Children's Hospital of Pittsburgh

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2011 – June 30, 2012

Formula Grant Overview

The Children's Hospital of Pittsburgh received $958,038 in formula funds for the grant award period January 1, 2009 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

A Genetic Model of Congenital Obstructive Nephropathy - End-stage kidney disease costs over 15 billion dollars annually in the United States and represents a major cause of death and impaired quality of life. The long-term objective of this project is to gain a better understanding of the most common cause of kidney disease in children - a blockage in urine flow. Completion of the studies outlined in this proposal will help us design new diagnostic tests and treatment strategies for children and adults with kidney disease.

Duration of Project

1/1/2009 - 9/15/2012

Project Overview

In utero obstructive nephropathy has the potential to interrupt normal renal development and represents the most common cause of chronic renal failure in children. The long-term objective of this project is to gain a better understanding of the pathophysiology of in utero obstructive nephropathy as it relates to normal kidney development in an effort to identify potential therapeutic pathways for intervention. We have identified a highly unique mutant mouse model, designated mgb, for megabladder, that develops overt signs of lower urinary tract obstruction in utero resulting in the development of hydroureteronephrosis and progressive renal failure. In this project, we will utilize the mgb mouse model to: 1) complete characterization of the development of in utero obstructive nephropathy in the mgb mouse including the identification of pathophysiological changes associated with kidney development, 2) identify and characterize the role that transforming growth factor β (TGF-β) signaling plays in mediating renal injury in the mgb mouse model of in utero obstructive nephropathy, and 3) develop and assess therapeutic strategies designed to ameliorate renal injury in the mgb mouse model of in utero obstructive nephropathy. The identification and characterization of the factors responsible for the pathogenic changes in renal function following the development of congenital bilateral obstructive nephropathy in mgb mice will provide a platform for the evaluation of pharmacological, surgical,
and gene therapy strategies designed to prevent and treat the pathogenic processes associated with the development of progressive renal failure in utero.

Principal Investigator

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Other Participating Researchers

None

Expected Research Outcomes and Benefits

At present, the only therapy for babies born with blockage of urine flow is surgery. Unfortunately, approximately 50% go on to complete kidney failure despite the surgery (due to damage that occurs in the womb). In addition to the high costs of kidney failure, 25-50% of babies born with complete kidney failure will die before their first birthday (despite dialysis or kidney transplantation).

We now have a unique mouse model that develops blockage of urine before birth. Remarkably, when we perform surgery to relieve the blockage, about 50% of them survive (just like children born with urinary blockage). This gives us an opportunity to learn more about why some children do well with surgery and others do not. It also gives us chances to explore what drugs we can use before birth to improve kidney survival after surgery. What we learn from this very unique model can be used to improve survival and quality of life of children born with blockage of urine flow.

Summary of Research Completed

As noted in the letter dated October 10, 2011 to the Department of Health, we had completed most of the original aims for this project and had utilized this data to successfully compete for additional funding with a colleague. There are therefore only limited progress report data for Project #1 for FY11. The majority of the progress data for the report are for Project #2 (see below).

During the remaining period for Project #1, we did additional characterization of bladder defects in megabladder (mgb) mice using 3D reconstructive imaging. First, we found that while total bladder volumes between mgb and controls were equivalent at embryonic day (E) 13.5, mgb volumes were well below control volumes at later developmental stages. Next, we compared developmental changes in volumes of the urothelium and detrusor smooth muscle in control and mgb mice. From E13.5-E17.5, controls had a relatively constant volume of urothelium at ~5%, while detrusor muscle volumes increased from 32% to 67%. In contrast, mgb bladders had an
increase in urothelial volume from 6% at E13.5 to 12% at E17.5, while detrusor volumes started at 6% and only increased to 18% (reflecting the poor smooth muscle differentiation in the mgb mice). Finally, we compared the pattern of smooth muscle differentiation in control and mgb mice. In controls, smooth muscle differentiation started at E13.5 at the upper bladder dome with a projection extending down the right posterior surface; by E14.5, controls had smooth muscle encompassing the upper half of the bladder; by E15.5 the entire bladder was encased in smooth muscle that increased in thickness with advancing age. In mgb mice, bladder muscle development initiated at E13.5 in a manner similar to controls with a projection of differentiation along the right posterior surface; however, by E15.5, the mgb muscle remained highly fenestrated and did not fully extend to caudal regions as it had in controls. Due to massive distension of the mgb bladders starting at E16.5 (when urine was present), it was technically not feasible to interrogate detrusor muscle development in mgb bladders.

**Project End Date**

Did the project end during this reporting period?

* Yes  □ No

If the project has ended, indicate the project end date:    __09__/__15__/__11___.

If the project has not yet ended, indicate the anticipated project end date:  ____/____/____.

**Human Embryonic Stem Cell Research**

Did this project involve, in any capacity, human embryonic stem cells?

□ Yes  x No

If Yes, were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?

□ Yes  □ No N/A

**Research Project 2: Project Title and Purpose**

*Novel Mouse Model of Vescicoureteral Reflux and Dysfunctional Voiding* – Vesicoureteral reflux (VUR) or backtracking of urine from the bladder back up into the kidneys, is the most common urinary tract birth defect. Reflux nephropathy or kidney injury associated with reflux is the 4th leading cause of kidney failure in children. Reflux is also associated with dysfunctional voiding (DV) (abnormal bladder contractions) and patients with both conditions have a high risk for reflux nephropathy. Despite the association between reflux and voiding dysfunction, no one has found a genetic link. Our lab has developed a genetic mouse model that has very high rates of reflux and also has evidence of voiding dysfunction (as well as evidence of long term injury resembling reflux nephropathy). Using this mouse, we can identify treatments for patients with VUR and DV to decrease the incidence of long term injury from reflux nephropathy.
Anticipated Duration of Project

10/19/2011 – 12/31/2012

Project Overview

Vesicoureteral reflux (VUR; backflow of urine from the bladder into the ureter) and dysfunctional voiding (DV) are associated with kidney scaring or reflux nephropathy, with the highest risks being in patients with both VUR and DV. Our laboratory has generated a unique genetic mouse model with both VUR and DV, called $Fgfr2^{St/-}$ (with deletion of fibroblast growth factor receptor 2 in stroma around the Wolffian duct and ureter and in the bladder). Preliminary analyses of $Fgfr2^{St/-}$ mice reveal high VUR rates likely from abnormal insertion of the ureter into the bladder (from aberrant ureteric induction off the Wolffian duct) and from aberrant ureteral peristalsis. $Fgfr2^{St/-}$ mice have evidence of abnormal voiding, likely from aberrant bladder muscle contractions. Our hypothesis is that $Fgfr2^{St/-}$ mice represent a genetic model linking VUR and DV that pose a significant risk for reflux nephropathy. We will test the hypothesis with the following Aims: Aim 1: To determine roles of $Fgfr2$ in stroma during Wolffian duct/ureteric bud, ureter, and bladder development. Aim 2: To determine mechanisms by which $Fgfr2$ stromal deletion leads to VUR and ureteric, ureteral peristaltic, and voiding defects.

Principal Investigator

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Other Participating Researchers

Kenneth Walker – employed by Children’s Hospital of Pittsburgh

Expected Research Outcomes and Benefits

At present, vesicoureteral reflux or backtracking of urine from the bladder back up into the kidneys, is the most common urinary tract birth defect. Reflux nephropathy or long term injury to the kidneys associated with reflux is the 4th leading cause of kidney failure in children and a leading cause of increased blood pressure. There is a clear association between reflux and voiding dysfunction (not urinating frequently enough, abnormal bladder contractions) and those patients with both conditions are at a much higher risk for reflux nephropathy than those with either condition alone. Despite the association between reflux and voiding dysfunction, there has never been a genetic link described.
We have a novel genetic mouse model that has very high rates of reflux and also has evidence of voiding dysfunction (as well as evidence of long term injury resembling reflux nephropathy). This gives us an opportunity to understand potential genetic links between these conditions. It also allows us to understand how these genetic abnormalities result in the combination of reflux and voiding dysfunction. Ultimately, we can learn insights into appropriate treatments for patients with these conditions to decrease the incidence of long term injury from reflux nephropathy.

Summary of Research Completed

The progress report will be divided according to the specific aims of the project:

Specific Aim 1: To determine roles of Fgfr2 in stroma during Wolffian duct/ureteric bud, ureter, and bladder development. We have made significant progress in completing this Aim.

*Fgfr2*St-/- mice have ureteric induction defects and high rates of reflux with abnormal ureterovesical junctions:

To identify the lineage responsible for the defects in the Pax3cre mutants, we generated Tbx18cre knock-outs of *Fgfr2* in the stroma (*Tbx18creTg/+Fgfr2Lox/Lox or Fgfr2St-/-*). *Fgfr2St-/-* mice were viable without limb defects and heterozygotes were normal. We assessed for ureteric bud induction defects by whole mount *in situ* hybridization for *Ret* at embryonic day (E) 11.0 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 (157.46µm ± 32.25) were not different than controls (156.55µm ± 30.42); however, while control duct lengths were tightly clustered around the mean, mutant duct lengths were nearly random with 64% (18/28) having ducts that were >1 standard deviation shorter or longer than the control mean (Figure 1). Thus, loss of *Fgfr2* in stroma leads to cranially or caudally shifted ureteric bud induction sites.

Given the ureteric bud induction defects in *Fgfr2*St-/- mice, we assayed for 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 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 \( \text{Fgfr2}^{St-/} \) ureters compared to non-refluxing control and \( \text{Fgfr2}^{St-/} \) ureters (Figure 3). Thus, \( \text{Fgfr2}^{St-/} \) 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.

\( \text{Fgfr2}^{St-/} \) mice have ureteral peristaltic defects:
Since tailbud derived stroma 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).

\( \text{Fgfr2}^{St-/} \) mice develop distended bladders and have dysfunctional voiding:
We next examined \( \text{Fgfr2}^{St-/} \) mice for bladder defects. Histological sections in embryos and in P1 and 1 month old mice after cystograms showed no obvious defects in \( \text{Fgfr2}^{St-/} \) bladders versus controls (Figure 5 and not shown). By 6 months of age, however, at least half of the \( \text{Fgfr2}^{St-/} \) mice had very grossly distended bladders prior to performing cystograms (not shown). Following cystograms, all 6 month old \( \text{Fgfr2}^{St-/} \) 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). \( \text{Fgfr2}^{St-/} \) 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, \( \text{Fgfr2}^{St-/} \) mice have evidence of dysfunctional voiding starting at 1 month that worsens with age.

Thus, Tbx18cre deletion of \( \text{Fgfr2} \) in tailbud stroma leads to ureteric bud induction defects with subsequent abnormal ureteral insertion into the bladder and vesicoureteral reflux. The mutants also have ureteral peristaltic defects and exhibit voiding dysfunction, despite normal appearing histology at early ages. The ureter and bladder defects likely contribute to the persistence of the reflux in older animals. This model provides a novel genetic link between reflux and ureter and bladder dysfunction.

Specific Aim 2: To determine mechanisms by which \( \text{Fgfr2} \) stromal deletion leads to VUR and ureteric, ureteral peristaltic, and voiding defects. We have made some progress in completing this Aim.

\( \text{Bmp4} \) and \( \text{Sprouty1} \) are likely downstream targets of \( \text{Fgfr2} \) signaling leading to the ureteric induction defects in \( \text{Fgfr2}^{St-/} \) mutant mice:
We performed *in situ* hybridization and real time PCR (qPCR) at E10.5 for molecules that affect 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 7). 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 7).

**Figures and Tables:**

![Figure 1: E11 Fgfr2St−/− mice have ureteric bud induction defects. In situ hybridization for Ret shows that compared with controls (A), Fgfr2St−/− 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) whereas the majority of Fgfr2St−/− are outside of that range. ***p<0.01 vs. control](image-url)
Table 1: Reflux rates, laterality and grades in Fgfr2<sup>St/-</sup> and control mice at P1 (*p<0.001)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Reflux (%)</th>
<th>Unilateral</th>
<th>Median grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 Control</td>
<td>2.6% (2/76)</td>
<td>100%</td>
<td>1</td>
</tr>
<tr>
<td>P1 Fgfr2&lt;sup&gt;St/-&lt;/sup&gt;</td>
<td>74.2% (23/31)*</td>
<td>95.7%</td>
<td>2</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Reflux (%)</th>
<th>Unilateral</th>
<th>Median grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 month Control</td>
<td>12.5% (2/16)</td>
<td>100%</td>
<td>1</td>
</tr>
<tr>
<td>6 month Fgfr2&lt;sup&gt;St/-&lt;/sup&gt;</td>
<td>90.9% (10/11)*</td>
<td>90%</td>
<td>2</td>
</tr>
</tbody>
</table>

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

Figure 3: P1 Fgfr2<sup>St/-</sup> refluxing ureters are displaced and have shortened intravesicular tunnels. a-c. Control without reflux (a) has similar ureteral insertion sites. Fgfr2<sup>St/-</sup> mouse with unilateral reflux (b) has high/lateral displacement of the ureter with reflux (*). c. Graph shows that Fgfr2<sup>St/-</sup> 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<sup>St/-</sup> with reflux).
Figure 4: P1 \(\text{Fgfr2}^{\text{St/-}}\) ureters have normal histology but abnormal peristalsis. a,b. H&E stains of ureters appear normal in control (a) and \(\text{Fgfr2}^{\text{St/-}}\) (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 \(\text{Fgfr2}^{\text{St/-}}\) (d) (blue=DAPI). e. Graph shows that mean contraction intervals are increased in mutants vs. controls (*p<0.001).

Figure 5: \(\text{Fgfr2}^{\text{St/-}}\) bladders appear normal at P1, but distended at 6 months. a,b. H&E stains of P1 control (Con) (a) & \(\text{Fgfr2}^{\text{St/-}}\) (R2St/-) (b) appear similar (bar=muscle thickness). c,d. Smooth muscle actin (red) and E-cadherin (green) staining appear similar in P1 control (c) & \(\text{Fgfr2}^{\text{St/-}}\) (d). e-g. Trichrome shows 6m \(\text{Fgfr2}^{\text{St/-}}\) bladders are mildly (f) to severely (g) distended with less muscle vs. controls (e) (arrowheads= muscle).

Figure 6: \(\text{Fgfr2}^{\text{St/-}}\) 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 \(\text{Fgfr2}^{\text{St/-}}\) (R2St/-) have multiple smaller voids closer to their food/water at 1 and 6 months (b,d). e. Graph shows 3 and 6 month \(\text{Fgfr2}^{\text{St/-}}\) have smaller maximum void spots (implying worsened voiding dysfunction) vs. controls. *p<0.01

Figure 7: \(\text{Bmp4}\) and \(\text{Sprouty1}\) expression is decreased in E10 \(\text{Fgfr2}^{\text{St/-}}\) urogenital ridges. a-d. \textit{In situ} hybridization for \(\text{Bmp4}\) (a, b, arrows) and \(\text{Sprouty1}\) (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 \(\text{Bmp4}\) and \(\text{Sprouty1}\) mRNA vs. controls (*p<0.05, **p<0.001). g. Proposed Bmp4 signaling from the stroma regulating Sprouty1 in the Wolffian duct.