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TITLE: Novel mTORC1 and 2 Signaling Pathways in Polycystic Kidney Disease (PKD)

PRINCIPAL INVESTIGATOR: Charles L. Edelstein MD, PhD

CONTRACTING ORGANIZATION: University of Colorado, Denver
Aurora, CO 80045

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Abstract: This proposal will study novel mTORC1 and 2 signaling pathways that mediate ADPKD and investigate the effects of mTORC1 (Raptor) knockout, mTORC2 (Rictor) knockout or combined mTORC1 and 2 knockout on cyst growth and kidney function. The overall hypothesis is that there is increased mTORC1 (4E-BP1) and mTORC2 (AktSer473, PKCα and SGK1) signaling in PKD kidneys and that combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice will slow cyst growth and improve kidney function more than mTORC1 (Raptor) knockout or mTORC2 (Rictor) knockout alone. We have made significant progress in the second year: We have further characterized 4E-BP1 signaling pathways in PKD kidneys and cells. We have continued to breed Pkd1-/-, mTORC2 (Rictor) -/- double knockout mice but have changed our breeding system. We have performed a head to head study to compare the therapeutic effect of the mTOR kinase inhibitor Torin-2 (that inhibits both mTORC1 and mTORC2) with sirolimus (that inhibits mTORC1) on cyst growth and kidney function. We have continued to use FISP-MRI scanning to obtain precise measurements of kidney and cyst volume and the number of cysts in live PKD mice.					
15. SUBJECT TERMS Polycystic kidney disease, PKD, mTORC1, mTORC2, Raptor, Rictor.					
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1. INTRODUCTION:

ADPKD is the most common life threatening hereditary disease in the USA. ADPKD accounts for about 5-10% of end-stage renal failure in the USA requiring dialysis and renal transplantation. There is no effective treatment for ADPKD. This proposal will study novel mTORC1 (pS6 and 4E-BP1) and 2 (Akt, PKC alpha, SGK1) signaling pathways that mediate ADPKD and investigate the effects of mTORC1 (Raptor) knockout, or the mTORC1 inhibitor (sirolimus), mTORC2 (Rictor) knockout or combined mTORC1 and 2 knockout or the mTOR kinase inhibitor drugs (TORKs) on cyst growth and kidney function. The overall hypothesis is that there is increased mTORC1 (4E-BP1) and mTORC2 (AktSer473, PKC α and SGK1) signaling in PKD kidneys and that combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 $-/-$ mice will slow cyst growth and improve kidney function more than mTORC1 (Raptor) knockout or mTORC2 (Rictor) knockout alone or that the mTOR kinase inhibitors will be more effective than sirolimus in decreasing kidney cyst growth. These studies will unequivocally address whether the mTORC1 (4E-BP1) or mTORC2 pathway or both pathways is important in PKD and whether it is worthwhile performing therapeutic interventions using the novel mTORC1 and 2 inhibitors that are in clinical trials in humans

2. KEYWORDS:

Autosomal dominant polycystic kidney disease

mTORC1

Raptor

mTORC2

Rictor

4E-BP1

mTOR kinase inhibitor

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

Major Task 1: To determine the effect of mTORC1 (Raptor) knockout in Pkd1 $-/-$ mice

Subtask 1: To determine whether Raptor knockout decreases the cystic phenotype

Subtask 2: To determine the effect of Raptor $-/-$ on 4E-BP1 signaling

Subtask 3: To determine the effect of 4E-BP inhibition on PKD

Major Task 2: To determine the effect of mTORC2 (Rictor) knockout in Pkd1 $-/-$ mice

Subtask 1: To determine whether Rictor knockout reduces the cystic phenotype in vivo

Subtask 2: AktSer473, SGK1 and PKC α phosphorylation in PKD in vivo

Subtask 3: AktSer473, SGK1 and PKC α phosphorylation in PKD in vitro

Major Task 3: To determine the effect of combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice

Subtask 1: To determine the effect of combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice on apoptosis and proliferation, cyst growth and kidney function.

SubTask 2: To determine the effect of TORCs versus sirolimus on PKD and kidney function

What was accomplished under these goals?

(1) Major activities:

Major Task 1: a) Development of Pkd1 -/- Raptor -/- double knockout mice using the tamoxifen-inducible ER2 Cre mice gave inconsistent results. The Cre did not always work leading to mice with a very variable form of the ADPKD disease. We are now using KSP 1.3 Cre mice that have an embryonic Cre that is spontaneous (does not need tamoxifen) and the Pkd1 -/- mice develop PKD and kidney failure at a much earlier age. We hope that this model will lead to a more reproducible and faster model of ADPKD. b) 4E-BP1 signaling is being studied in ADPKD kidneys and PKD cells in vitro.

Major Task 2: mTORC2 (Rictor) knockout in Pkd1 -/- mice. We are also now using the KSP 1.3 Cre rather than the tamoxifen-inducible ER2 Cre to develop these double knockout mice.

Major task 3: Treatment of Pkd1 -/- mice with mTOR kinase inhibitors that inhibit mTORC1 and mTORC2 (TORCs) versus Sirolimus (mTORC1 inhibitor).

Major Tasks 1, 2 and 3: We are using FISP-MRI scans to determine polycystic kidney volume and cyst volume and number of cysts per kidney at different time points of the disease.

2) Specific objectives:

Major Task 1: To determine whether Raptor knockout decreases the cystic phenotype. To determine the effect of Raptor -/- on 4E-BP1 signaling

Major Task 2: To determine whether Rictor knockout reduces the cystic phenotype in vivo.

Major Task 3: To determine the effect of TORCs versus sirolimus on PKD and kidney function.

3) Major results:

Major Task 1: We have restarted the breeding process using KSP 1.3 Cre mice instead of tamoxifen-inducible KSP Cad Cre ER2 mice. We have obtained Raptor floxed mice and are in the process of breeding the Raptor floxed mice with KSP 1.3 Cre mice to develop Raptor^{-/-}, Pkd1^{-/-} double knockout mice. We are in the second stage of the breeding process and have 70 cages of mice for this breeding process.

Major task 1: A major finding is that we have determined that there is increased 4E-BP1 in PKD kidneys and that 4E-BP1 signaling is dysregulated in PKD kidneys. Study is summarized below:

Dysregulated 4E-BP1 pathway in Polycystic Kidney Disease

Background: Unchecked proliferation of cystic epithelial cells is a major contributor to cyst growth in PKD. The 4E-BP1 pathway is a crucial checkpoint in translation initiation and cellular proliferation, regulated by multiple stimulatory factors like PKC α , ERK, and AKT. The aim of this study was to 1) determine whether the 4E-BP1 pathway is dysregulated in human PKD1^{-/-} cells, 2) examine the effects of a 4E-BP1, blind to AKT and mTOR (F113A) on the 4E-BP1 pathway, protein translation, proliferation, and 3) assess the feasibility of *in vivo* gene therapy of F113A 4E-BP1 adeno-associated virus (AAV9) in neonatal and adult mice.

Methods: Western blot, proliferation, and firefly-renilla assays were performed on human primary epithelial cells from normal renal cortical tubular epithelium (PKD1^{+/+}) and ADPKD cyst-lining epithelium (PKD1^{-/-}) transfected with pCAG-TdTomato or pCAG-F113A. AAV9 F113A, and AAV9-TdTomato vectors were prepared and administered from D3 and to D17, or D120 to D180.

Results: There was increased phospho (p4E-BP1) species and increased pPKCa(3.6 $\pm$ 0.2 vs 0.6 $\pm$ 0.0DU***), pERK(3.6 $\pm$ 0.2 vs 0.5 $\pm$ 0.2DU***), and pAKT(0.6 $\pm$ 0.1 vs 0.2 $\pm$ 0.1DU*), in PKD1^{-/-} vs. PKD1^{+/+} cells respectively. *In vitro*, F113A expression reduced p4E-BP1 S65 (1.4 $\pm$ 0.2 vs 0.02 $\pm$ 0.01DU***), reduced cyclin D1 (0.65 $\pm$ 0.04 vs 0.51 $\pm$ 0.10DU*) and increased autophagosome marker, LC3-II, (0.02 $\pm$ 0.00 vs 0.31 $\pm$ 0.13DU*) expression in PKD1^{-/-} cells. Stimulation with insulin resulted in maintained p4E-BP1 S65 suppression with F113A in PKD1^{-/-} (2.1 $\pm$ 0.3 vs 0.2 $\pm$ 0.1DU***). F113A also reduced cap-dependent protein translation (by 37%**), and 72hr proliferation (250 $\pm$ 4 vs 180 $\pm$ 5 480/528nm O.D***). In neonatal and adult mice, administration of AAV9 resulted in detectable F113A RNA in the heart, kidney, and liver, and reduced p4E-BP1 S65 expression in the heart (2.0 $\pm$ 1.3 vs 0.6 $\pm$ 0.4DU*). *p<0.05, **p<0.01, *** p<0.001 vs to control.

Conclusions: In PKD, a setting of cystic tubular epithelial hyperproliferation, the 4E-BP1 pathway is dysregulated, with increased phosphoregulation of 4E-BP1 by multiple overactive kinases such as; PKCa, ERK, AKT. *In vitro*, F113A expression in PKD1^{-/-} cells, results in a shift towards hypophosphorylated 4E-BP1 species, reduced cap dependent protein translation, reduced proliferation, and increased autophagosomes. 4E-BP1 F113A gene therapy to counter the dysregulated 4E-BP1 pathway *in vivo* models of PKD is feasible to inhibit a pathway seemingly integral to the pathobiology of PKD.

Major Task 2: To determine whether Rictor knockout reduces the cystic phenotype in vivo

We have restarted the breeding process using KSP 1.3 Cre mice instead of tamoxifen-inducible KSP Cad Cre ER2 mice. We have obtained Rictor floxed mice and are in the process of breeding the Rictor floxed mice with KSP 1.3 Cre mice to develop Rictor^{-/-}, Pkd1^{-/-} double knockout mice. We are in the second stage of the breeding process and have 70 cages of mice for this breeding process.

Activation of the mTORC1 (Raptor) or 2 (Rictor) pathway, as occurs in ADPKD, is known to inhibit autophagy. We have found that autophagy is suppressed in PKD possibly due to activation of the mTOR pathway. We have found that agents that potentially inhibit the mTOR pathway like metformin or 2 deoxy glucose are able to rescue suppressed autophagy in PKD. Study is summarized below:

Rescue of suppressed autophagy in polycystic kidney disease

Background: Autophagy maintains proteostasis by sequestering damaged organelles and proteins into autophagosomes for delivery to the lysosome where cargo is degraded and recycled. Several treatments like mTOR inhibitors that have been shown to ameliorate PKD in mice are potent activators of autophagy. The aims of the study were to determine the effects of several novel autophagy inducers (that also have the potential to activate mTOR) on autophagy, apoptosis, and proliferation markers in PKD kidneys and to determine whether kidney-specific autophagy knockout results in a cystic phenotype.

Methods; Mice were treated with 2-deoxyglucose (2DG), trehalose (TRE), or metformin (MET), that are also potentially mTORC1 or 2 activators, followed by bafilomycin (BAF) to measure autophagic flux. p62 (autophagy-specific tag for degradation & marker of autophagy inhibition), LC3-II (marker of autophagosomes), Atg12-5 (marker of autophagosome elongation), cleaved caspase 3 (a marker of apoptosis), were measured by immunoblot. Kidney-specific Atg7^{-/-} and wild-type (WT) controls received MRIs at 180D of age to precisely measure cyst volume and number.

Results: PKD kidneys had significantly reduced autophagic flux compared to WT, which was rescued by 2DG. Further, PKD kidneys had less Atg12-5 complex vs WT, an effect blocked by TRE. PKD kidneys had more cleaved caspase 3, a marker of apoptosis, compared to WT, which was decreased by MET, 2DG, and TRE. cMyc phosphorylation was greater in PKD compared to WT, an effect blocked by 2DG. On MRI scan, mean number of cysts (0.5mm) in 180 day old Atg7^{-/-} mice was 5±2 vs. 1±0.5 in WT (p<0.05). One Atg7^{-/-} kidney was massively cystic.

Relative densitometry	FLUX	Atg12-5	p cMyc	CC3
WT VEH	0.4±0.05*	0.4±0.05*	0.8±0.1 *	1.0±0.05*
PKD VEH	0.1±0.03	0.2±0.02	1.3±0.1	1.6±0.09
PKD 2DG	0.3±0.06*	0.2±0.08	0.9±0.1*	1.0±0.15*
PKD TRE	0.1±0.08	0.3±0.07	1.2±0.2	0.6±0.1*
PKD MET	0.1±0.07	0.2±0.02	-	0.9±0.08*

*p<0.05 vs PKD VEH

Conclusion

In summary, 2DG, that has the potential to activate mTORC1 or 2, restored autophagic flux in PKD kidneys. Autophagy inducers increased expression of autophagy-related proteins and decreased apoptosis and proliferation markers. Kidney-specific knockout of autophagy in 180 day old mice resulted in a cystic phenotype.

Major Task 3: To determine the effect of TORCs versus sirolimus on PKD and kidney function.

We have found that in a mouse model of autosomal dominant polycystic kidney disease (ADPKD), both sirolimus and an mTOR kinase inhibitor decrease cyst volume and improve kidney function despite differential effects signaling and apoptosis. Study is summarized below:

In a mouse model of autosomal dominant polycystic kidney disease (ADPKD), both sirolimus and an mTOR kinase inhibitor decrease cyst volume and improve kidney function despite differential effects signaling and apoptosis.

Background: Sirolimus indirectly inhibits mTORC1 and reduces cyst growth in rodent models of autosomal dominant polycystic kidney disease (ADPKD). The novel ATP competitive mTOR kinase inhibitors (TORCs) e.g. Torin2 directly inhibit mTOR kinase resulting in inhibition of both mTORC1 and 2. The purpose of the study was to perform a head-to-head comparison of Torin2 versus sirolimus in a hypomorphic mouse model of PKD.

Methods: C57Bl/6 *Pkd1* p.R3277C (ADPKD) mice were treated with 0.5mg/kg Sirolimus (SIR) or 10mg/kg Torin2 (TORK) daily from 50 to 120 days of age. Serum BUN was analyzed using an enzymatic assay. Cyst area was quantified from H&E stained kidneys. Kidneys were immunoblotted for mTORC1 and mTORC2 substrates, pS6Ser235/236 and pAktSer473. IHC was performed for mTORC1 substrate 4E-BP1, in addition to proliferation (PCNA) and apoptosis (TUNEL) assessments.

Results: Kidney weight, BUN and cyst area were significantly decreased by both SIR and TORK compared to vehicle (VEH). pAKT was decreased in both TORK and SIR treatments. pS6 staining, p4E-BP1 isoform T70, and PCNA staining were significantly decreased by both SIR and TORK in PKD kidneys. TORK but not SIR significantly decreased p4E-BP1Thr 37/46 isoform and increased TUNEL staining in PKD kidneys. No side effects were noted on gross examination in either treatment group.

	KW/BW (%)	BUN (mg/dL)	Cyst area (%)	pS6	pAKT	pS6 Staining (%)	4E-BP1 T70 Staining (%)	4E-BP1 Thr 37/47 staining (%)	PCNA Staining (%)	TUNEL Staining (%)
PKD VEH	2.6	63	31.6	1.3	0.8	9.9	6.6	7.3	2.25	5.5
PKD TORK	2.1*	41*	22.1*	1	0.3*	0.8*	2.1*	4.1*	1.5*	9.5*
PKD SIR	2.3*	39*	20.2*	0.7*	0.2*	1.3*	2.6*	6.6	1.4*	5.3

*p<0.05 vs VEH, n=4-5 per group. Staining (%)=% of non cystic and cystic areas staining positive

Conclusions: For the first time, we have shown in a head-to-head study that the TORK, Torin2, is as effective as sirolimus in decreasing kidney size and BUN in a hypomorphic ADPKD mouse model. Both drugs inhibited pAKT, p4E-BP1T70 isoform and proliferation. TORK but not SIR significantly decreased p4E-BP1Thr 37/46 isoform and increased apoptosis staining in PKD kidneys. In conclusion, both SIR and TORK are equally effective in decreasing PKD and improving kidney function, but have differential effects on 4E-BP1 signaling and apoptosis.

4) Other achievements: Major tasks 1, 2 and 3.

We have used FISP-MRI scanning to obtain measurements of kidney and cyst volume in live PKD mice at 70 days of age (See Figures 1, 2 and 3).

FIGURE 1
Mouse Red #139
01/04/2018
Severe Cyst Development

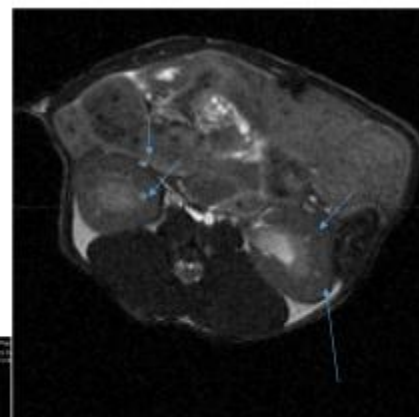
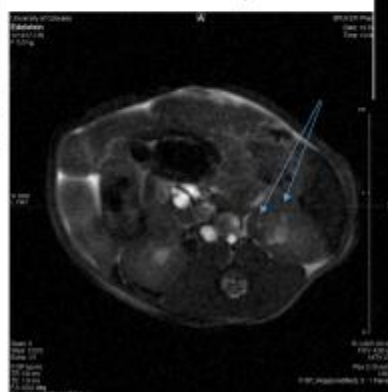
Figure 1 consists of three coronal MRI scans of the abdomen, labeled (a), (b), and (c). Each scan shows the kidneys and surrounding structures. (a) is a T1-weighted image, (b) is a T2-weighted image, and (c) is a T2-weighted image. The scans show a hyperintense lesion in the right kidney in (b) and a hyperintense lesion in the left kidney in (c).



Mouse Red #143
01/04/2018
Mild Case of Cyst

Figure 3

Mouse Red #135
12/19/2017
Mild Case of Cyst



Mouse Red #141
12/19/2017
Mild Case of Cyst

FISP-MRI will allow us to perform longitudinal imaging of cysts and determination of kidney volume over a period of time in the same animal. See data below for kidney volume, number (#) of cysts, maximum cyst diameter, cyst volume (%) in mouse #Red16 with ADPKD that shows an increase in all cyst indices over a 2 month period (Figures 1, 2 and 3).

Mouse #Red16

5/31/2018

Kidney Volume,
mm³

Cyst

max cyst diam,
mm

Cyst % volume

Right Kidney

198

3

0.6

3%

Left Kidney

174

7

0.5

5%

Body Weight
[g]

22.1

7/31/2018

Kidney Volume, mm ³	# Cyst	max cyst diam, mm	Cyst % volume
241	19	1.9	51%
253	31	2.9	62%
29.7			

What opportunities for training and professional development has the project provided?

The grant was not meant to provide training or professional development. However, during the summer there were 3 high school students and 3 college students working in the Laboratory as volunteers to advance their professional skills in laboratory medical research. Dr Holditch attended the PKD Foundation Conference in Kansas City in July 2018. Dr Holditch presented a poster at this conference, got feedback on the poster and attended lectures that resulted in increased knowledge and skill in the area of PKD. Dr Holditch has submitted a K grant to the NIH to further study gene therapy in PKD animals.

How were the results disseminated to communities of interest?

Our results to date will be presented at the annual American Society of Nephrology Meeting in New Orleans in November, 2018 and at the Western Medical Research Conference meetings in Carmel, California in January 2019.

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

- 1) We have replaced the kidney-specific tamoxifen-inducible Pkd1 Cre mice with Ksp 1.3 Cre mice that have an embryonic Cre. We have obtained Raptor floxed mice and are in the process of breeding the Raptor floxed mice with mice with a Ksp 1.3 Cre mice to develop Raptor^{-/-}, Pkd1^{-/-} double knockout mice.
- 2) We have performed pilot studies to use a novel intervention; utilizing adeno-associated viral vectors (AAV) expressing the F113A 4E-BP1, in a murine model of PKD. F113A 4E-BP1 is a mutant that cannot be phosphorylated and should block the 4E-BP1 signaling pathway in PKD kidneys. We have found that the viral vectors target the kidney and have the desired effect on 4E-BP1 signaling. We have started a treatment study of the AAV expressing F113A 4E-BP1 in Pkd 1^{-/-} mice. We will determine whether the treatment reduces cyst growth and improves kidney function in Pkd1^{-/-} mice.
- 3) In the last year we have developed more Pkd1^{-/-} Rictor^{-/-} double knockout mice using the kidney-specific tamoxifen-inducible Pkd1 Cre. This gave inconsistent results and it looked like the kidney-specific tamoxifen-inducible Pkd1 Cre was working inconsistently. We have started breeding the Ksp

1.3 Cre mice with the Rictor floxed mice and Pkd1 floxed mice to develop a more rapid model of PKD in Pkd1 ^{-/-} Rictor ^{-/-} mice. We will perform MRI scans every 2-3 months in these mice and at sacrifice, measure kidney function, cysts volume and mTORC1 and mTORC2 signaling pathways. We expect that Rictor knockout will improve the cystic phenotype of Pkd1 ^{-/-} mice.

- 4) We plan to repeat the sirolimus (mTORC1 inhibitor) vs. Torin-2 (mTORC1 and mTORC2 inhibitor) study in Pkd1 ^{-/-} mice and administer the Torin2 via an Alzet osmotic mini pump as Torin2 has a short half life (<6 hrs). We will determine the effects of therapy on PKD and kidney function and store kidneys for study of mTORC1 and 2 signaling pathways.
- 5) The mTOR pathway is integral to autophagy. We will determine the effect of treatment of Pkd1 ^{-/-} mice with autophagy inducers e.g. 2 deoxy glucose, trehalose, Beclin-1 peptide, on mTORC1 and 2 signaling and PKD.
- 6) We have hearts from Pkd1 ^{-/-} mice. If time permits, we will study mTORC1 and 2 signaling pathways in hearts from Pkd1 ^{-/-} mice. It will be novel to discover that mTORC1 or 2 signaling is activated in the hearts of Pkd1 ^{-/-} mice.
- 7) We will continue to study mTORC1 and 2 signaling pathways especially 4E-BP1 in human Pkd1 ^{-/-} cells

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

A major finding is that we have determined that phosphorylation insensitive 4E-BP1 reduces hyperproliferative phenotype in vitro. Based on these data, we have performed pilot studies to use a novel intervention; utilizing adeno-associated viral vectors (AAV) expressing the F113A 4E-BP1, in a murine model of PKD in vivo. Use of this phosphorylation-insensitive 4E-BP1 may provide a future rationale for treatment to decrease PKD

Another major finding is that we have determined that the mTOR kinase inhibitor Torin2 is as effective as sirolimus to treat PKD in Pkd1 ^{-/-} mice. These findings provide a rationale for the use of combined mTORC1 and 2 inhibitors (the mTOR kinase inhibitors or TORCs) as future treatments in PKD.

Another major finding is that activation of the mTORC1 and 2 pathway in Pkd1 ^{-/-} mice is associated with suppressed autophagy in the kidney. We have determined that the suppressed autophagy in Pkd1 ^{-/-} kidneys can be rescued with autophagy inducers like 2 deoxy glucose.

What was the impact on other disciplines?

Many cancers are driven by increased 4E-BP1 signaling. Use of this phosphorylation-insensitive 4E-BP1 may provide a future rationale for treatment to decrease certain cancers.

Many cancers are driven by increased mTORC2 signaling. Demonstration that mTOR kinase inhibitors e.g. Torin2 decrease proliferation and have an effect on apoptosis may provide a future rationale for the use of mTOR kinase inhibitors to certain cancers.

Suppressed autophagy and increased mTOR signaling plays a role in disease processes like cancer and aging. Our studies of the effect of autophagy inducers on mTOR signaling and PKD will help in the understanding of the pathogenesis and treatment of other processes like aging and cancer.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

The study of mTOR kinase inhibitors like Torin2 or autophagy inducers to treat patients with ADPKD.

The genetic studies (Pkd1 ^{-/-} mice with additional knockout of mTORC1 or 2 or both) will offer mechanistic insights into novel mTORC1 and 2 signaling pathways in ADPKD.

The pharmacological studies (4E-BP1 inhibition, AktSer473, PKC α or SGK1 inhibition and TORKs) and gene therapy studies (AAV gene therapy against 4E-BP1) will provide insights into future therapies for PKD.

Discovery of new treatments for PKD could improve the quality of life and extend the life span of people with ADPKD.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

The tamoxifen-inducible ER2 Cre did not always work leading to mice with a very variable form of the ADPKD disease. We are now using KSP 1.3 Cre mice that have an embryonic Cre that is spontaneous (does not need tamoxifen) and the Pkd1 ^{-/-} mice develop PKD and kidney failure at a much earlier age. This model will lead to a more reproducible and faster model of ADPKD.

Actual or anticipated problems or delays and actions or plans to resolve them

The use of the tamoxifen-inducible ER2 Cre has lead to a delay in the development of a consistent and reproducible model of Pkd1 ^{-/-} mice and Pkd1, Raptor or Rictor double knockout mice. We are now using Ksp 1.3 Cre mice and have more than 70 breeding cages to breed the Ksp 1.3 Cre mice with Pkd1, Rictor and Raptor floxed mice. Thus we are attempting to speed the breeding process by increasing the number of breeding cages.

Changes that had a significant impact on expenditures

Purchase of Ksp 1.3 Cre mice and increased number of breeding cages has increased expenses.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

Publications, conference papers, and presentations

Nothing to report (yet)

Journal publications.

Nothing to report (yet)

Books or other non-periodical, one-time publications.

Nothing to report (yet)

Other publications, conference papers, and presentations.

Three abstracts accepted for presentation at the annual American Society of Nephrology meeting in San Diego in October 2018

1. **Dysregulated 4E-BP1 pathway in polycystic kidney disease**
2. **Rescue of suppressed autophagy in polycystic kidney disease**
3. **Head-to-head study of sirolimus and an mTOR kinase inhibitor (TORK) in a hypomorphic model of polycystic kidney disease**

Website(s) or other Internet site(s)

Nothing to report (yet)

Technologies or techniques

Nothing to report (yet)

Inventions, patent applications, and/or licenses

Nothing to report (yet)

Other Products

Nothing to report (yet)

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name:	Charles Edelstein
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month	12

worked:	
Contribution to Project:	Dr. Edelstein has overseen the design, performance and analysis of the studies
Funding Support:	DOD funding. VA Merit award funding.
Name:	<i>Sara Holditch</i>
Project Role:	<i>Post-doctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	<i>4E-BP1 studies, Creation of double knockout mice, Mouse treatment studies,</i>
Funding Support:	DOD funding. PKD Foundation Fellowship Award funding
Name:	Dan Atwood
Project Role:	PRA
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Breeding of single and double knockout mice. Genotyping of mice. Performing immunohistochemistry
Funding Support:	50% DOD funding. 50% VA Merit Award funding
Name:	<i>Nicole Brown</i>
Project Role:	<i>PRA</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6

Contribution to Project:	<i>Autophagy studies. Genotyping animals, animal injections, Performing immunoblots, General Lab Manager duties</i>
Funding Support:	50% DOD funding. 50% VA Merit Award funding

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

- 1) VA Merit Award VA Merit Award to Charles Edelstein . 1I01BX001737-01A1. The IL-33/ST2/CD4 cell system in acute kidney injury. No Cost extension 01/01/2017-01/01/2018. Bridge funding 01/01/18-04/01/18
- 2) VA Merit Award VA Merit Award to Charles Edelstein. BX003803-01A1. Autophagy in polycystic kidney disease. (Edelstein) 07/01/18-07/01/22.

What other organizations were involved as partners?

Nothing to report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable

QUAD CHARTS:

Not applicable

APPENDICES:

Attached are abstracts that have been accepted as poster presentations for the annual American Society of Nephrology meeting in October 2018

1. Dysregulated 4E-BP1 pathway in polycystic kidney disease
2. Rescue of suppressed autophagy in polycystic kidney disease
3. Head-to-head study of sirolimus and an mTOR kinase inhibitor (TORK) in a hypomorphic model of polycystic kidney disease

Dysregulated 4E-BP1 pathway in Polycystic Kidney Disease

Sara J Holditch¹, Dan J Atwood¹, Carolyn N Brown¹, Charles L Edelstein¹

¹Univ Colorado Denver;

Intro: Unchecked proliferation of cystic epithelial cells is a major contributor to cyst growth in PKD. The 4E-BP1 pathway is a crucial checkpoint in translation initiation and cellular proliferation, regulated by multiple stimulatory factors like PKC α , ERK, and AKT. The aim of this study was to 1) determine whether the 4E-BP1 pathway is dysregulated in human PKD1^{-/-} cells, 2) examine the effects of a 4E-BP1, blind to AKT and mTOR (F113A) on the 4E-BP1 pathway, protein translation, proliferation, and 3) assess the feasibility of *in vivo* gene therapy of F113A 4E-BP1 adeno-associated virus (AAV9) in neonatal and adult mice.

Methods: Western blot, proliferation, and firefly-renilla assays were performed on human primary epithelial cells from normal renal cortical tubular epithelium (PKD1^{+/+}) and ADPKD cyst-lining epithelium (PKD1^{-/-}) transfected with pCAG-TdTomato or pCAG-F113A. AAV9 F113A, and AAV9-TdTomato vectors were prepared and administered from D3 and to D17, or D120 to D180.

Results: There was increased phospho (p4E-BP1) species and increased pPKC α (3.6 $\pm$ 0.2 vs 0.6 $\pm$ 0.0DU***), pERK(3.6 $\pm$ 0.2 vs 0.5 $\pm$ 0.2DU***), and pAKT(0.6 $\pm$ 0.1 vs 0.2 $\pm$ 0.1DU*), in PKD1^{-/-} vs. PKD1^{+/+} cells respectively. *In vitro*, F113A expression reduced p4E-BP1 S65 (1.4 $\pm$ 0.2 vs 0.02 $\pm$ 0.01DU***), reduced cyclin D1 (0.65 $\pm$ 0.04 vs 0.51 $\pm$ 0.10DU*) and increased autophagosome marker, LC3-II, (0.02 $\pm$ 0.00 vs 0.31 $\pm$ 0.13DU*) expression in PKD1^{-/-} cells. Stimulation with insulin resulted in maintained p4E-BP1 S65 suppression with F113A in PKD1^{-/-} (2.1 $\pm$ 0.3 vs 0.2 $\pm$ 0.1DU***). F113A also reduced cap-dependent protein translation (by 37%**), and 72hr proliferation (250 $\pm$ 4 vs 180 $\pm$ 5 480/528nm O.D***). In neonatal and adult mice, administration of AAV9 resulted in detectable F113A RNA in the heart, kidney, and liver, and reduced p4E-BP1 S65 expression in the heart (2.0 $\pm$ 1.3 vs 0.6 $\pm$ 0.4DU*). *p<0.05, **p<0.01, *** p<0.001 vs to control.

Conclusions: In PKD, a setting of cystic tubular epithelial hyperproliferation, the 4E-BP1 pathway is dysregulated, with increased phosphoregulation of 4E-BP1 by multiple overactive kinases such as; PKC α , ERK, AKT. *In vitro*, F113A expression in PKD1^{-/-} cells, results in a shift towards hypophosphorylated 4E-BP1 species, reduced cap dependent protein translation, reduced proliferation, and increased autophagosomes. 4E-BP1 F113A gene therapy to counter the dysregulated 4E-BP1 pathway *in vivo* models of PKD is feasible to inhibit a pathway seemingly integral to the pathobiology of PKD.

Rescue of suppressed autophagy in polycystic kidney disease

Carolyn N Brown¹, Sara Holditch¹, Natalie Serkova, Andrew Thorburn¹, Charles Edelstein¹

Background: Autophagy maintains proteostasis by sequestering damaged organelles and proteins into autophagosomes for delivery to the lysosome where cargo is degraded and recycled. Several treatments that have been shown to ameliorate PKD in mice are potent activators of autophagy. The aims of the study were to determine the effects of several autophagy inducers on autophagy, apoptosis, and proliferation markers in PKD kidneys and to determine whether kidney-specific autophagy knockout results in a cystic phenotype.

Methods: Mice were treated with 2-deoxyglucose (2DG), trehalose (TRE), or metformin (MET) followed by bafilomycin (BAF) to measure autophagic flux. p62 (autophagy-specific tag for degradation & a marker of autophagy inhibition), LC3-II (a marker of autophagosomes), Atg12-5 (a marker of autophagosome elongation), cleaved caspase 3 (a marker of apoptosis), and AMBRA1 (Activating molecule in Beclin1-regulated autophagy) that links autophagy to cell proliferation by promoting dephosphorylation and degradation of cMyc (a transcription factor that activates pro-proliferative genes) were measured by immunoblot. Kidney-specific Atg7^{-/-} mice received MRIs at 180D of age to precisely measure cyst volume and number.

Results: PKD kidneys had significantly reduced autophagic flux compared to wild-type (WT), which was rescued by 2DG. Further, PKD kidneys had less Atg12-5 complex vs WT, an effect blocked by TRE. PKD kidneys had more cleaved caspase 3, a marker of apoptosis, compared to WT, which was decreased by MET, 2DG, and TRE. cMyc phosphorylation was greater in PKD compared to WT, an effect blocked by 2DG. Trehalose increased AMBRA expression in PKD kidneys. On MRI scan, 180 day old Atg7^{-/-} mice had a mean cyst volume of 6±0.4 and a mean number of cysts per kidney 3.8±1.5. Age-matched WT controls did not show any cysts on MRI.

Relative densitometry	FLUX	Atg12-5	p cMyc	AMBRA	CC3
WT VEH	0.4±0.05*	0.4±0.05*	0.8±0.1 *	0.7±0.1	1.0±0.05*
PKD VEH	0.1±0.03	0.2±0.02	1.3±0.1	0.7±0.1	1.6±0.09
PKD 2DG	0.3±0.06*	0.2±0.08	0.9±0.1*	1.3±0.1	1.0±0.15*
PKD TRE	0.1±0.08	0.3±0.07	1.2±0.2	1.2±0.1*	0.6±0.1*
PKD MET	0.1±0.07	0.2±0.02	-	-	0.9±0.08*

*p<0.05 vs PKD VEH

In summary, 2DG restored autophagic flux in PKD kidneys. Autophagy inducers increased expression of autophagy-related proteins and decreased apoptosis and proliferation markers. Kidney-specific knockout of autophagy resulted in a cystic phenotype.

Head-to-head study of sirolimus and an mTOR kinase inhibitor (TORK) in a hypomorphic model of polycystic kidney disease

Sara J Holditch¹, Dan J Atwood¹, Carolyn N Brown¹, Charles L Edelstein¹

¹Univ Colorado Denver;

Background: Sirolimus indirectly inhibits mTORC1 and reduces but does not completely inhibit cyst growth in rodent models of autosomal dominant polycystic kidney disease (ADPKD). Sirolimus was not effective in human PKD and was associated with side effects. The novel ATP competitive mTOR kinase inhibitors (TORKs) e.g. Torin2 directly inhibit mTOR kinase that results in inhibition of both mTORC1 and 2. TORKs, which increase liver enzymes, have a different side effect profile to sirolimus, which causes mucositis. We report a head-to-head comparison of Torin2 versus sirolimus in a hypomorphic mouse model of PKD.

Methods: C57Bl/6 *Pkd1* p.R3277C (PKD) mice were treated with 0.5mg/kg sirolimus (SIR) or 10mg/kg Torin2 (TORK) daily from 50 to 120 days of age. Serum BUN was analyzed using an enzymatic assay. Cyst area was quantified from H&E stained kidneys. Kidneys were immunoblotted for mTORC1 substrates, pS6 and p4E-BP1 (S65), mTORC2 substrate, pAkt (S473), LC3-II (autophagosome marker), and p62 (autophagy-specific ubiquitin-binding protein). Autophagic flux was defined as the difference between LC3-II before and after administration of the lysosomal inhibitor bafilomycin.

Results: Kidney weight and BUN were significantly decreased in TORK and SIR-treated mice compared to vehicle (VEH). CVD showed a tendency to decrease with both TORK and SIR. pAKT was decreased in both TORK and SIR treatments. pS6 was significantly decreased in SIR-treated mice only. TORK significantly increased autophagic flux (FLUX). No side effects were noted on gross examination in either treatment group.

	KW/BW (%)	BUN (mg/dL)	pAKT	pS6	FLUX	p62	Cyst area (%)	p4EBP1
PKD VEH	2.6±0.1	63±2	0.8±0.2	1.3±0.1	0.02±0.1	1.03±0.05	31.6±3.2	0.7±0.1
PKD TORK	2.1±0.2*	41±4*	0.3±0.1*	1.0±0.2	0.82±0.2*	0.88±0.02	22.1±4.8	0.4±0.1
PKD SIR	2.3±0.02*	39±4*	0.2±0.1*	0.7±0.04*	0.10±0.2	0.86±0.01	20.2±3.2	0.4±0.1

*p<0.05 vs VEH, n=4

Conclusions: For the first time, we have shown in a head-to-head study that the TORK, Torin2, is as effective as sirolimus in decreasing kidney size and BUN in a hypomorphic ADPKD mouse model. Both drugs inhibited pAKT. SIR had a better effect on pS6 than TORK. Interestingly, TORK resulted in a significant increase in autophagic flux. As Torin2 has a short half-life (~1 hr), further studies are warranted to determine whether more frequent dosing of Torin2 will be more effective than sirolimus.