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):** 1/1/2009 - 12/31/2012

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

4. **Grant Contact Person's Telephone Number:** 412-692-8071

5. **Grant SAP Number:** 4100047629

6. **Project Number and Title of Research Project:** 2 – Novel Mouse Model of Vesicoureteral Reflux and Dysfunctional Voiding

7. **Start and End Date of Research Project:** 10/19/2011 – 12/31/2012

8. **Name of Principal Investigator for the Research Project:** Carlton M. Bates, 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:

   $ 265,787.42

   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	Position Title	% of Effort on Project	Cost
Bates, C	Principal Investigator		98,835.48
DiGiovanni, V	Post-Doc		37,561.15
Bushnell, D	Lab Mgr		27,120.89

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	Position Title	% of Effort on Project
Shaefer, C	Research Technician	10

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
Inverted Epi-Fluorescence Microscope	This allowed us to image fluorescently labeled whole kidney and urinary tracts.	9,620

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:
A Genetic Model of Vesicoureteral Reflux and Reflux Nephropathy."	x NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	March, 2013	$250,000 (direct costs)	pending

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:

As described in 11(A) above, we intend to continue pursuing NIH funding and have an application currently under review.

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

We used the data supported by this grant to apply for an NIH grant that is currently under review. This has become a major project in the lab that we hope to continue for years to come. This is particularly important given that few people are studying lower urinary tract development. Data we obtain will be critical for new treatments for structural lower urinary tract disease, which is a leading cause of chronic and end stage kidney disease 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
Female				2
Unknown				
Total				**3**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				3
Unknown				
Total				**3**

	Undergraduate	Masters	Pre-doc	Post-doc
White				3
Black				
Asian				
Other				
Unknown				
Total				**3**

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

    Yes_____ No____x_____

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

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

    Yes____x_____ No_____

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

    Much of the work described in this project was done as a collaborative effort between my laboratory and other investigators at the University of Pittsburgh. All of the physiology studies in the bladder (cystometry and bladder sheet assays) were conducted in the laboratory of Dr. Tony Kanai, Associate Professor in the Department of Pharmacology. Prior to working with us, he has worked exclusively in injury models and not genetic models. Our

skill sets have clearly complemented each others' research programs, which is something that should continue moving forward.

In addition, the computational analysis for the RNA-Seq data was done largely by another investigator, Dr. Dennis Kostka, who is an Assistant Professor in the Developmental Biology Department here at the University of Pittsburgh. Again, these collaborative efforts have been of great benefit to both of our programs, and would likely not have happened without the support of this grant.

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 <u>for the period that the project was funded (i.e., from project start date through end date).</u> 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. <u>Provide detailed results of the project.</u> 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.

The progress report will be divided according to the specific aims of the project. Figures and Tables will be placed at the end of this section:

Specific Aim 1: To determine roles of *Fgfr2* in stroma during Wolffian duct/ureteric bud, ureter, and bladder development.

Fgfr2$^{St-/-}$ mice have ureteric induction defects and high rates of reflux with abnormal ureterovesical junctions:
To determine the roles of fibroblast growth factor receptor 2 (Fgfr2) in peri-Wolffian duct stroma, we generated Tbx18cre conditional knockouts of *Fgfr2* in that tissue (*Tbx18cre*$^{Tg/+}$*Fgfr2*$^{Lox/Lox}$ *or Fgfr2*$^{St-/-}$). *Fgfr2*$^{St-/-}$ mice were viable without limb defects and heterozygotes were normal. We assessed for ureteric bud induction defects by whole mount immunostaining for pan-cytokeratin at E11.0 to measure the length of the common nephric duct (Wolffian duct segment between the ureteric bud base and the cloaca) (Figure 1). Mean mutant common nephric duct lengths (170.75µm ± 32.25) were not different than controls (182.74 ± 53.94); however, while control duct lengths were tightly clustered around the mean, mutant duct lengths were nearly random with 69% having ducts that were >1 standard deviation shorter or longer than the control mean ($p<0.01$) (Figure 1). Thus, loss of *Fgfr2* in stroma leads to cranially or caudally shifts in ureteric bud induction sites.

Given the ureteric bud induction defects in *Fgfr2*$^{St-/-}$ mice, we tested the mice for vesicoureteral reflux by performing cystograms with methylene blue dye infused into bladders as described in the Research Design and Methods section of the application. Postnatal day (P) 1 and 6 month old *Fgfr2*$^{St-/-}$ mice had high rates of mostly unilateral, grade 2 (out of 5) reflux relative to controls with no gender bias (Figure 2, Table 1, Table 2 and not shown). Thus, deletion of *Fgfr2* in the stroma leads to high rates of reflux.

Given the ureteric induction defects and high reflux rates, we assessed *Fgfr2*$^{St-/-}$ ureterovesical junctions by 3D reconstruction of the lower urinary tract as described in the Research Design and Methods section of the application. Briefly, we traced P1 H&E stained serial sections of ureteral and bladder tissues into layers, aligned the layers and rendered 3D images (Stereoinvestigator, MBF). When we superimposed triangles formed by the external ureteral insertion points and bladder neck, we found that non-refluxing controls have similar insertional angles with an average difference of <15° between the left and right angles (Figure 3). In contrast, P1 *Fgfr2*$^{St-/-}$ mice with unilateral reflux have an average difference of >30° between the side without reflux and the side with reflux, indicating asymmetric ureteral insertion and displacement of the refluxing ureter. P1 *Fgfr2*$^{St-/-}$ mice with no reflux have similar angles at the ureteral insertion points like controls. The 3D reconstructions also revealed that intravesicular tunnel lengths were shortened in refluxing *Fgfr2*$^{St-/-}$ ureters compared to non refluxing control and *Fgfr2*$^{St-/-}$ ureters (Figure 3). Thus, *Fgfr2*$^{St-/-}$ mice have abnormal ureteral insertion in the bladder (malpositioned ureters and shortened intravesical tunnels) on the sides of reflux, consistent with what has been described in humans and animal models of reflux.

<u>*Fgfr2*$^{St-/-}$ mice also have ureteral peristaltic defects:</u>
Since Tbx18-positive stroma also gives rise to ureteral and bladder mesenchyme including muscle, we next examined mutant mice for ureter defects. Embryonic and P1 ureters appear normal H&E staining and by immunofluorescence for E-cadherin (urothelium) and smooth muscle actin (muscle) (Figure 4 and not shown). However, E13.5 embryonic ureteral cultures grown for 4 days at an air-fluid interface have aberrant peristalsis with decreased frequency and abnormal coordination of contractions including failure of propagation of proximal contractions, simultaneous proximal and distal contractions, and prolonged contractions (Figure 4 and not shown).

<u>*Fgfr2*$^{St-/-}$ mice develop distended bladders and have dysfunctional voiding:</u>
We next examined *Fgfr2*$^{St-/-}$ mice for bladder defects. Histological sections in embryos and in P1 and 1 month old mice after cystograms showed no obvious defects in *Fgfr2*$^{St-/-}$ bladders versus controls (Figure 5 and not shown). By 6 months of age, however, at least half of the *Fgfr2*$^{St-/-}$ mice had very grossly distended bladders prior to performing cystograms (not shown). Following cystograms, all 6 month old *Fgfr2*$^{St-/-}$ bladders were variably distended (without gender bias) with what appeared to be less compact and/or thinner muscle layers by trichrome staining compared with controls (Figure 5).

We then tested the mice for voiding dysfunction by void stain on paper (VSOP) tests, placing filter paper under mice in metabolic cages for 4 hours and examining spots under ultraviolet light. Controls typically had 1-2 large void stains distant from their food and water source (Figure 6). *Fgfr2*$^{St-/-}$ male and female mice had many smaller voids and dribbles of urine trailing away from the food and water site, even at 1 month of age (when the bladder histology was normal) (Figure 6). Mutant dysfunctional voiding appeared to worsen with age, with reduced maximum void spot volumes at 3 and 6 months vs. controls (and equivalent volumes at 1 month). Thus, *Fgfr2*$^{St-/-}$ mice have evidence of dysfunctional voiding starting at 1 month that worsens with age.

Fgfr2$^{St-/-}$ bladders have poor compliance, high pressures, decreased contractile forces and less ability to relax:

Based on the VSOP data, we then sought to more thoroughly interrogate bladder dysfunction in the mutants. Thus, we performed decerebrate cystometry in live animals, by infusing saline at 10ml/minute and noting bladder pressures and voiding responses over time. Compared to controls, mutants had higher baseline and threshold pressures and decreased intercontraction intervals (Figure 7).

We then isolated bladder sheets, finding that mutants had attenuated relaxation responses to β_3-adrenergic agonists vs. controls (not shown). In contrast, *Fgfr2*$^{St-/-}$ bladders relaxed normally in response to forskolin, which increases cAMP levels as happens in response to β-adrenergic agonists; this suggests that *Fgfr2*$^{St-/-}$ bladders maintain a partially contracted state. Also, the contractile force generated by *Fgfr2*$^{St-/-}$ bladders was markedly attenuated with electrical field stimulation, cholinergic agonists (10 mM Carbachol), purinergic agonists (10 mM α-β-methylene ATP) and KCl (120 mM) (Figure 7 and not shown). We stretched bladder sheets at 500mm intervals and plotted baseline (passive) tension and active force generated by electrical stimulation; *Fgfr2*$^{St-/-}$ bladders had increased passive tension (poor compliance) and decreased active force (attenuated contractions) with increasing stretch vs. controls (Figure 7). Thus, 1 month old *Fgfr2*$^{St-/-}$ bladders have poor compliance, high pressures and reduced contractile forces, likely secondary to an inability to relax normally.

Fgfr2$^{St-/-}$ mice have evidence of progressive chronic kidney disease:

Given the constellation of lower urinary tract defects in *Fgfr2*$^{St-/-}$ mice detailed above, we examined the mutants for signs of renal injury. At 1 month of age, ~75% of *Fgfr2*$^{St-/-}$ mice (n=4) had mild to moderate hydronephrosis on H&E stained sections and renal ultrasound (US) without an obvious gender bias; there was no evidence of renal fibrosis on trichrome stained sections (not shown). By 6 months of age, 100% of the *Fgfr2*$^{St-/-}$ mice (n=10) had moderate to severe hydronephrosis on H&E sections and US, without an obvious gender bias (Figure 8 and not shown). Trichrome staining shows that compared with controls, 6 month old *Fgfr2*$^{St-/-}$ mice had fibrosis within glomeruli and the peritubular interstitium and in the deep medulla adjacent to the renal pelvis (Figure 8). Thus, with advancing age, *Fgfr2*$^{St-/-}$ mice develop worsening hydronephrosis and renal fibrosis.

We then examined serum and urinary biomarkers of chronic kidney disease. At 1 month, *Fgfr2*$^{St-/-}$ mice have normal serum markers including blood urea nitrogen (BUN), phosphorous, bicarbonate, and potassium (not shown). By 6 months, however, both male and female *Fgfr2*$^{St-/-}$ mice had high BUN and phosphorous levels compared to controls (Figure 9). Male mutants had lower bicarbonate levels and trends for increased potassium levels versus controls (Figure 9) (females have not yet been assayed). Also, 6 month male and female *Fgfr2*$^{St-/-}$ mice have trends for increased 24 hour urine protein/creatinine ratios (n=4) (not shown). Thus, *Fgfr2*$^{St-/-}$ mice have evidence of evolving chronic kidney disease based on serum biomarkers and proteinuria.

Specific Aim 2: To determine mechanisms by which *Fgfr2* stromal deletion leads to VUR and ureteric, ureteral peristaltic, and voiding defects.

Bmp4 and *Sprouty1* are likely downstream targets of Fgfr2 signaling leading to the ureteric induction defects in E10.5 *Fgfr2*$^{St-/-}$ mutant mice:
Given that early embryonic *Fgfr2*$^{St-/-}$ mice had ureteric induction defects, we performed *in situ* hybridization and real time PCR (qPCR) at E10.5 for molecules known to regulate ureteric induction. We detected no changes in *Ret*, *Robo2*, or *Slit2* expression (not shown). We did however note ~50% decreases in *Bmp4* and *Sprouty1* expression in *Fgfr2*$^{St-/-}$ mice (Figure 10). Similar reductions in Bmp4 levels have been associated with ureteric induction defects in other models (Miyazaki et al, J Clin Invest. 105(7):863-73, 2000). We hypothesize that Bmp4 signals via non canonical pathways (Erk) to regulate Sprouty1 levels in the Wolffian duct (Figure 10).

Older Fgfr2$^{St-/-}$ embryonic bladders and ureters have evidence of increased hedgehog signaling:
Given that the Tbx18cre lines also deleted *Fgfr2* in ureter and bladder mesenchyme at later embryonic stages, we assessed E16.5 *Fgfr2*$^{St-/-}$ bladders and ureters for signaling defects. Given critical roles of hedgehog (Hh) in the mesenchyme, we examined Hh activity. By *in situ* hybridization, Sonic hedgehog (*Shh)* expression appears normal in *Fgfr2*$^{St-/-}$ bladder urothelium; however, Hh signaling readouts, *Patched 1* and *Bmp4*, appear enhanced and expanded in the mesenchyme (Figure 11).

We then confirmed similar *Shh* levels in E16.5 *Fgfr2*$^{St-/-}$ and control bladders by real time PCR (qPCR); however, multiple Hh activity readouts including *Patched 1*, *Gli1*, hedgehog interacting protein (*Hhip*), and *Bmp4* were (or trended to be) elevated by 45-70% vs. controls (Figure 12). Also, qPCR in E16.5 ureters revealed similar *Shh* levels, but increased levels of Hh readouts (Figure 12). Thus, E16.5 *Fgfr2*$^{St-/-}$ bladders and ureters appear to have increased Hh activity, which could mispattern the muscle and/or lamina propria. Since *Shh* is unaltered, Fgfr2 likely represses factors that enhance Shh signaling and/or blocks expression of other ligands (e.g. Indian Hh).

We then performed qPCR for factors that enhance Hh activity. E16.5 *Fgfr2*$^{St-/-}$ bladders had increased *Boc* and trends for more *Cdo* (related to *Boc)*, while *Gas1* was unaltered (Figure 6). We saw similar patterns in E16.5 ureters (Figure 13). Thus, loss of *Fgfr2* may increase factors such as Cdo and Boc that enhance Hh activity (Figure 13).

P1 *Fgfr2*$^{St-/-}$ bladders appear to have dysregulation of genes regulating proliferation/differentiation, muscle and connective tissue patterning, and receptors/channels*:*
We then examined P1 bladders for changes in gene expression that may be mediating many of the physiological defects seen in *Fgfr2*$^{St-/-}$ mice (Figure 7). Thus, we performed RNA-Seq on P1 *Fgfr2*$^{St-/-}$and cre negative control bladder mRNA (n=3 per group). We made cDNA libraries (Illumina) and performed 100 base single end sequencing (~20 million reads/sample) (Tufts Genomics Core). We then identified 98 differentially expressed genes in *Fgfr2*$^{St-/-}$ bladders versus controls, using a high degree of stringency (false discovery rate < 0.2). Many of the top genes encoded for proteins that regulate proliferation/differentiation, muscle or connective tissue patterning, and channels or receptors (Table 3). We have begun validating differential expression of the genes by qPCR (Table 3, blue font). Although

hedgehog signaling has dropped dramatically after E16.5, many of the apparently upregulated genes at P1 such as *Nmyc* and *Sox9* are known hedgehog targets (Table 3); it is unclear whether this represents sustained expression in the absence of ongoing Hh signaling. Thus, despite overtly normal histology in P1 *Fgfr2*$^{St-/-}$ bladders, we have begun identifying genes that may be driving functional defects.

<u>Summary:</u>

Tbx18cre deletion of *Fgfr2* in mesenchyme around the Wolffian duct, ureter and bladder, leads to ureteric induction defects with subsequent abnormal ureteral insertion into the bladder and vesicoureteral reflux. The mutants also have ureteral peristaltic defects and voiding dysfunction, despite normal appearing histology at early ages. The voiding defects are further characterized by high pressure and low compliant bladders. This model provides a novel genetic link between reflux and ureter and bladder dysfunction. Furthermore, this constellation of lower urinary tract dysfunction leads to fibrotic chronic kidney injury that appears to mimic the renal injury seen in patients with reflux nephropathy. In addition, we have begun to elucidate the aberrant signaling pathways downstream of Fgfr2 leading to these defects. Together, these data may lead to novel therapies to treat children with chronic kidney disease from structural lower urinary tract disorders.

Figures and Tables:

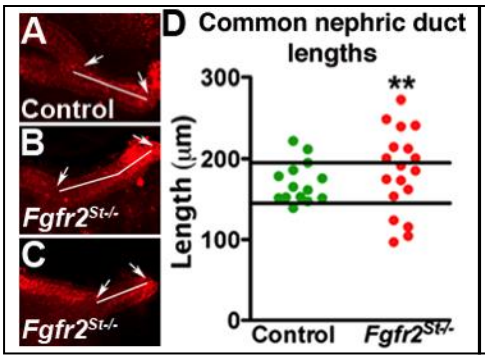

Figure 1: E11 *Fgfr2*$^{St-/-}$ mice have ureteric bud induction defects. Pan-cytokeratin staining shows that compared with controls (A), *Fgfr2*$^{St-/-}$ mice often have shorter (B) or longer (C) common nephric ducts. Graph of duct lengths (D) shows that controls are mostly within 1 standard deviation of the mean (solid bars) where-as most *Fgfr2*$^{St-/-}$ are outside of that range. **p<0.01.

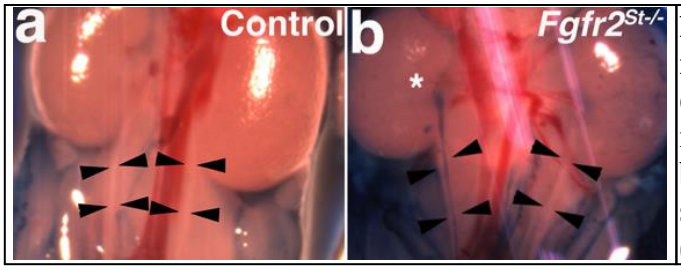

Figure 2: P1 *Fgfr2*$^{St-/-}$ mice have high rates of reflux a. Control mouse has no reflux. **b**. *Fgfr2*$^{St-/-}$ has right sided grade 2 reflux (*). arrows=ureters

Table 1: Reflux rates, laterality and grades in *Fgfr2*$^{St-/-}$ and control mice at P1 (*p<0.001)

Genotype	Reflux	Unilateral	Median grade
P1 Control	2.6% (2/76)	100%	1
P1 *Fgfr2*$^{St-/-}$	74.2% (23/31)*	95.7%	2

Table 2: Reflux rates, laterality and grades in *Fgfr2*$^{St-/-}$ and control mice at 6 months (*p<0.001)

Genotype	Reflux	Unilateral	Median grade
6 month Control	12.5% (2/16)	100%	1
6 month *Fgfr2*$^{St-/-}$	90.9% (10/11)*	90%	2

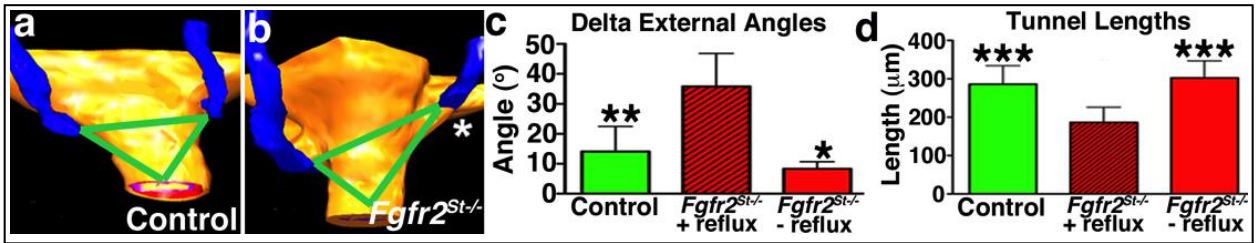

Figure 3: P1 *Fgfr2$^{St-/-}$* refluxing ureters are displaced and have shortened intravesicular tunnels. a-c. Control without reflux (a) has similar ureteral insertion sites. *Fgfr2$^{St-/-}$* mouse with unilateral reflux (b) has high/lateral displacement of the ureter with reflux (*).**c.** Graph shows that *Fgfr2$^{St-/-}$* mice with unilateral reflux have mean differences of >30° between insertional angles while mutants and controls without reflux have differences <15°. **d**. Graph shows intravesicular tunnel lengths in mutant ureters with reflux are shorter than non-refluxing control and mutant ureters. (*p<0.05; **p<0.01; ***p<0.001 vs. *Fgfr2$^{St-/-}$* with reflux).

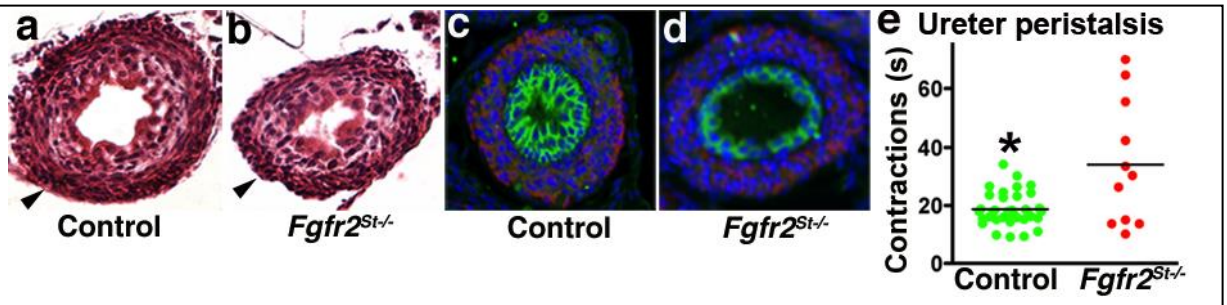

Figure 4: P1 *Fgfr2$^{St-/-}$* ureters have normal histology but abnormal peristalsis. **a,b**. H&E stains of ureters appear normal in control (a) and *Fgfr2$^{St-/-}$* (b) (arrowheads= muscle layers). **c,d**. Ureter staining for E-cadherin (green, urothelium) and smooth muscle actin (red, muscle) appears normal in control (c) and *Fgfr2$^{St-/-}$* (d) (blue=DAPI). **e.** Graph shows that mean contraction intervals are increased in mutants vs. controls (*p<0.001).

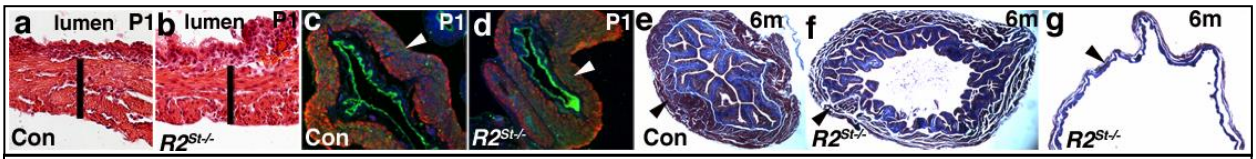

Figure 5: *Fgfr2$^{St-/-}$* bladders appear normal at P1, but distended at 6 months. a,b. H&E stains of P1 control (Con) (a) & *Fgfr2$^{St-/-}$* (*R2$^{St-/-}$*) (b) appear similar (bar=muscle thickness). **c,d**. Smooth muscle actin (red) and E-cadherin (green) staining appear similar in P1 control (c) & *Fgfr2$^{St-/-}$* (d). **e-g**. Trichrome shows 6m *Fgfr2$^{St-/-}$* bladders are mildly (f) to severely (g) distended with less muscle vs. controls (e) (arrowheads= muscle).

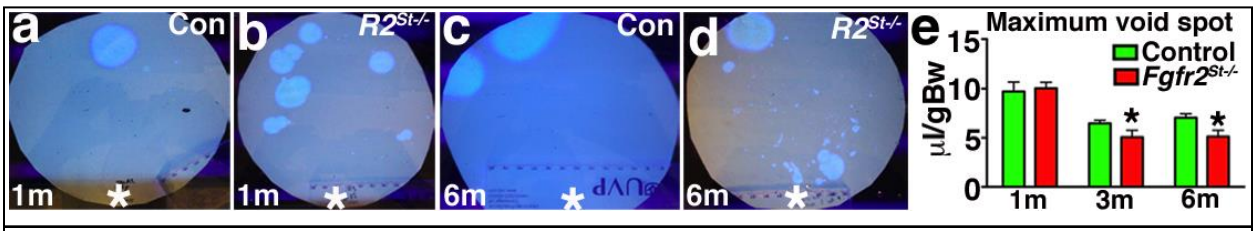

Figure 6: *Fgfr2$^{St-/-}$* mice have dysfunctional voiding worsening with age: a-d. VSOP shows that controls (Con) have 1-2 large void spots distant from their food/water (*) at 1 and 6 months (a,c) while *Fgfr2$^{St-/-}$* (*R2$^{St-/-}$*) have multiple smaller voids closer to their food/water at 1 and 6 months (b,d). **e**. Graph shows 3 and 6 month *Fgfr2$^{St-/-}$* have smaller maximum void spots (implying worsened voiding dysfunction) vs. controls. *p<0.01

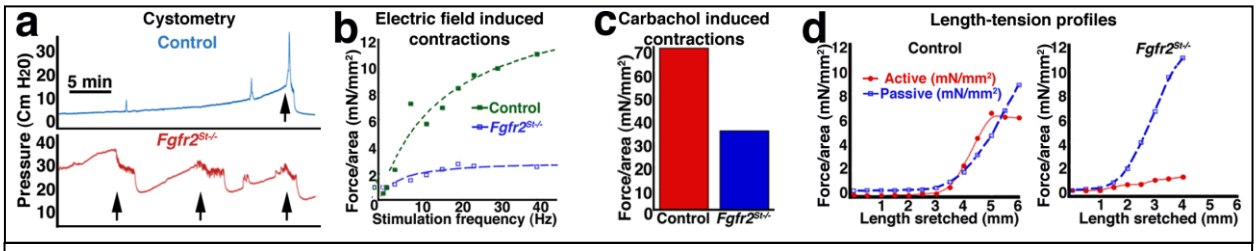

Figure 7: One month old *Fgfr2$^{St-/-}$* bladders have poor compliance and reduced force of contractions. a. Cystometry shows that *Fgfr2$^{St-/-}$* have high baseline and threshold pressures and shortened intercontraction intervals vs. controls (arrows=voiding). **b**. Electrical field stimulation in isolated bladder sheets shows that force generated is attenuated in mutants vs. controls. **c**. Contractions evoked by Carbachol are attenuated in mutants vs. controls. **d**. With increasing stretch, *Fgfr2$^{St-/-}$* bladders have increased passive tension (poor compliance) and decreased active force from electrical stimulation (attenuated contractions) vs. controls.

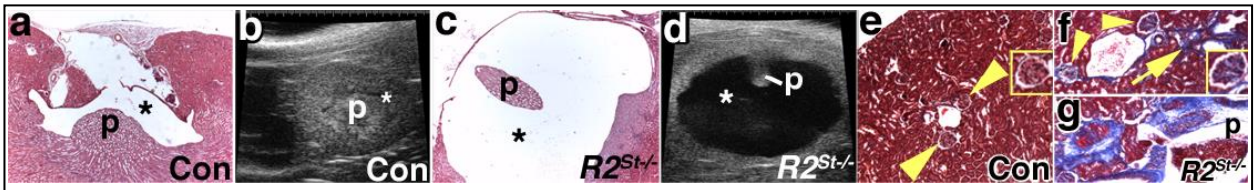

Figure 8: Six month old *Fgfr2$^{St-/-}$* kidneys have hydronephrosis and fibrosis. a-d. H&E stained sections (a,c) and ultrasound images (b,d) show a normal control (a,b) and a *Fgfr2$^{St-/-}$* mouse with severe hydroneph-rosis (c,d) (p= papilla, *= pelvis). **e-g**. Trichrome stained sections show a normal control (e) including glomeruli (arrowheads) and a *Fgfr2$^{St-/-}$* kidney with blue staining indicating glomerular fibrosis (f, arrowheads), interstitial fibrosis (f, arrow) and peri-pelvic fibrosis (g) (p=pelvis) (insets in e and f show higher power views of glomeruli).

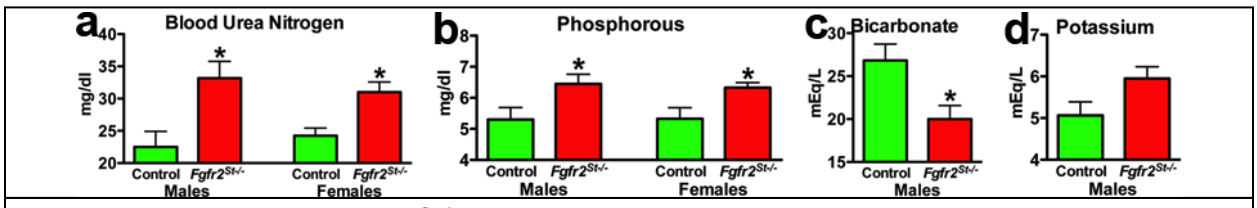

Figure 9: Six month old *Fgfr2*$^{St-/-}$ mice have serum biomarkers consistent with chronic kidney disease. a,b. Compared with controls, male and female *Fgfr2*$^{St-/-}$ mice have increases in BUN (a) and phosphorous (b). **c,d**. Male *Fgfr2*$^{St-/-}$ mice have decreased bicarbonate and trend for increased potassium vs. controls (*p<0.01).

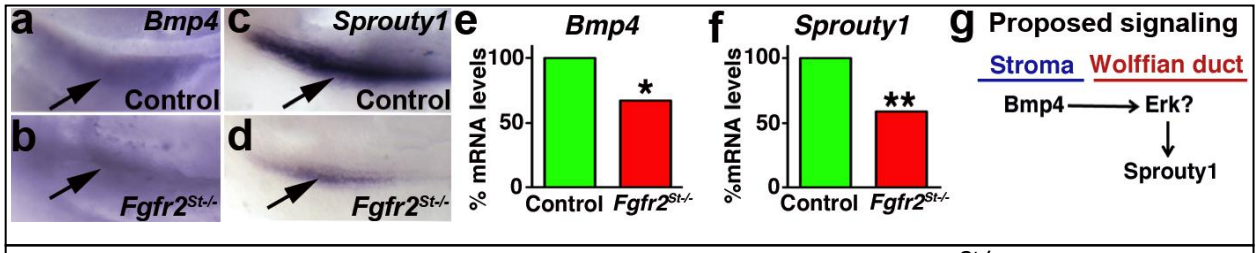

Figure 10: *Bmp4* and *Sprouty1* expression is decreased in E10 *Fgfr2*$^{St-/-}$ urogenital ridges. a-d. *In situ* hybridization for *Bmp4* (a, b, arrows) and *Sprouty1* (c, d, arrows) shows decreased levels in mutants (b,d) vs. controls (a,c). **e,f** Graphs of qPCR assays show 40-50% reductions in *Bmp4* and *Sprouty1* mRNA vs. controls (*p<0.05, **p<0.001). **g**. Proposed Bmp4 signaling from the stroma regulating Sprouty1 in the Wolffian duct.

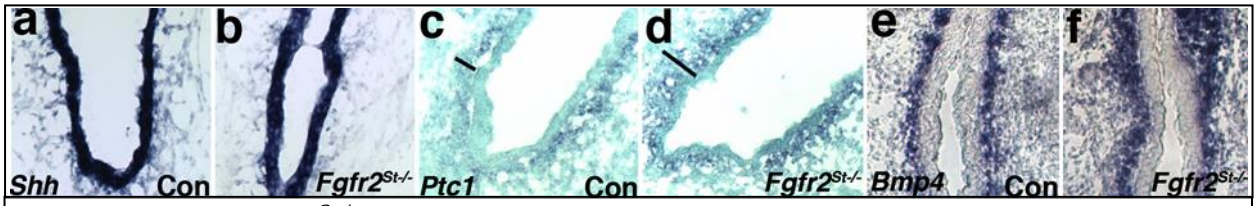

Figure 11: E16.5 *Fgfr2*$^{St-/-}$ bladders have normal *Shh* but increased *Ptc1* and *Bmp4* expression: a,b. In situ reveals similar *Shh* levels in control and *Fgfr2*$^{St-/-}$ urothelium. **c-f**. In situ shows enhanced and expanded *Ptc1* and *Bmp4* staining in submucosa of *Fgfr2*$^{St-/-}$ mice (d,f) vs. controls (c,e) (see lines in c,d). all= 200x mag.

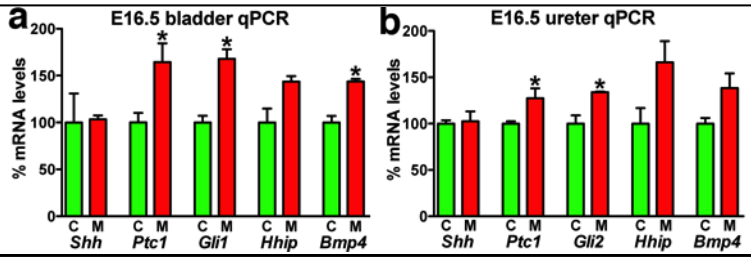

Figure 12: Real time PCR reveals equivalent *Shh* but increased readouts of Hh activity in E16.5 *Fgfr2*$^{St-/-}$ bladders and ureters: E16.5 bladders (a) and ureters (b) have similar *Shh* mRNA but increases in Hh readouts in mutant (M) tissues vs. controls (C). n=3 per genotype. *p<0.05. Bladder *Hhip* p=0.06; Ureter *Hhip* p=0.09, *Bmp4* p=0.08.

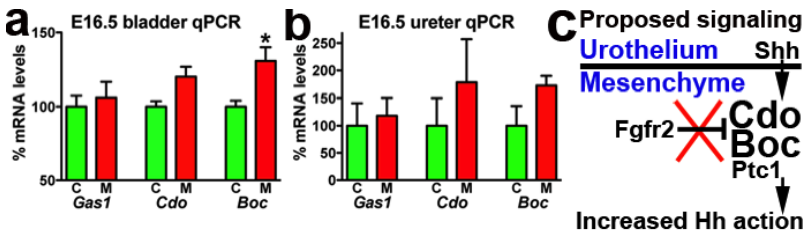

Figure 13: qPCR shows likely increases in *Boc* and *Cdo* in E16.5 *Fgfr2*$^{St-/-}$ bladders & ureters: a,b. E16.5 bladders (a) and ureters (b) have similar *Gas1* levels but increased (or trends for) *Cdo* and *Boc*. n=3 per genotype. *p<0.05. Bladder *Cdo* p=0.06; Ureter *Boc* p=0.1. **c**. Proposed signaling with *Fgfr2* loss.

Table 3: Differential expression of genes in P1 *Fgfr2*$^{St-/-}$ bladders relative to controls

Proliferation/Differentiation		**Muscle/connective tissue patterning**		**Channels/receptors**	
Increased	Decreased	Increased	Decreased	Increased	Decreased
Nmyc, Gdf10, *Gdf1, Osr2, Eya2, Fz10, Dkk2, Scx, Kif26b*	*Tbx20, Klf9, Klf15, Rnf17, Prune2, Zbtb16*	***Mylpf, Actn2***, *Sox9, Col9a2/17a1/23a1/6a4, Adamts15/19, Ankrd1, Myl6b, Neb, Hdc, Emilin3*	***Myocardin***, *Sorbs1*	***Troponin I2/C1,*** *Troponin I1, Dpp6, Clca3, Trpm5*	***Cholinergic receptor M2, Hcn1,*** *Mrgprd*

Blue font = qPCR confirms differential expression

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
___x___ 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, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

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. None				☐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 are planning on submitting peer reviewed publications on the constellation of findings outlined in this grant application. We will likely first submit the data on vesicoureteral reflux, including the aberrant ureteric induction, ureterovesical junction defects, and abnormal Bmp4 and Sprouty1 signaling in the E10.5 mutants as a first manuscript. We will likely then follow up with a manuscript on the ureter and bladder muscle defects including the abnormal hedgehog signaling outlined above.

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.

As this is a basic science project, there has not yet been any impact directly on any diseases. That said, the data strongly suggests that there are likely genetic links between vesicoureteral reflux and bladder dysfunction in many children. Furthermore, most children with dysfunctional voiding and reflux do not have formal cystometry performed to look for organic bladder disease including poor compliance and high pressure (which may then lead to kidney injury). We anticipate that we (and/or others) will initiate clinical studies in the future examining possible genetic defects in humans with reflux and dysfunctional voiding and examining the incidence of organic bladder disease by cystometry in these patients.

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.

None.

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.

NAME	POSITION TITLE
Carlton M. Bates, M.D.	Associate Professor
eRA COMMONS USER NAME BATESCM	

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
Otterbein College, Westerville, OH.	B.S.	1982-1986	Chemistry, Life Science
The Ohio State University College of Medicine, Columbus, OH.	M.D.	1986-1991	Medicine
Pediatric Residency Program, The Ohio State University, Columbus, OH.	Residency	1991-1994	Pediatrics
Pediatric Nephrology Fellowship Program, Univ of Texas Southwestern Medical Center, Dallas, TX.	Fellowship	1994-1997	Pediatric Nephrology

A. Personal Statement:

My research is focused on the role of fibroblast growth factor receptors (Fgfrs) in kidney and urinary tract development, using mouse models. I have an extensive track record of publications and previous funding manipulating expression of Fgfrs 1 and/or 2 and their adapter proteins using a combination of conditional and global knockout models; these approaches have revealed many novel roles for the receptors in multiple lineages and at different stages of kidney and urinary tract development. These mouse lines often mimic many of the congenital forms of kidney disease that are leading causes of renal disease in children, of which I am familiar given my history as a clinical Pediatric Nephrologist for over 15 years. My research track record in renal and urinary tract maldevelopment and post-natal consequences coupled with my clinical experience, make me uniquely qualified to be the PI on this application.

B. Positions and Honors

Positions

1997-1998 Assistant Instructor, Dept. of Pediatrics, Division of Nephrology, University of Texas Southwestern Medical Center, Dallas, TX.

1998-1999 Assistant Professor, Dept. of Pediatrics, Division of Nephrology, University of Texas Southwestern Medical Center, Dallas, TX.

1999-2007 Assistant Professor, Dept. of Pediatrics, Section of Nephrology, The Ohio State University College of Medicine and Public Health, Columbus, OH.

2007-11/08 Associate Professor, Dept. of Pediatrics, Section of Nephrology, The Ohio State University College of Medicine, Columbus, OH.

12/08- Associate Professor, Department of Pediatrics, University of Pittsburgh School of Medicine, Division Chief of Nephrology, Fellowship Program Director, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA.

Reviewer Activities (grant applications):

2001 *Ad hoc* reviewer for The Wellcome Trust funding agency

2005- *Ad hoc* reviewer for the Urologic and Kidney Development and Genitourinary Diseases NIH study section (9/2005, 2/2006, 6/2006, 10/2006)

2008 Reviewer for NIH/NIDDK Special Emphasis Panel ZDK1 GRB-7 (01) M.

2009 Reviewer for NIH Challenge Grant Editorial Panel 18 ZRG1 DKUS-A (58) (07/20/2009).

2009 Reviewer for NIH Special Emphasis Panel ZRG1 DKUS-F (02) (11/04/2009).

2011- Member, Basil O'Connor Research Advisory Committee (2011-2016)

Reviewer/Editorial Activities (journals/abstracts):

Ad hoc reviewer for the journals Human Molecular Genetics, Oncogene, Pediatric Nephrology, American Journal of Kidney Diseases, American Journal of Physiology-Renal Physiology, The Pediatric Infectious Disease Journal, Journal of the American Society of Nephrology, Toxicology and Applied Pharmacology, Kidney International, Methods and Findings in Experimental and Clinical Pharmacology, Pediatric Research, Clinical Journal of the American Society of Nephrology, BMC Developmental Biology, Developmental Dynamics, Journal of Urology, Life Sciences, Biochimica et Biophysica Acta, PLoS One

2004- Editorial board member for the journal Pediatric Nephrology, Journal of the American Society of Nephrology

2010- Associate Editor (Educational Reviews) of the journal Pediatric Nephrology

Professional (last 5 years):

2008 Chair of the Program Committee of the American Society of Pediatric Nephrology

2008- co-Chair of the Research Committee of the American Society of Pediatric Nephrology

2009- Member, Organizing Committee, International Workshop on Developmental Nephrology, New York (8/10)

2009- Member, Organizing Committee, International Pediatric Nephrology Association meeting, New York (9/10)

2010-11 Member, Program Committee of the American Society of Nephrology

2010-13 co-Chair, Organizing Committee, International Workshop on Developmental Nephrology

2010-13 Member, Scientific Committee for the International Pediatric Nephrology Association 2013 Congress

2010- Member, Council of the International Pediatric Nephrology Association

Invited talks/oral presentations (last 5 years)

2008 Invited lecture: “Role of fibroblast growth factor receptors in the kidney” at the Department of Zoology, Miami University, Oxford, Ohio on September 18th, 2008.

2009 Invited Lecture: “Role of fibroblast growth factor receptors in the kidney” at the O’Brien Center lecture, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas (September 22, 2009).

2009 Invited lecture: “Congenital Diseases of the Kidney and Urinary Tract” at Pediatric Grand Rounds, Children’s Medical Center at Dallas, Dallas, Texas (September 23, 2009).

2010 Invited lecture: “Role of fibroblast growth factor receptors in the kidney” at Magee-Womens NICU Grand Rounds, Pittsburgh, PA (January 14, 2010)

2010 Invited lecture: "Role of fibroblast growth factor receptors in the kidney" at Magee-Womens Research Institute's Work-in-Progress (WIP) Conference & Research Seminar, Pittsburgh, PA (February 2, 2010).

2010 Invited lecture: “Role of fibroblast growth factor receptors in the kidney” at the Mount Sinai School of Medicine, New York, NY (April 12, 2010).

2011 Invited lecture: “Role of fibroblast growth factor receptors in the kidney” at the Renal Research Seminar, Renal-Electrolyte Division, University of Pittsburgh, Pittsburgh, PA (May 11, 2011)

2012 Invited lecture: “Roles of Mesenchymal Fgfr Signaling in Urogenital Development” at Einstein Renal Grand Rounds, New York, NY (April 5, 2012).

2012 Invited lecture: “Roles of fibroblast growth factor receptors in models of structural kidney disease” at Children’s Hospital of Pittsburgh Pediatric Grand Rounds, Pittsburgh, PA (July 12, 2012).

2013 Invited lecture: “Roles of fibroblast growth factor receptors in models of structural kidney disease” at University of California-San Francisco Renal Grand Rounds, San Francisco, CA (February 27, 2013).