Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Children's Hospital of Pittsburgh of the UPMC Health System

2. **Reporting Period (start and end date of grant award period):** 1/1/2011-12/31/2013

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):**David H. Perlmutter, MD

4. **Grant Contact Person's Telephone Number:** 412-692-6081

5. **Grant SAP Number:** 4100054844

6. **Project Number and Title of Research Project:** Project #2 Defining the Role of microRNAs in Podocyte Function and the Renal Stroma

7. **Start and End Date of Research Project:** 1/1/2011-12/31/2013

8. **Name of Principal Investigator for the Research Project:** Jacqueline Ho, MD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 200,305.18

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Ho, Jacqueline	Principal Investigator	10% Yrs 1-3	$37,143.55
Bodnar, Andrew J.	Research Technician	50% Yr 1-2	$36,630.15
Bodnar, Andrew J.	Research Technician	14% Yr 3	$5,478.72
Tumir, Christopher	Student Researcher	100% Yrs 1-2	$1,791.38

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Chu, Jessica YS	Postdoctoral fellow	50%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
MicroRNAs in the renal stroma.	☐NIH ☐ Other federal (specify:_____ _____) X Nonfederal source (specify: March of Dimes)	June 2013	$150,000	$150,000
	☐NIH ☐ Other federal (specify:_____ _____) ☐Nonfederal source (specify:)		$	$
.	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify:)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

We plan to submit an R01 based on our work investigating a role for miRNAs in the renal stroma in approximately 1 year (in the interim, the project is being funded by a March of Dimes Basil O'Connor Starter Scholar Award).

The preliminary data for the Satellite Health care grant was obtained from results being generated for the CURE project.

12. Future of Research Project. What are the future plans for this research project?

The future plans for this research project are based on two goals. Firstly, we will use *in vitro* and *in vivo* approaches to validate predicted miRNA targets in podocytes. Ultimately, we aim to determine the functional significance of these miRNA-mRNA target interactions in podocyte structure and function, as a means of understanding why mis-regulation of miRNAs in renal disease may result in renal pathology. Secondly, we aim to define a role for miRNAs in the renal stroma, with a specific focus on its role in patterning nephron progenitors and the glomerular mesangium. Both of these goals will enable an improved understanding of congenital and glomerular diseases of the kidney, leading causes of renal failure in children.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male	1	0	0	0
Female	0	0	0	1
Unknown	0	0	0	0
Total	**1**	**0**	**0**	**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic	0	0	0	0
Non-Hispanic	1	0	0	1
Unknown	0	0	0	0
Total	**1**	**0**	**0**	**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White	1	0	0	0
Black	0	0	0	0
Asian	0	0	0	1
Other	0	0	0	0
Unknown	0	0	0	0
Total	**1**	**0**	**0**	**1**

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

    Yes____X_____ No_____

    If yes, please list the name and degree of each researcher and his/her previous affiliation:

    Jessica YS Chu, PhD, University of Hong Kong

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

    Yes_____ No____X_____

    If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. **Collaboration, business and community involvement.**

    16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

    Yes_____ No____X_____

    If yes, please describe the collaborations:

    16(B) Did the research project result in commercial development of any research products?

    Yes_____ No____X_____

    If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

MicroRNAs (miRNAs) are a novel class of small noncoding RNAs that regulate gene expression via the post-transcriptional repression of specific target mRNAs. The long-term goal of our laboratory is to characterize the molecular pathways regulated by miRNAs in kidney development and disease. The renal stroma plays a crucial role in nephron formation, and given its close proximity to nephron progenitors, likely forms an important microenvironment or

"niche" for these cells. However, the interaction between nephron progenitors and their presumed "niche" in the determination of congenital nephron number remains poorly defined. Our laboratory has data suggesting that loss of miRNAs in the renal stroma results in an expanded nephron progenitor pool and mis-regulated nephrogenesis.

The glomerulus acts as the filtering unit of the kidney. The filtration barrier is composed of fenestrated endothelial cells, the glomerular basement membrane, and the podocyte, a highly specialized epithelial cell that is often injured or lost in glomerular diseases. Our work demonstrates that miRNA function in the podocyte is critical in maintaining this filtration barrier. In this research project, we wish to characterize the molecular pathways that regulate the podocyte and renal stroma during kidney development. *We hypothesize that miRNAs regulate key mRNA transcripts required for podocyte structure and function, and the renal stroma.* To test this hypothesis, we propose the following aims:

Revised Specific Aims: **Note that this project was revised. On 11/21/2011 the Department of Health approved a request from Children's Hospital of Pittsburgh to revise this project. Based on preliminary data obtained in the first year of funding for the project, the Children's Hospital of Pittsburgh obtained funding from the Norman S. Coplon Extramural Grant to complete the remainder of the specific aims outlined in the original proposal. The revised strategic plan expanded on the goals of the original project to address specific miRNA-mRNA target interactions identified in their recent work, which was not currently funded by the Coplon grant. The intent of the revised research project is to collect data over the remaining grant period to define the requirement for miRNAs in the renal stroma.*

(Revised) Specific Aim 1: To validate and determine the function of miRNA-mediated regulation of Ulk1 in podocytes.
(Revised) Specific Aim 2: To define the role of miRNAs in regulating developing nephron progenitors in the early mesenchyme and the renal stroma.

Original Specific Aims:
Specific Aim 1: To identify podocyte-specific miRNA-mRNA target interactions.
Specific Aim 2: To define the function of miRNAs in podocytes using complementary *in vitro* and *in vivo* approaches.

Summary of Research Completed

Progress on Specific Aim 1 (Original):
The goals in Specific Aim 1 (Original) were almost fully completed, as we identified several plausible candidate podocyte-specific miRNA-mRNA target interactions that we continue to validate. The remainder of the validation experiments were subsequently funded by the Norman S. Coplon Extramural Grant, and a revised proposal was submitted as noted above.

We have profiled the expression of miRNAs in the glomerulus using miRNA microarrays. The top twenty glomerular miRNAs were selected for further bioinformatics analysis to identify potential miRNA targets (in descending order: mmu-let7a-e, mmu-let-7c, mmu-let-7b, mmu-miR-143, mmu-miR-23b, mmu-miR-23a, mmu-miR-26a, mmu-miR-126-3p, mmu-miR-30c,

mmu-miR-744, mmu-let-7i, mmu-miR-10a, mmu-miR-125a, mmu-miR-214, mmu-miR-24, mmu-miR-30b, mmu-miR-503, mmu-miR-16, mmu-miR-26b, mmu-miR-191). In collaboration with Dr. Priyanka Pandey, possible targets were individually analyzed using four publicly available bioinformatics miRNA target prediction tools: TargetScan, miRBase/miRanda, microT and MAMI. Custom-written Perl scripts were used to generate lists of predicted miRNA targets based on their identification by two, three, or all four, target prediction algorithms. These targets were then mapped against a published 'meta-analysis' of previous glomerular transcriptome profiling experiments (He et al, 2008, JASN, 19: 260-268). This analysis identified 85 miRNA-mRNA target pairs that were predicted by all four algorithms and 929 target pairs that were predicted by three or more tools (data not shown).

The list of possible targets was narrowed to a subset of miRNA-mRNA target interactions for further study based on the bioinformatics scores for the predicted interactions, predicted and/or known function(s) of the encoded proteins and expression of the miRNAs in the glomerulus. Initially, we focused on potential targets of mmu-miR-23b, mmu-miR-24 and mmu-miR-26a (Table 1), since we had previously defined their glomerular expression by LNA in situ hybridization. We anticipated that biologically important targets of these miRNAs would also be expressed in glomeruli, and were specifically interested in those that may be implicated in the regulation of podocyte structure and function. Thus, we confirmed the expression of nine out of ten candidate miRNA target genes using reverse-transcriptase polymerase chain reaction (RT-PCR) on total RNA from wild-type mouse glomeruli (Figure 1). Semaphorin-6D was the only transcript that was not identified in glomeruli.

Table 1: Potential glomerular targets of mmu-miR-23b, mmu-miR-24 and mmu-miR-26a. Targets were predicted using the following bioinformatic algorithms: TargetScan, miRBase/miRanda, microT and MAMI. Predicted targets were mapped against a 'meta-analysis' of previous glomerular transcriptome profiling experiments (He et al, 2008, JASN, 19:260-268). TS, Target Scan; MI, miRBase. The context score refers to the score assigned by TargetScan (the higher the score, the more likely the target represents a true biologic target).

Gene ID	Gene	Expression data	Target Prediction	No of miRNA sites	Context Score	Biological Relevance
RAP1A	small GTPase RAP	Affymetrix	TS, MI	2 23a; 1 24; 1 26a	-0.35	integrin-med cell adhesion
MRC1	mannose receptor, C type 1	EST, GlomChip	TS, MI	3 miR23b	-0.58	dendritic cells (APCs)
MRC1L1	mannose receptor, C type 1-like 1	EST, GlomChip	TS, MI	3 miR23b	-0.58	
ULK1	unc-51-like kinase	Affymetrix	TS, MI	2 miR26a; 1 miR24	-0.49	axon branching
SEMA6D	semaphorin 6D	Affymetrix, RT	TS, MI	2 miR23b; 1 miR26a	-0.41	axon guidance
PLOD2	procoll-lys, 2-oxoglut 5-dioxygen 2	Affymetrix	TS	1 miR24;2 miR26a	-0.37	collagen synthesis
TMEM2	transmembrane protein 2	Affymetrix	TS, MI	1 miR23b; 1miR26a	-0.52	deafness/PKD
PITPNC1	phosphatidylinositol transfer prot	Affymetrix	TS, MI	1 miR23b; 1 miR26a	-0.41	retinal neurons; cytokinesis
RAP1B	small GTPase RAP	EST	TS	1 miR24; 1 miR23b	-0.33	mesangial expression
ADM	adrenomedullin	Affymetrix, T Podo	TS	1 miR26a, 1 miR-24	-0.30	podocyte injury (anti-O)
PKP4	plakophilin 4	SAGE	TS, MI	1 miR23b	-0.50	cell-cell junctions
TOB1	transducer of ERBB2, 1	EST	TS, MI	1 miR26a	-0.49	tumor suppressor
ZDHHC6	zinc finger, DHHC type	SAGE, array	TS, MI	1 miR26a	-0.47	novel
PTP4A2	protein tyrosine phosphatase 4A2	EST; ISH	MI	1 miR23b	-0.37	tumorigenesis
PTPN13	protein tyrosine phosphatase	EST	TS, MI	1 mir26a	-0.35	apoptosis; axon branching
ZNF608	zinc finger 608	Glomchip	TS, MI	1 mIR26a	-0.35	DNA damage
DLG5	discs, large homolog 5	Affymetrix; ISH	TS, MI	1 miR26a	-0.33	cysts in KO; CD
CD200	CD200	GlomChip; Affy; IHC	TS	1 miR26a	-0.32	immune modulation
EPHA2	EPH receptor A2	GlomChip	TS, MI	1 mir26a	-0.30	axon guidance

To exclude those transcripts that were expressed by other cell types present in glomeruli such as endothelial or mesangial cells, RT-PCR was performed on total RNA isolated from a conditionally immortalized podocyte cell line that was grown under conditions that would permit

differentiation (37°C) or ongoing replication (33°C). Both Sema-6D and Mrc-1 were not expressed in the podocyte cell line. The putative miRNA targets (present in wild type glomeruli and the podocyte cell line) include transcripts that have been implicated in integrin-mediated cell-adhesion (Rap1a), cell-cell junctions (Pkp4), axon branching (Ulk1), collagen synthesis (Plod2), polycystic kidney disease (Tmem2), cytokinesis (Pitpnc1), mesangial cell function (Rap1b), and podocyte injury (Adm).

Biologically relevant miRNA targets would be predicted to be up-regulated in *NPHS2-cre*, *Dicer*$^{Flx/Flx}$ podocytes. Of the targets listed above, our preliminary results suggest that unc-51-like kinase-1 (Ulk1) and procollagen-lysine, 2- oxoglutarate 5-dioxygenase 2 (Plod2) are up-regulated in *NPHS2-cre*, *Dicer*$^{Flx/Flx}$ glomeruli by real-time PCR (Figure 2). Interestingly, knockdown of Ulk1/2 has been shown to result in excessive axon arborization and stunted axon elongation, processes akin to foot process formation and remodeling in the podocyte. Ulk1 and Ulk2 are two of the most closely related members of the mammalian homologues of yeast Atg1, which is known to be critical for autophagy. Moreover, recent work has demonstrated that podocyte-specific deletion of Atg5 results in a glomerulopathy, thought to be related to the dependence of podocytes on autophagy to maintain cellular homeostasis in response to stress. In contrast, Plod2 is a lysyl hydroxylase that catalyzes the hydroxylation of lysine residues in collagen, influencing the stability of the extracellular matrix. In the kidney, two isoforms of Plod2 are expressed, and the differing specificities of these two isoforms for lysine residues are thought to affect collagen strength.

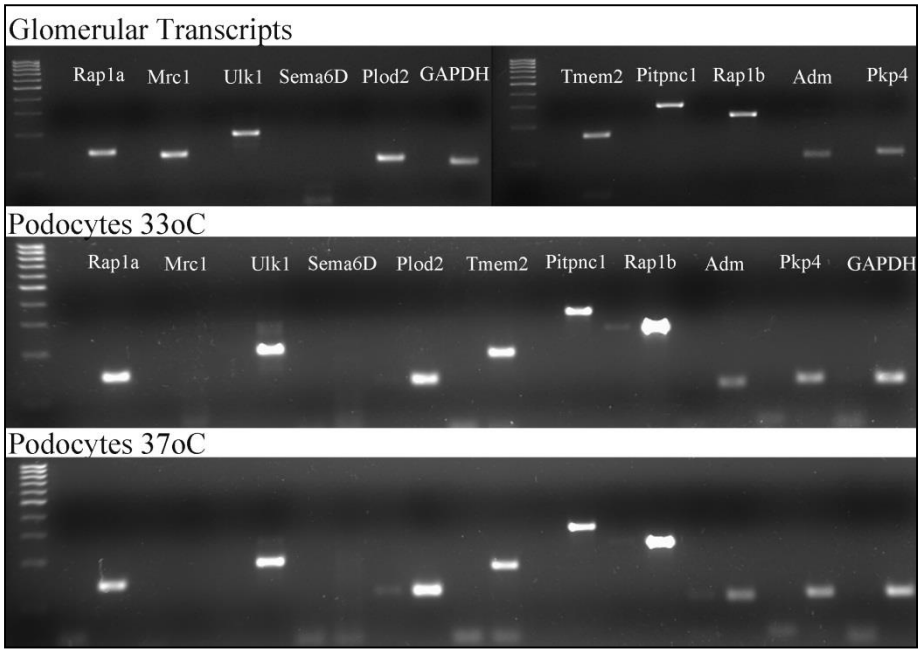

Figure 1. RT-PCR for putative targets of mmu-miR-23b, mmu-miR-24 and mmu-miR-26a using total RNA isolated from mouse glomeruli (top), undifferentiated podocyte cell line (middle) and differentiated podocyte cell line (bottom). For each sample, a no RT control was run to the left of the transcript of interest. GAPDH was used as the positive control.

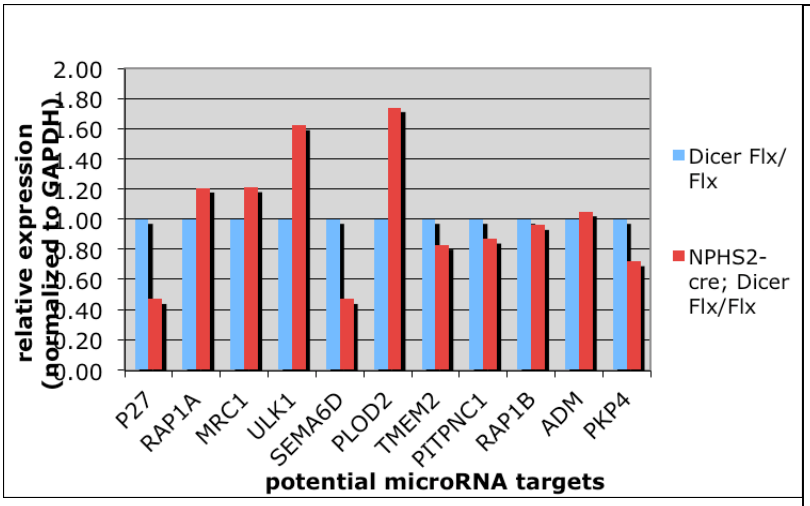

Figure 2. Real-time PCR demonstrating increased expression of Ulk1 and Plod2 in *NPHS2-cre, Dicer*$^{Flx/Flx}$ when compared to control glomeruli. The expression of p27 and Sema6D is down-regulated in the mutants, and in the remaining candidate miRNA targets, the expression is not significantly changed (Rap1A, Mrc1, Tmem2, PITPNC1, Rap1b, Adm and Pkp4).

Progress on Specific Aim 2 (Original):

The goals of Specific Aim 2 were partially achieved, as the remainder of this aim was subsequently funded by the Norman S. Coplon Extramural Grant, as noted above. In addition, we continue to have technical challenges with transfecting the immortalized podocyte cell line with high efficiency.

We obtained a conditionally immortalized murine podocyte cell line that was recently characterized by Dr. Valerie Schumacher at Children's Hospital of Boston, Harvard Medical School. When these cells are grown under nonpermissive conditions (37°C), the podocytes undergo cell cycle arrest, express numerous podocyte-specific proteins and form cellular processes with an ordered array of actin fibers and microtubules, reminiscent of podocyte foot processes *in vivo*. The expression of glomerular miRNAs in this cell line was confirmed by Northern blot (Figure 3). Total RNA was isolated from podocytes grown at 33°C and 37°C, run on a polyacrylamide gel, transferred to a nylon membrane, and hybridized overnight with P^{32}-labeled oligonucleotides complementary to the mature miRNA sequence. Interestingly, expression of all three glomerular miRNAs of interest (mmu-miR-23b, mmu-miR-24 and mmu-miR-26a) was up-regulated following differentiation of the podocyte cell line. Although mmu-let-7c and mmu-miR-10a were also induced in differentiated podocytes, mmu-let-7c is expressed ubiquitously and mmu-miR-10a in tubular elements of the adult kidney based on our previous LNA in situ hybridization data. Several miRNAs that are expressed in the developing mouse kidney were either not detected by Northern (mmu-miR-335; mmu-miR-30c) or down-regulated in differentiated podocytes (mmu-miR-130a). We continue to optimize techniques for miRNA knockdown in this podocyte cell line to evaluate the requirement for specific miRNAs in podocyte structure *in vitro*, as this will serve as an important *in vitro* model.

Ultimately, we aim to test these potential novel miRNA targets in podocytes using an *in vitro* luciferase reporter assay, confirm whether they are indeed upregulated in podocytes of the mutant glomeruli, and determine whether the mRNA-miRNA interaction is functionally significant. This work is continues to be in progress for those transcripts identified in Specific Aim 1, original.

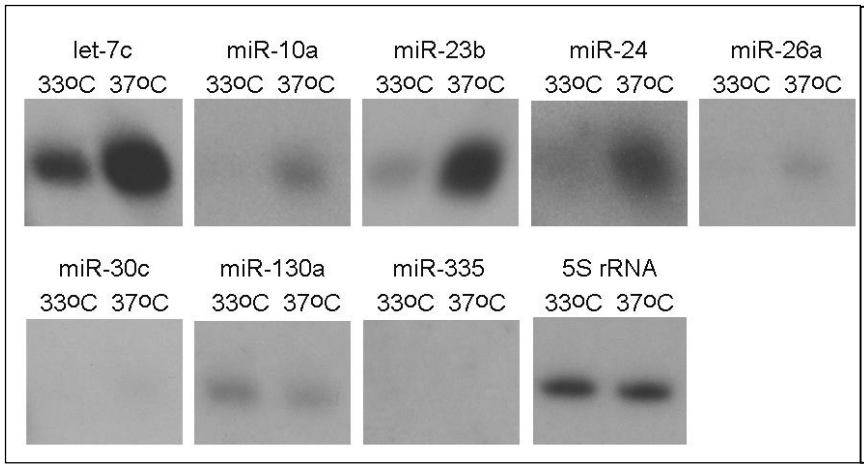

Figure 3: Northern blot analysis for miRNA expression in podocytes grown under permissive (33°C) or nonpermissive conditions (37°C). 5S rRNA was used as a loading control.

Progress on Specific Aim 1, revised:
This aim was partially achieved, and was limited by several Ulk1 antibodies that were tested that did not work well with immunostaining or immunoblotting.

We are currently confirming the regulation of Ulk1 using an *in vitro* luciferase reporter assay. The Ulk1 antibodies that we have tested to date have not worked well in adult kidney sections or Western blot, so these are ongoing experiments.

Progress on Specific Aim 2, revised:
This aim was fully achieved, and formed the basis of one publication (Chu et al, 2014) and one successful grant application during the funding period (March of Dimes Basil O'Connor Starter Scholar Award).

To define the requirement for miRNAs in the early metanephric mesenchyme (which gives rise to nephron progenitors and the renal stroma during kidney development), we generated a transgenic mouse model that lacks mature miRNAs in this lineage: *Pax3CreTg, Dicer*$^{Flx/Flx}$. The early loss of miRNAs results in severe renal agenesis by embryonic day 14 (E14). This occurs despite normal ureteric bud outgrowth from the Wolffian duct, as visualized by calbinden staining, and normal condensation of the metanephric mesenchyme to form the cap mesenchyme (or nephron progenitors) at E11 (Figure 4). The initial specification of this lineage appears intact when evaluated by staining for transcription factors known to be expressed in nephron progenitors, including Six2, Pax2 and NCAM (Figure 4). There is no gross difference in proliferation in control and mutant metanephric mesenchyme (data not shown); however, there is significantly increased apoptosis in the mutant metanephric mesenchyme as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining at E11 (Figure 5). By E12, the metanephric mesenchyme is markedly abnormal with increased apoptosis and failure of the ureteric bud to branch, likely secondary to the mesenchymal defects (data not shown).

We have subsequently shown that there was a significant increase in the expression of the pro-apoptotic protein Bim in the mutant mesenchyme (Figure 6). Bim often interacts with the pro-survival protein, Bcl2, which was unchanged between control and mutant kidneys (Figure 6).

Two miRNAs that have either been shown to target Bim or are predicted to target Bim, mmu-miR-10a and mmu-miR-17, are expressed in the developing kidney at E11.5 by locked nucleic acid *in situ* hybridization (LNA-ISH) (Figure 7). We also show that there was loss of these miRNAs in *Pax3CreTg, Dicer*$^{Flx/Flx}$ mutant kidneys by both LNA-ISH and quantitative real-time PCR using Taqman miRNA assays, as would be expected in this model (Figure 7). Overall, these data are the first to demonstrate an early requirement for miRNAs in the metanephric mesenchyme, and suggests a crucial role for miRNAs in regulating the survival of this lineage (Am J Physiol Renal Physiol, in press).

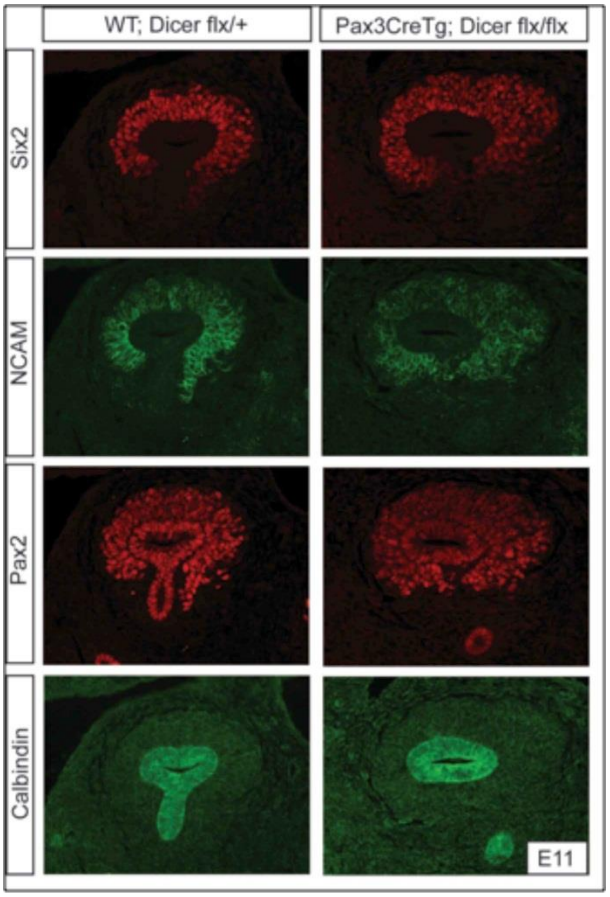

Figure 4. Immunofluorescence staining for E11 control and *Pax3CreTg, Dicer*$^{Flx/Flx}$ embryos for Six2, NCAM and Pax2 showing normal initial specification of the metanephric mesenchyme. There is also normal expression of calbindin and Pax2 in the ureteric bud.

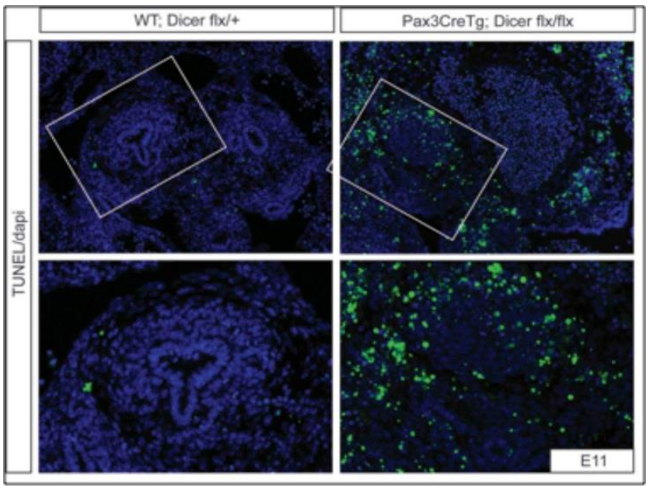

Figure 5. TUNEL staining for E11 control and *Pax3CreTg, Dicer*$^{Flx/Flx}$ embryos demonstrating increased apoptosis in mutant kidneys. The bottom panels are higher magnification images from the outlined boxes in the upper panels.

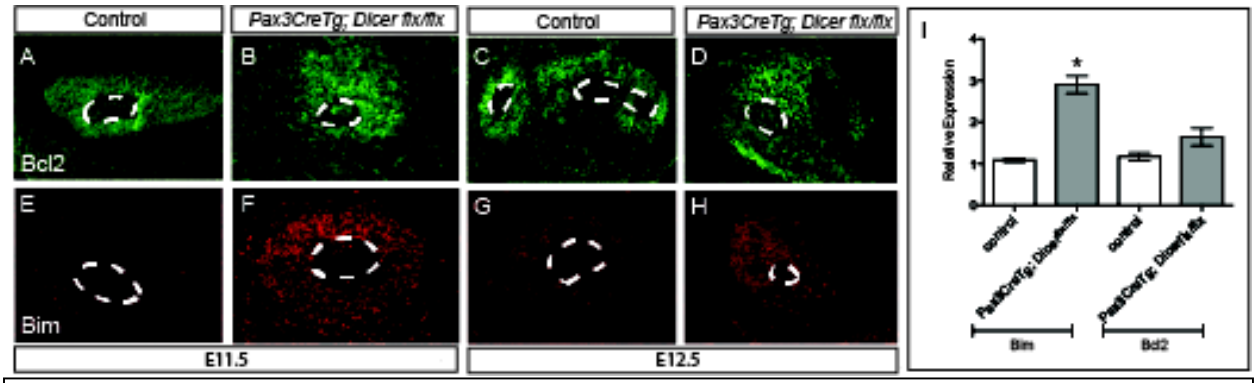

Figure 6. Immunofluorescent staining demonstrates increased Bim and unchanged Bcl2 expression in the metanephric mesenchyme of *Pax3Cre*^{Tg}, *Dicer*$^{flx/flx}$ E11.5 and E12.5 kidneys. (A-D) Bcl2 staining in control (A, C) and *Pax3Cre*^{Tg}, *Dicer*$^{flx/flx}$ kidneys (B, D) is unchanged at E11.5 and E12.5. (E-H) Bim staining in *Pax3Cre*^{Tg}, *Dicer*$^{flx/flx}$ kidneys (F, H) is increased at E11.5 and E12.5 when compared to controls (E, G). Magnification, 20X. (I) Quantitative real-time PCR performed on total RNA isolated from E11.5 kidneys confirms increased expression of Bim ($p<0.05$, paired t-test, *), and no significant difference in Bcl2 expression ($p > 0.05$, paired t-test). Bars represent standard errors of the mean.

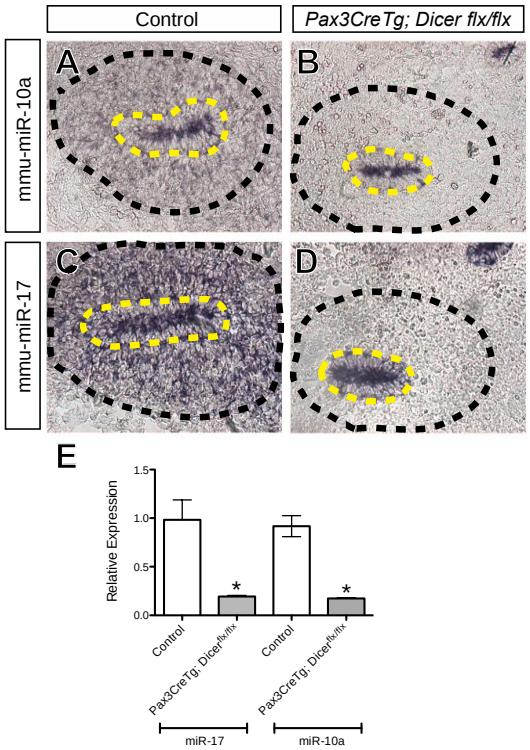

Figure 7. Cre-mediated excision of the conditionally floxed *Dicer* allele via the *Pax3Cre*^{Tg} allele results in loss of miRNA expression in the metanephric mesenchyme at E11.5. (A-D) LNA in situ hybridization demonstrates loss of mmu-miR-10a and mmu-miR-17-5p expression in the metanephric mesenchyme of *Pax3Cre*^{Tg}, *Dicer*$^{flx/flx}$ kidneys (B, D) compared to controls (A, C). Black dashed line, metanephric mesenchyme; yellow dashed line, ureteric bud. The magnification is 20X. (E). Quantitative real-time PCR performed on total RNA isolated from E11.5 kidneys confirms decreased expression of mmu-miR-10a and mmu-miR-17-5p ($p<0.05$, paired t-test, *). The relative expression of mmu-miR-10a and mmu-miR-17-5p denotes the fold change in delta CT in mutants normalized to controls. Bars represent standard errors of the mean.

The renal stroma plays a critical role in nephron formation, and given its close proximity to nephron progenitors, likely forms an important microenvironment or "niche" for these cells during kidney development. To examine the requirement for miRNAs in the renal stroma, we generated mice that lack functional miRNAs in this lineage using a conditionally floxed allele of *Dicer*, which is required to form mature miRNAs, and *Foxd1cre*, which drives the loss of *Dicer* expression in renal stroma and its derivatives. Histological examination of the mutant kidneys during development reveals an expansion of the "cap" of nephron progenitors around ureteric bud tips, and disorganization of the developing nephron structures just below the renal capsule, in the area termed the nephrogenic zone. These persist through to postnatal day 0 (P0), at which point the loss of mature miRNAs in the renal stroma results in significantly smaller kidneys and perinatal lethality (data not shown).

The renal stroma is thought to give rise to the supporting network of mesangial cells within the glomerulus, a subset of the renal vasculature, and the renal interstitium. To further define the impact of loss of mature miRNAs in the renal interstitium, whole mount immunofluorescence was performed for several markers of early renal lineages at E15 and E18: FoxD1 for the renal stroma itself, Six2 for the cap mesenchyme, and calbindin for ureteric buds. This demonstrated a decrease in FoxD1-expressing renal stromal cells, increased Six2-expressing nephron progenitors, and abnormal ureteric branching in *FoxD1cre; Dicer*$^{flx/flx}$ kidneys, as early as E15 (Figure 8A). The decrease in FoxD1 and increase in Six2 expression was confirmed by quantitative PCR (qPCR) (Figure 8B). Overall, the Six2-positive nephron progenitors appear less compact around the ureteric bud tips. Together, these data support the notion that miRNA loss in the renal stroma influences the stromal signals that normally regulate nephron progenitor proliferation, nephron differentiation, and ureteric branching.

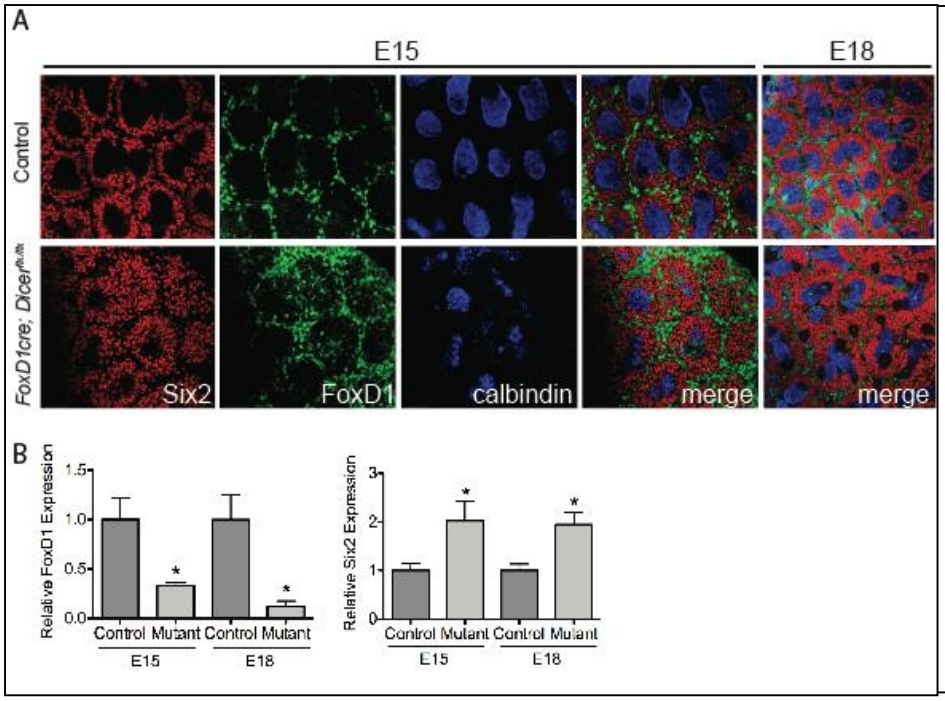

Figure 8A. Confocal images of wholemount immunostaining for Six2, FoxD1 and calbindin in control and *FoxD1cre; Dicer*$^{flx/flx}$ E15 and E18 kidneys. **B.** qPCR using Taqman assays confirmed increased Six2 and decreased FoxD1 expression in control and mutant kidneys.

The decreased FoxD1 expression suggests there may be a cell-autonomous defect in FoxD1-expressing cells and their descendents in *FoxD1cre; Dicer*$^{flx/flx}$ kidneys. Immunofluorescence

was performed to evaluate early smooth muscle cells, which are derived from the renal stroma, using an anti-SM22 antibody (Figure 9, left panels). There was a marked decrease in SM22-positive cells in E18 mutant kidneys. To determine if this was related to increased cell death, terminal deoxynucleotidyl transferase dUTP nick end label (TUNEL) staining was performed. This demonstrated increased apoptosis in the renal stroma as early as E15 in *FoxD1cre; Dicer*$^{flx/flx}$ kidneys (Figure 9, right panels). Recent work has implicated the stroma in vascular development in the kidney, both in terms of giving rise directly to a subset of renal vessels, and in providing the pericytes that serve as key supportive cells for the renal vasculature. PECAM staining reveals abnormal vascular patterning in the mutant kidneys, including the presence of blood vessels in the renal capsule (Figure 9, middle panels). Thus, miRNAs are likely to play an important role in regulating the molecular mechanisms underlying the development of the renal stroma itself.

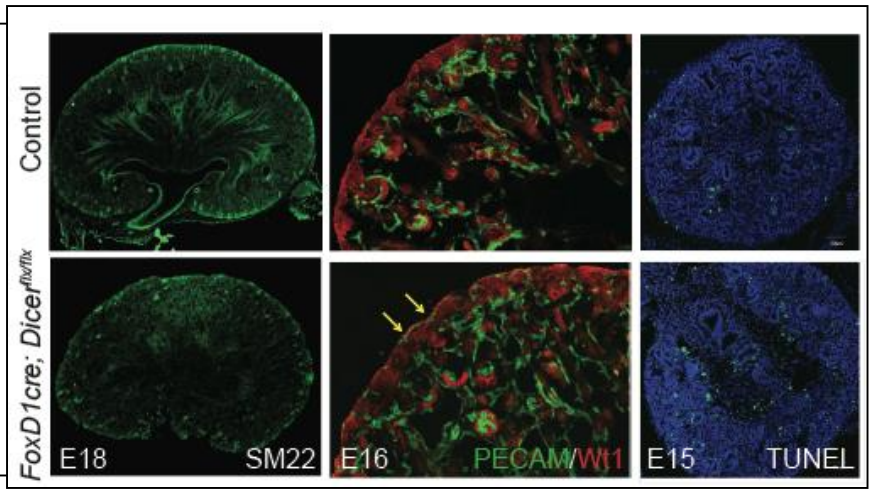

Figure 9.
Immunofluorescent staining for SM22 (early smooth muscle marker), PECAM (endothelial cells), Wt1 (cap mesenchyme and early podocytes) and TUNEL in control and *FoxD1cre; Dicer*$^{flx/flx}$ E15 and E18 kidneys. Vessels in the renal capsule, yellow

Glomerular mesangial cells are also derived from the renal stroma. Interestingly, histological examination of E18 *FoxD1cre; Dicer*$^{flx/flx}$ mutant kidneys demonstrated disorganized or absent capillary loops in the developing glomerulus (Figure 10, left panel). This phenotype is reminiscent of several mutations in signaling pathways or transcription factors that are thought to be critical in glomerular mesangial development, including: PDGFRβ, PDGFβ, EphrinB2, GPR4, Notch2, CXCR4, Pod1 and Foxc2. Immunofluorescent staining for the podocyte marker, Wt1, revealed normal glomerular expression (Figure 10, red staining). In contrast, there were aneurysmal capillary loops identified in the mutant glomeruli via PECAM staining for endothelial cells (Figure 10, left panels). In addition, mesangial cell loss was observed, given the absence of the early mesangial cell marker, PDGFRβ (Figure 10, middle panels). Finally, fewer parietal cells were observed with SM22 staining (Figure 10, right panels). These data imply a crucial role for miRNAs in glomerular mesangial cell development, a process which continues to be relatively undefined.

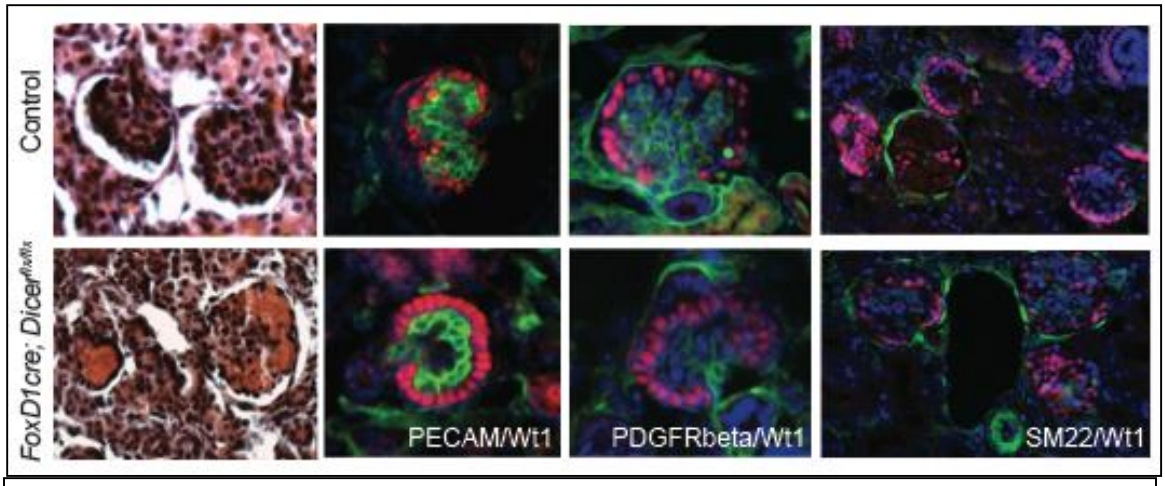

Figure 10. Histology (left panels) and immunofluorescent staining for PECAM (endothelial cells), PDGFRβ (mesangial cells) and SM22 (parietal epithelial cells) (green) in control and *FoxD1cre; Dicer*$^{flx/flx}$ E18 glomeruli. All sections were co-labeled with Wt1 (red) to mark podocytes.

To identify miRNAs expressed in the renal stroma, we crossed *FoxD1cre; Dicer*$^{flx/+}$ mice with a Rosa-CAG-dTomato reporter mouse line, which will label renal stroma and its derivatives in fluorescent Tomato red. We performed fluorescence-activated cell sorting (FACS) to isolate control and mutant FoxD1-derived cells from dissociated kidneys in collaboration with Dr. Sunder Sims-Lucas, Department of Pediatrics, University of Pittsburgh. We plan to compare the profile of small RNAs and mRNA transcripts in control and *FoxD1cre; Dicer*$^{flx/flx}$ CAG-positive renal stroma to identify miRNAs expressed in the renal stroma, and define changes in the renal stromal transcriptome of mutant kidneys. Thus, we have prepared total RNA for high-throughput RNA sequencing on three independent sets of pooled total RNA from CAG-positive stromal cells isolated from control and mutant kidneys, and sent these samples to BGI Americas. For mRNA deep sequencing, BGI Americas is generating cDNA libraries following amplification. BGI Americas will then perform 50 base pair single-end sequencing reads for small RNAs, or 100 base pair paired-end sequencing reads for mRNA transcripts (with a goal of 10 million reads/sample for small RNAs and 30-40 million reads/sample for mRNA transcripts to achieve adequate depth of sequencing coverage) using the Illumina HiSeq 2000 sequencing system. We are currently awaiting the sequencing data.

Published Abstracts, Presentations

Ho J et al, 2012, "MicroRNAs in the metanephric mesenchyme are critical for early kidney development." American Society for Nephrology meeting, San Diego, CA [poster presentation].

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American

_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X___ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase

Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Dicer function is required in the metanephric mesenchyme for early kidney development.	Jessica Y.S. Chu, Sunder Sims-Lucas, Daniel S. Bushnell, Andrew J. Bodnar, Jordan A. Kreidberg and Jacqueline Ho	Am J Physiol Renal Physiol	July 2013	☐Submitted XAccepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans:

We plan to submit a paper regarding the initial phenotypic description of the *FoxD1cre; Dicer*$^{flx/flx}$ mice within the next three months.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project.

Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Overall, these data are the first to demonstrate an early requirement for miRNAs in the metanephric mesenchyme during kidney development, and suggests a crucial role for miRNAs in regulating the survival of this lineage. Moreover, our data also describe a novel role for miRNAs in the renal stroma and developing glomerular mesangium, that we are currently characterizing in greater detail.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Ho, Jacqueline	POSITION TITLE Assistant Professor
eRA COMMONS USER NAME (credential, e.g., agency login) JACQUELINEHO	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Western Ontario London, ON, Canada	BSc (Hon)	May 1995	Genetics
University of Toronto Toronto, ON, Canada	MSc	Oct 1997	Developmental Biology
University of Western Ontario; London, ON, Canada	MD	May 2001	Medicine
University of British Columbia Vancouver, BC, Canada	Residency	June 2005	Pediatrics
Children's Hospital Boston Boston, MA, USA	Fellowship	June 2010	Pediatric nephrology

A. Personal Statement.

My career goal is to be a successful, independently funded physician-scientist in an academic pediatric nephrology division. I currently hold a position as a physician-scientist in the Division of Pediatric Nephrology at the University of Pittsburgh. My research focus is in understanding the role of microRNAs (miRNAs) in kidney disease and development. In collaboration with Dr. Butterworth, we have evaluated miRNA expression in response to aldosterone *in vivo* and generated transgenic mice which lack miRNAs in the collecting duct to study the role of miRNAs in kidney sodium regulation.

B. Positions.

Positions and Employment

Sept 1995 - Aug1997	Graduate student (full-time) Department of Molecular Genetics, University of Toronto, Canada Supervisor: Janet Rossant, PhD
Sept 1997 - May 2001	Medical student (full-time) University of Western Ontario, Canada
July 2001 - June 2005	Pediatric resident (full-time) Department of Pediatrics, British Columbia Children's Hospital & University of British Columbia, Canada Program Director: Andrew Macnab, MD
July 2005 - June 2010	Clinical fellow and postdoctoral research fellow (full-time) Division of Nephrology, Children's Hospital Boston & Harvard Medical School, Boston, MA Supervisor (research): Jordan A Kreidberg, MD PhD Division Head (clinical): William E Harmon, MD
Aug 2010- June 2012	Instructor in Pediatrics (full-time) Division of Nephrology, Children's Hospital of Pittsburgh of UPMC & University of Pittsburgh School of Medicine Pittsburgh, PA Mentor (research): Carlton M Bates, MD Division Head (clinical): Carlton M Bates, MD
July 2012 – present	Assistant Professor of Pediatrics (full-time) Division of Nephrology, Children's Hospital of Pittsburgh of UPMC & University of Pittsburgh School of Medicine Pittsburgh, PA Division Head: Carlton M Bates, MD