our one-year report, the villin enhancer-driven expression of cre was not sufficient to induce recombination of the MADM5 allele at any stage analyzed (newborn, 2 week old and 4 week old mice). Indeed, no fluorescence was observed in those kidneys. However, as shown above, the embryonic induction of recombination did result in fluorescent clones with no severe phenotype, so we continued our studies using the Six2cre; MADM5 tg/gt; Pkd2+/- mouse model.

The above-mentioned models (Six2cre and Villincre) proved to induce either low or no recombination respectively in our MADM system. This severely compromises the use of these samples in FACS purification, as only a very small percentage of cells could be recovered for further

RNAseq analysis. In order to increase the level of recombination we generated a third mouse model using CMV-cre (Tg(CMV-cre)1Cgn/J). This mouse expresses cre under the control of the cytomegalovirus promoter in all cells of the embryo and the adult mouse. The rationale for this approach was that by being ubiquitously expressed, cre would induce higher levels of recombination than those achieved by the nephron progenitor-specific cre. To our surprise, the number of recombined clones (either Green/red or yellow) at all stages analyzed (newborn, 2, 4 and 12 weeks) was even lower than that of the Six2cre model. Therefore, we have limited the analysis to the study of the clones generated by the Six2cre model.

As stated in our one-year report, we had observed differences in cell morphology of the mutant vs. wildtype clones at 12 weeks of age. We have extended this study to analyze more 12 weeks old mice as well as 52 weeks old. All the analyzed mice had several fluorescent clones (either green/red or yellow). Several mice at 12 weeks and all mice at 52 weeks presented green (mutant) clones with their red (wild-type) counterparts that showed significant morphological differences. Remarkably, many green clones were in cystic tubules (Fig.3), while some green cells that were not cystic were not in epithelial tubes (instead they were in glomeruli and were consistent with a podocytes identity). Of note, all cystic structures were GFP-positive. **Therefore, we have shown that cysts do form in mice that recapitulate the two-hit model**.

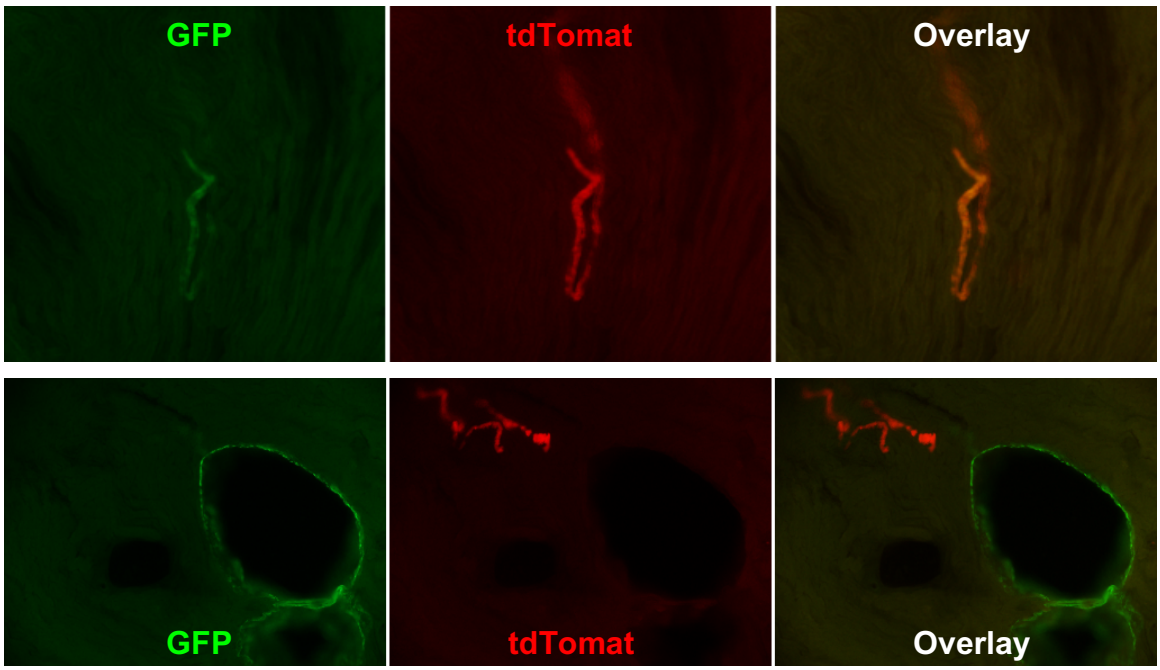

Fig.3. Two examples of clones observed in 12 week-old MADM kidneys. The upper panel shows a yellow clone in which all cells are green and red, and therefore heterozygous for Pkd2. The lower panel shows a clone with green (Pkd2-/-) and red (Pkd2 wildtype) cells. While the red cells present a normal epithelial morphology, the green cells have formed a cyst.

When we plot the size of the cystic clones (in number of cells) compared to counterpart wildtype clones we observed that the cystic phenotype was already present when mutant and control clones have on average the same number of cells (Fig.4), demonstrating that **extensive proliferation is not a requirement for the initiation of cysts**.

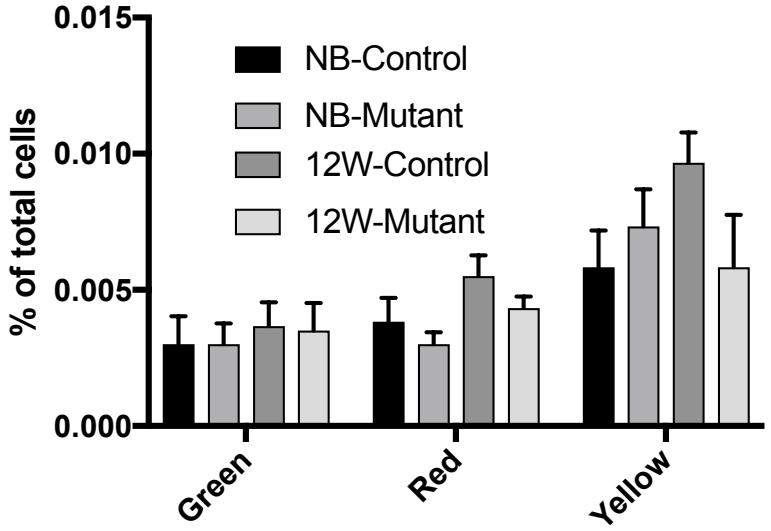

Fig.4. Kidneys were isolated, digested to a single-cell suspension and analyzed by flow cytometry. The percentage of fluorescent cells over total kidney cells are represented. No significant difference is observed between the number of green and red cells. n=6 samples per group

<u>Stated goals not met</u>:

Of all the stated goals of the project, only one has been compromised: Major Task 3.1: Identify the fate of Ddx11-/- cells in our model of ADPKD (weeks 1-50). The objective of this task was to characterize how ablating mutant cells affected kidney function compared to cyst development. This approach was based on our original proposal to use Pkd1 mutant alleles (in chromosome 17) together with MADM17 (in chromosome 17) and Ddx11 (also in chromosome 17). The inter-chromosomal recombination induced by cre would generate cells that are Pkd1 -/- and also Dds11-/- leading to the death of the cell. As explained in section 5, the MADM17 mouse model got delayed in production while the MADM5 mouse model became available. Pkd2 is in chromosome 5 and, as we stated on the alternative approach section of the proposal, the study of Pkd2 is equally relevant to ADPKD. Hence, we move forward with the goals of the project using MADM5 and Pkd2; however, in this genetic set up the use of Ddx11 became uninformative for the ultimate goal of the project.

Another problem we have encountered that prevented us from meeting our goal was the low percentage of fluorescent cells per kidney. The recombination level was very good for us to identify individual clones when visualizing the kidney sections but was too low to make FACS a feasible approach to collect cells and isolate RNA for RNAseq. We then used laser capture microdissection (LCM)on frozen and paraffin sections of sample kidneys. However, the processing of the tissue for

either frozen or paraffin sectioning either resulted in significant autofluorescence of the tissue or bleaching of the endogenous GFP/tdTomato fluorescence or both. While we are still working on alternative methods to purify these cells, this goal could not be completed within the timeframe of the award.

- **What opportunities for training and professional development have the project provided?**

Nothing to Report.

- **How were the results disseminated to communities of interest?**

Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to Report.

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

The objectives and the scope of the proposal did not change. We have implemented the following experimental changes to our approach:

- **Exchange of Pkd1 by Pkd2 alleles:**

The MADM approach to generate single cells with the desired genotype relies on the availability of a MADM construct in the same chromosome where the gene of interest is. Our original approach proposed the use of MADM17 with Pkd1 (in chromosome 17). However, our collaborator Dr. Simon Hippenmeyer experienced a delay in the generation of MADM17. On the other hand, they had just generated MADM5, in chromosome 5 where Pkd2 is located. Pkd1 and Pkd2 function coordinately

and disruptions in either gene affect the same pathway results in severe cystic phenotype. Only the frequency of mutations is significantly higher in Pkd1 than Pkd2. As we aim at understanding the developing of cysts once the mutation occurs, we are confident that the exchange of Pkd1 by Pkd2 is not detrimental to the final outcome of the project.

- **Introduction of CMV-cre:**

As mentioned on our one-year report, we have introduced the CMV-cre in our model with the objective of increasing the number of recombinant cells. Unfortunately this approach failed to provide the desired outcome.

- **Isolation of cells using laser capture vs. FACS:**

Given the low frequency of recombinant cells in our Six2cre; MADM5 tg/gt; Pkd2+/- mice, it is not feasible to use fluorescence-activated cell sorting (FACS) to purify those cells as they represent less than 0.01% of the total kidney cells. We have attempted laser capture microdissection to isolate cells from frozen and paraffin sections. However, that approach also failed as the processing of the tissue for those techniques resulted in increased autofluorescence of the kidney and/or severe reduction in endogenous fluorescence (from GFP and TdTomato).

Actual or anticipated problems or delays and actions or plans to resolve them: Nothing to Report.

Changes that had a significant impact on expenditures: Nothing to Report.

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: Nothing to Report.

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:	Cristina Cebrián-Ligero
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	No change
Funding Support:	No change

Name:	Yuanyuan Xiao
Project Role:	Laboratory technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	No change
Funding Support:	No change

Name:	Bryan Torres-Collazo
Project Role:	Undergraduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.25
Contribution to Project:	No change
Funding Support:	No change

Name:	Austin Kokoruda
Project Role:	Laboratory technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12 months
Contribution to Project:	Mouse colony maintenance
Funding Support:	PR140202

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

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: n/a

QUAD CHARTS: n/a

9. APPENDICES: No appendices