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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Focal segmental glomerulosclerosis (FSGS) is a kidney disease characterized by the abnormal leak of protein into the urine (proteinuria) and eventual kidney failure. Nuclear factor 2 erythroid 2 (Nrf2) is a transcription factor that may play a role in FSGS. Nrf2 is classically thought to protect cells from injury, but clinical trials using Nrf2 enhancers actually worsened proteinuria. Our own studies in animal models of FSGS also show increased proteinuria and kidney injury when Nrf2 activity is increased. **We hypothesize that increasing Nrf2 activity actually increases injury during FSGS.** In specific aim 1, we will perform animal experiments that will tell us how Nrf2 affects the different types of cells in the kidney during FSGS disease. To do this, we will conditionally knockout or activate Nrf2 in specific kidney cell types in vivo (tubules or endothelium) and examine progression of FSGS. We will also perform single cell/nucleus RNA sequencing in mouse kidneys to provide comprehensive cell-specific transcriptomes in response FSGS and to Nrf2 activation or deletion. In specific aim 2, we will comprehensively and definitively test whether drugs that increase or inhibit Nrf2 activity will be beneficial in FSGS. In this reporting period we successfully generated tubule-specific Keap1 knockout mice and exposed them to disease. These mice did not exhibit differences in FSGS disease but require additional study. If confirmed, these findings suggest that tubular Nrf2 may not play a role in FSGS pathogenesis and we will focus on endothelial-specific knockouts instead.

15. SUBJECT TERMS

Focal segmental glomerulosclerosis, proteinuria, glomerulosclerosis, Nrf2, Keap1

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1. Introduction

Focal segmental glomerulosclerosis (FSGS) is a debilitating kidney disease characterized by the abnormal leak of blood proteins into the urine (proteinuria). Nuclear factor 2 erythroid 2 (Nrf2) is a transcription factor in kidney cells that upregulates protective mechanisms in cells to prevent injury. In spite of this known function, Nrf2 enhancement in animal models of kidney disease actually worsened proteinuria and kidney disease. Our own studies in animal models of FSGS also show increased proteinuria and kidney injury when Nrf2 activity is increased. Therefore, we hypothesize that increasing Nrf2 activity actually increases injury during FSGS. In specific aim 1, we will perform animal experiments that will tell us how Nrf2 expression in specific kidney cells affects disease. We do this by inducing FSGS disease in mice that carry a mutation affecting Nrf2 activity only in specific kidney cell types. In this way we can understand whether the presence of Nrf2 in a specific type of kidney cell has therapeutic or harmful effects during disease. This knowledge can aid in the better understanding of Nrf2 biology and in future development of therapeutic drugs, which can then be targeted to specific kidney cells for maximal benefit. In specific aim 2, we will comprehensively and definitively test whether drugs that increase or inhibit Nrf2 activity will be beneficial in FSGS in our mice. Drugs will be given at low or high doses, or delivered early or late in disease, since there is prior evidence suggesting dose- and time-dependent effects of Nrf2. Filling in this gap in knowledge will help to design future clinical trials in humans.

2. Keywords

Focal segmental glomerulosclerosis
Proteinuria
Albuminuria
Nuclear factor 2 erythroid 2 (Nrf2)
Kelch like ECH associated protein 1 (Keap1)
Oxidative stress
Kidney Fibrosis

3. Accomplishments

The **major goals** of the project are to 1) determine the cell-specific effects of Keap1 and Nrf2 knockout in FSGS models; and 2) evaluate the effects of pharmacologic Nrf2 targeting in FSGS. In this initial award period, we have made important progress in pursuit of these goals.

Our **accomplishments** in year one have been focused predominantly on determining cell-specific effects of Keap1 and Nrf2 in FSGS. Our prior work showed that global Nrf2 enhancement by either pharmacologic or genetic means (in which a mutation leads to hyperactive Nrf2 in all cells and tissues of the mouse) leads to increased proteinuria and kidney injury. This aim is intended to determine which cell types in the kidney are most important for this Nrf2 effects through selective activation or deletion in certain kidney cell types. Our first task was to acquire both IACUC and ACURO approval for our studies. We then generated conditional knockout mice for both the Keap1 and Nrf2 genes in which these genes were deleted in renal tubules or in endothelial cells.

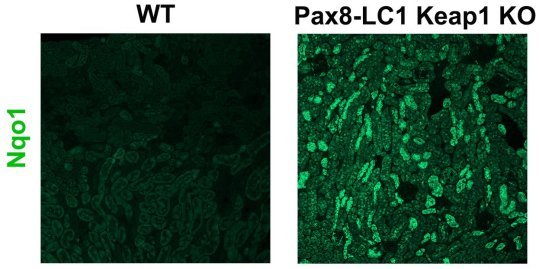

Figure 1. Tubular-specific knockout of Keap1 increases Nrf2 activity. Immunostaining for the Nrf2 target Nqo1 (green) was performed in kidney and reveal a significant upregulation of Nqo1 in the tubular-specific Keap1 knockout mice compared to wild type (WT) littermates.

We generated a tubule-specific Keap1 knockout mouse. Since Keap1 is the inhibitor of Nrf2, these mice will have enhanced Nrf2 expression in kidney tubular epithelia. These mice were generated by breeding Keap1 floxed animals with mice expressing the Pax8-rtTA and LC1-Cre transgenes to generate a triple transgenic mouse. Since three transgenes were required, breeding was complicated, but ultimately we successfully generated a colony of the appropriate mice. In this

system, the Pax8 promoter is only active in renal tubules from the proximal tubule to the collecting duct, and activates expression of the reverse tetracycline transactivator (rtTA). In the presence of doxycycline, the rtTA will induce the expression of Cre recombinase that leads to elimination of the Keap1 gene in the renal tubules only.

We treated these mice with doxycycline in their drinking water for two weeks duration (2g/L doxycycline). In Figure 1, we are staining for an Nrf2 gene target known as Nqo1 (a surrogate for Nrf2 activity). With doxycycline there is a significant increase in Nqo1 immunostaining in kidney tubules (but not in glomeruli or blood vessels) from Keap1 knockout mice compared to littermate controls, confirming that Nrf2 activity was indeed greater in the kidney tubules.

We exposed these mice to adriamycin (doxorubicin) in a classic animal model of FSGS. These mice are subjected to unilateral nephrectomy prior to injection with adriamycin (18mg/kg). The nephrectomy is a necessary step to sensitize the relatively resistant C57Bl/6 background strain to adriamycin-induced injury. Our results demonstrate that the tubular activation of Nrf2 does not affect proteinuria compared to normal littermates (Figure 2).

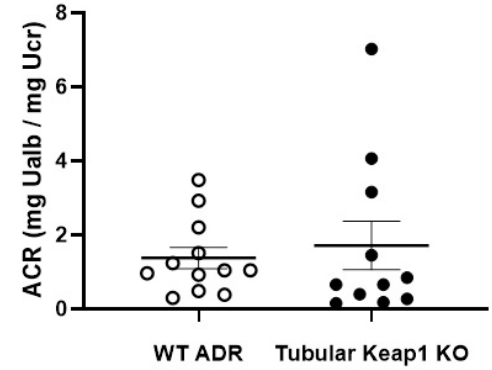

Figure 2. Tubular-specific knockout of Keap1 does not affect proteinuria in adriamycin-induced FSGS. Urine was collected from mice treated with adriamycin to induce FSGS. Proteinuria was not significantly different between knockout and wild-type animals.

We went on to examine a second model of FSGS induced by exposure to angiotensin II. In this study, osmotic minipumps secreting angiotensin II (1.5mg/kg/day) are subcutaneously implanted in the mice, which causes glomerular injury mimicking glomerulosclerosis as well as proteinuria. In this case, we again did not see a significant difference in proteinuria between tubular specific Keap1 knockout and wild-type littermates, but there appeared to be a trend towards a difference. Our results may have been affected by low numbers of animals (Figure 3).

If we find that the angiotensin II model of FSGS is affected by tubular Keap1 knockout while the adriamycin model is not, it suggests that the two disease models proceed through different pathways differentially affected by Nrf2. This would be an interesting finding that we would investigate further.

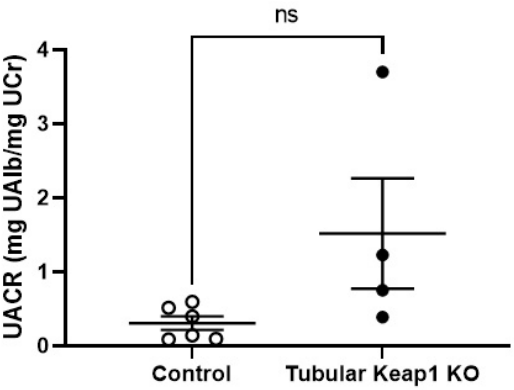

Figure 3. Tubular-specific knockout of Keap1 does not significantly affect proteinuria in angiotensin II-induced FSGS. Urine was collected from mice treated with a chronic infusion of angiotensin II to induce FSGS. Proteinuria was not significantly different between knockout and wild-type animals but did show a trend that may become significant with more animals.

Plans for the next reporting period are to repeat the angiotensin II experiment with a larger set of animals. We will also proceed with work in which Nrf2, not Keap1, is knocked out in the tubular compartment, which could lead to protection against injury. As described in our proposal, we also plan to use endothelial-specific Keap1 and Nrf2 knockout mice, since evidence suggests that endothelial injury precedes other injury in the kidney. All of these mice are under development presently. Interestingly, in work not funded by this award we have discovered an important role for podocyte-specific Nrf2 expression in the adriamycin, but not the angiotensin II model of FSGS. This affirms important cell- and disease-specific roles of Nrf2 in the kidney.

Additional studies for the upcoming award period include the performance of single nucleus RNA sequencing as an alternative method to determine cell-specific Nrf2 effects in the kidney. We will utilize the angiotensin II-induced FSGS model and examine cellular changes during disease and during exposure to Nrf2-enhancing

drugs. By identifying cell-specific changes in gene transcription in response to Nrf2 activation, this experiment will provide an important basis by which we can understand Nrf2 activity in the kidney during disease.

This award has afforded **training and professional development opportunities**. Hannah Hartman is a technician who has worked on this project and gained experience in mouse handling, surgery, and manipulation as well as the evaluation of kidney disease through molecular and histologic means. She will participate in this year's Kidney Week at the American Society of Nephrology as a poster presenter.

Our results were not **disseminated to communities of interest** (nothing to report).

4. Impact

The **impact of this award on the principal discipline of the project** is to better our understanding of Nrf2 biology in the kidney. Clinical trials have examined the role of Nrf2 enhancers on chronic kidney disease but have not been successful in treating disease. Our study sheds light on the pathologic mechanisms by revealing how Nrf2 plays a role in specific cell types in the kidney. In the process, we are also generating useful tools including unique transgenic mice that can be used by the scientific community for the study of Nrf2 biology in the kidney.

There is nothing to report for **impact on other disciplines, technology transfer, or society**.

5. Changes/Problems

We do not report any changes in overall scientific approach or use of vertebrate animals in this award. However, we do report that there were delays in finding necessary technical staff for this project, leading to a lower financial expenditure and scientific progress than anticipated. We have now hired a laboratory technician starting in late September 2023 that, once trained, will participate in this project.

Additional delays occurred in the breeding of some of the animals listed in this protocol. Namely, the endothelial-specific Keap1 and Nrf2 knockouts did not breed to the expected numbers required for experimentation early in this project period, but numbers have now improved and we should have animals for experimentation within the next few months. Additionally we are still validating whether these animals exhibit adequate knockout of these genes. We anticipate that both of these issues will be resolved over the course of this reporting period.

6. Products

Work supported by this project is being presented and the American Society of Nephrology 2023 Kidney Week in poster format:

Podocyte specific Nrf2 activity protects against adriamycin-induced kidney injury. Hartman HL, Bondi CD, Wang J, and Tan RJ. *J Am Soc Nephrol* [poster abstract accepted]

We are also generating unique animal models for the study of Nrf2. As described above, these include tubular- and endothelial-specific knockout mice for Keap1 or Nrf2. These will allow investigators to study genetically elevated, or reduced, Nrf2 activity in tubules and endothelium in kidney and other diseases.

7. Participants & Other Collaborating Organizations

Name:	Roderick J. Tan
Project Role:	PD/PI

Research Identifier:	0000-0003-1012-1722
Nearest person month worked:	4
Contribution to project:	Overall conceptualization, organization, and execution of the project.
Funding support:	USMRAA (this project) NIH R01 DK131991 Effect of Renal Nerves on Chronic Kidney Disease NIH R01 DK064005 Tenancin-C as a major component of the fibrogenic niche NIH U54 DK137329 Pittsburgh Center for Kidney Research VHA I01 BX005680 The role of Nrf2 in proteinuric chronic kidney disease

Name:	Hannah Hartman
Project Role:	Laboratory Technician
Research Identifier:	
Nearest person month worked:	2
Contribution to project:	Execution of research project under supervision of Roderick Tan
Funding support:	USMRAA (this project) NIH R01 DK131991 Effect of Renal Nerves on Chronic Kidney Disease VHA I01 BX005680 The role of Nrf2 in proteinuric chronic kidney disease

Current support for PD/PI Roderick Tan (changes from prior reporting in bold, overall effort for this award remains unchanged)

Pros and Cons of Nrf2 in FSGS Pathogenesis (PD/PI: Tan)
USMRAA, Dept of Defense PR211476
8/15/22 – 8/14/26

The Role of Nrf2 in Proteinuric Chronic Kidney Disease (PD/PI: Tan)
Veterans Affairs Administration I01 BX005680
4/01/22 – 3/31/26

Effect of Renal Nerves on Chronic Kidney Disease (PD/PI: Tan)
NIH/NIDDK R01 DK131991
6/1/22 – 3/31/27

Tenancin-C as a major component of the fibrogenic niche (PD/PI: Tan)
NIH/NIDDK R01 DK064005

7/1/21 – 6/30/23 (pending NCE until 6/30/24)

Pittsburgh Center for Kidney Research (PD/PI: Kleyman)
NIH/NIDDK **U54 DK137329**
9/1/23 – 6/30/28

Renal endothelium and the development of chronic kidney disease in sickle cell disorders (PD/PI: Ghosh)
NIH/NIDDK R01 DK132145
4/1/23 – 3/31/28

Molecular and cellular pathogenesis of kidney disease in sickle cell disorders (PD/PI: Ghosh)
NIH/NIDDK R01 DK124426
1/1/21 – 12/31/24

ENaC regulation by biliary factors (PD/PI: Kashlan)
NIH/NIDDK R01 DK125439
7/1/20 – 6/30/24

RNA binding proteins in end-organ autoimmune pathology (PD/PI: Gaffen / Biswas)
NIH/NIAID R01 AI162616
2/8/22 – 1/31/27

No other organizations were involved as partners in this research (nothing to report).

8. Special Reporting Requirements

Nothing to report

9. Appendices

Nothing to report