Deletion of fibroblast growth factor receptor 2 from the peri-Wolffian duct stroma leads to ureteric induction abnormalities and vesicoureteral reflux

Kenneth A. Walker  
*Children's Hospital of Pittsburgh of UPMC, Pittsburgh*

Sunder Sims-Lucas  
*Children's Hospital of Pittsburgh of UPMC, Pittsburgh*

Valeria E. Di Giovanni  
*Children's Hospital of Pittsburgh of UPMC, Pittsburgh*

Caitlin Schaefer  
*Children's Hospital of Pittsburgh of UPMC, Pittsburgh*

Whitney M. Sunseri  
*Children's Hospital of Pittsburgh of UPMC, Pittsburgh*

*See next page for additional authors*

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

**Recommended Citation**

Walker, Kenneth A.; Sims-Lucas, Sunder; Di Giovanni, Valeria E.; Schaefer, Caitlin; Sunseri, Whitney M.; Novitskaya, Tatiana; de Caestecker, Mark P.; Chen, Feng; and Bates, Carlton M., "Deletion of fibroblast growth factor receptor 2 from the peri-Wolffian duct stroma leads to ureteric induction abnormalities and vesicoureteral reflux." PLoS One. 8,2. e56062. (2013).  
[https://digitalcommons.wustl.edu/open_access_pubs/1393](https://digitalcommons.wustl.edu/open_access_pubs/1393)

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact [vanam@wustl.edu](mailto:vanam@wustl.edu).
Abstract

Purpose: Pax3cre-mediated deletion of fibroblast growth factor receptor 2 (Fgfr2) broadly in renal and urinary tract mesenchyme led to ureteric bud (UB) induction defects and vesicoureteral reflux (VUR), although the mechanisms were unclear. Here, we investigated whether Fgfr2 acts specifically in peri-Wolffian duct stroma (ST) to regulate UB induction and development of VUR and the mechanisms of Fgfr2 activity.

Methods: We conditionally deleted Fgfr2 in ST (Fgfr2ST−/−) using Tbx18cre mice. To look for ureteric bud induction defects in young embryos, we assessed length and apoptosis of common nephric ducts (CNDs). We performed 3D reconstructions and histological analyses of urinary tracts of embryos and postnatal mice and cystograms in postnatal mice to test for VUR. We performed in situ hybridization and real-time PCR in young embryos to determine mechanisms underlying UB induction defects.

Results: We confirmed that Fgfr2 is expressed in ST and that Fgfr2 was efficiently deleted in this tissue in Fgfr2ST−/− mice at embryonic day (E) 10.5. E11.5 Fgfr2ST−/− mice had randomized UB induction sites with approximately 1/3 arising too high and 1/3 too low from the Wolffian duct; however, apoptosis was unaltered in E12.5 mutant CNDs. While ureters were histologically normal, E15.5 Fgfr2ST−/− mice exhibit improper ureteral insertion sites into the bladder, consistent with the ureteric induction defects. While ureter and bladder histology appeared normal, postnatal day (P) 1 mutants had high rates of VUR versus controls (75% versus 3%, p = 0.001) and occasionally other defects including renal hypoplasia and duplex systems. P1 mutant mice also had improper ureteral bladder insertion sites and shortened intravesicular tunnel lengths that correlated with VUR. E10.5 Fgfr2ST−/− mice had decreases in Bmp4 mRNA in stromal tissues, suggesting a mechanism underlying the ureteric induction and VUR phenotypes.

Conclusion: Mutations in FGFR2 could possibly cause VUR in humans.

Introduction

Murine metanephric kidney development starts at E10.5 when an outgrowth from the Wolffian duct, the ureteric bud, is induced by the adjacent metanephric mesenchyme (MM) [1]. The UB induction site is constrained to its proper position by tailbud mesenchymal derived stroma (ST) lying between the Wolffian duct and MM [2]. Perturbed growth factor signaling within the ST such as haploinsufficiency of bone morphogenetic protein 4 (Bmp4) causes a shift in the UB induction site leading to urinary tract anomalies including ectopic ureters and renal hypoplasia [2]. In addition, aberrant ureteric induction sites are associated with abnormal positioning of the ureteral insertion site into the bladder and increased likelihood of vesicoureteral reflux in humans and animal models [3,4].

VUR may be present in up to 2% of children and is associated with the development of reflux nephropathy, a leading cause of pediatric chronic kidney disease [5]. In addition to abnormal position of ureteral insertion into the bladder, VUR has been associated with a number of other defects including shortened intravesicular ureteral tunnel lengths [5,6,7] and bladder and ureteral muscle defects [8]. Despite some insights into pathophysiology, the genetics of VUR remain unclear. VUR follows a
dominant inheritance pattern, however no single gene defect has been shown to cause the majority of VUR cases [5].

Fibroblast growth factor receptors 1 and 2 are critical for different stages and lineages of kidney development [9–17]. While conditional deletion of Fgfr1 with the transgenic Pax5Cre line in the peri-Wolffian duct stroma and MM resulted in no kidney defects, Pax5Cre-mediated deletion of Fgfr2 led to UB induction abnormalities and high rates of VUR [9,10,11]. Despite these data, it is unclear whether deletion of Fgfr5 in the ST or the MM drives the abnormalities and what downstream targets mediate the actions of Fgfr2.

To determine whether loss of Fgfr2 in the ST alters ureteric induction leading to VUR, we utilized a Tbx18cre mouse line [18] to delete Fgfr2 expression in peri-Wolffian duct stroma but not in MM. E11.5 Fgfr2+/− mice exhibit aberrant ureteric induction with approximately 1/3 arising too high and 1/3 too low from the Wolffian duct. 3D imaging of E15.5 mutants revealed abnormal ureteral insertion sites into the bladder, consistent with the ureteric induction defects. P1 mutants had high rates of VUR associated with improper ureteral insertion sites into the bladder and shortened intravesicular tunnel lengths. Rarely Fgfr2+/− mice had other anomalies such as renal hypoplasia or duplex systems. Loss of Fgfr2 in ST led to a decrease in Bmp4 expression at E10.5 that is likely the reason for the ureteric induction defects.

Materials and Methods

Mice

Tbx18cre Te+/+ mice that drive cre expression in the peri-Wolffian duct stroma were bred with Fgfr2lox/lox mice, a gift from Dr. David Ouritz [19], to produce Tbx18creTe+/+ Fgfr2lox/lox mice with deletion of Fgfr2 in the ST (Fgfr2ST−/−). To detect cre expression, Tbx18cre Te+/+ and Fgfr2ST−/− mice were bred with Cag:RFP+/+ reporter mice (Cag-Tbx18cre Te+/+ and Cag-Fgfr2ST−/−) to generate offspring in which red fluorescent protein (RFP) is expressed at the site of cre recombinase [20]. For embryonic studies, timed overnight matings were generated; when a vaginal plug was identified, noon of that day was deemed E0.5. All experiments were carried out with approval of the University of Pittsburgh Institutional Animal Care and Use Committee.

Genotyping

DNA was extracted from tail clippings or embryonic tissues for PCR genotyping, as described [9]. The following genotyping primers were used to detect respective gene expression: Tbx18cre - 5'-CCATCCTAACAGCACC TGGGCCAGCTCAACA-3' and 5'-CCACCATCGGTGCGGGAGATGTCCTTCACT-3', Fgfr2 - 5'-GTCACATCTCAAGCCACTGTCGTGCC-3' (wildtype allele), 5'-CTCCACCTGATACATCTAAAGGCC-3' (boxed allele), CAG - 5'-AAG GGA GCT GCA GTG GAG TA-3', 5'-CCG AAA ATC TGT GGG AAG AGG TC-3' (wildtype allele), 5'-GCC ATT AAA GCA GCG TAT CC-3', 5'-CTG TTC CTG TAC GGC ATG G-3' (Cag allele).

Gene Expression Analysis

Whole-mount in situ hybridization (ISH) was performed on E10.5 control and Fgfr2ST−/− tissues as described [9]. Briefly, digoxigenin UTP-labeled antisense RNA probes were generated against Bmp4 (Gene Accession number: NM007554.2), Fgfr2 (NM021601.2) and Ret (NM001080708.1).

Fluorescence Activated Cell Sorting (FACS)

Six E10.5 Cag-Tbx18cre Te+/+ and Cag-Fgfr2ST−/− urogenital ridges were dissected and then manually dissociated with 0.3% collagenase into a cellular suspension in preparation for flow cytometry. Tbx18cre Te+/+ cells were FACsorted from other cells via the presence of RFP. Following flow cytometry, cells from each genotype were pooled for subsequent Real-time PCR analysis.

Real-time PCR

For quantitative analysis mRNA expression, six E10.5 Fgfr2ST−/− and control urogenital ridges, or pooled cells as mentioned previously, were collected and RNA extracted using a Mini-prep kit (Qiagen, Valencia, CA). To analyze gene expression, TaqMan® Gene Expression assays for Fgfr2, Ret and Bmp4 and Gapdh (NM_000804.2; endogenous control) were utilized (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed as described [21] on an Applied Biosystems ABI 7900 HT (Foster City, CA).

Common nephric duct length, proliferation and apoptosis

To assess common nephric duct (CND) length E11.5 urogenital ridges from Fgfr2ST−/− and cre negative littermate control embryos were dissected into ice cold methanol, washed in PBS, blocked and stained whole mount with a pan-cytokeratin antibody (1:10; Sigma Aldrich) followed by goat anti-mouse Alex Fluor 594 (1:500; Sigma Aldrich) to visualize the ureteric bud and Wolffian duct. Stained urogenital ridges were mounted and imaged using a confocal microscope. CND length from the caudal end of the nephric duct at the cloaca to the cranial edge of the ureteral bud stalk origin was measured using Imaris software (Bitplane, Zurich, Switzerland).

To investigate proliferation in the CND, E12.5 Fgfr2ST−/− and littermate control embryos were fixed in 4% PFA/PBS, embedded in paraffin and serially sectioned at 10 μm through the urogenital sinus and CND. Sections were then labeled with antibodies against phospho-histone H3 (pH3: 1:200; Sigma Aldrich) and E-Cadherin (Ecad: 1:250; Sigma Aldrich) to visualize the ureteric bud and Wolffian duct. Stained urogenital ridges were mounted and imaged using a confocal microscope. CND length from the caudal end of the nephric duct at the cloaca to the cranial edge of the ureteral bud stalk origin was measured using Imaris software (Bitplane, Zurich, Switzerland).

At the completion of the assay, to examine apoptosis of the CND, methanol fixed E12.5 Fgfr2ST−/− and littermate control urogenital blocks (including mesonephros, metanephros, ureter, gonads and cloaca) were examined using whole mount immunofluorescence with antibodies against pan-cytokeratin (1:10; Sigma Aldrich) to visualize CNDs and activated caspase-3 (1:250, Invitrogen) to detect apoptosis, as described previously [22]. We detected cytokeratin with goat anti-rabbit Alex Fluor 594 and E-Cadherin with donkey anti-rabbit Alex Fluor 488 (1:500; Molecular Probes). A minimum of three sections through the CND, at 20 μm intervals were assessed for each embryo of each genotype.

To assess for VUR, cystograms were performed in P1 Fgfr2ST−/− and littermate control pups as described [22]. Briefly, mice were euthanized and abdominal walls were reflected to expose the bladder. A 30-gauge needle was inserted into the bladder and 1% methylene blue dye was gravity filled into the bladder via a 50 ml syringe. Starting at a height of 0 cm, the syringe was raised 30 cm at 5-second intervals to a final height of 150 cm, at which time the syringe was maintained for 15 seconds. The presence of dye ascending from the bladder towards the kidney was determined as VUR. All VUR was graded according to international classifications [23]. At the completion of the assay,
all kidneys were photographed for assessment of kidney long axis via Image J software (National Institute of Health).

Three-Dimensional (3D) Reconstructions
Whole E15.5 embryos and lower body P1 (post-cystogram) tissues from control and Fgfr2ST−/− mice were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated and paraffin embedded. Tissues were serially sectioned at 10 μm through the ureters and bladder, and stained with hematoxylin and eosin (H&E). Bladders and ureteral insertion sites were reconstructed into three-dimensional models as previously described [10].

Histology and Immunohistochemistry
All tissues were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated and paraffin embedded. Tissues were sectioned at either 6 or 10 μm and stained with H&E to evaluate tissue morphology. For immunohistochemistry sections were dewaxed, antigen retrieved using citrate buffer, permeabilized using 5% tween in PBS, blocked with 1% donkey serum in PBS, and incubated overnight with alpha smooth muscle actin (αSMA; Sigma Aldrich) at 4°C (Figure 1A). Sections were then washed and incubated for 1 hour at room temperature with secondary antibodies (Rabbit anti-Mouse 594: 1:500; Donkey anti-Rat 488: 1:500; Molecular Probes) and DAPI (1:10,000) diluted in 1% donkey serum in PBS. Sections were then washed, mounted and visualized.

Statistics
One-way ANOVA followed by Bonferroni’s post-hoc analysis, student’s T-tests and fisher’s exact test analyses were conducted where appropriate, using GraphPad Prism™ 5 (GraphPad Software Inc, 1992–2007).

Results
Tbx18cre and Fgfr2 expression in the peri-Wolffian duct stroma
To confirm expression of the Tbx18cre during ureteric induction deletion we crossed Tbx18cre reporter mouse line (Figure 1A). Robust cre expression was observed in peri-wolffian duct stroma on the dorsal surface of the urogenital tract at E10.5 (Figure 1B). To confirm deletion of Fgfr2 in peri-Wolffian duct stroma in Fgfr2ST−/− mice, we performed whole-mount in situ hybridization for Fgfr2 in E10.5 embryos. In control embryos, a wide band of Fgfr2 mRNA expression was visualized in both the Wolffian duct and peri-Wolffian duct stroma (Figure 2A, 2C). In Fgfr2ST−/− embryos, we observed linear Fgfr2 expression in the Wolffian duct epithelium, but absent expression in the peri-Wolffian duct stroma (Figure 2B, 2D). We also dissected, dissociated, and FACsorted E10.5 Cag-Tbx18creTg/+ (control) and Cag-Fgfr2ST−/− urogenital ridge cells; after isolating mRNA, we confirmed a nearly complete loss of Fgfr2 expression in the Tbx18cre expressing urogenital ridge cells in Fgfr2ST−/− mice (5% Fgfr2 expression in Fgfr2ST−/− mice relative to controls; Figure 2E). Thus, these data confirm the Fgfr2ST−/− mice as a model of conditional deletion of Fgfr2 in the peri-Wolffian duct stroma.

Figure 1. Expression of the Tbx18cre in E10.5 mouse embryos and urogenital tracts. A. E10.5 Cag-Tbx18cre+/+ mouse demonstrate cre expression noted by RFP staining (red) in numerous tissues including fore- and hindlimbs, spine and urogenital ridges. B. Ventral view of dissected urogenital ridges illustrates robust cre expression in the peri-Wolffian duct stroma at E10.5 (highlighted between arrowheads). Scale bars = 100 μm. ***p<0.001 vs. control embryos. doi:10.1371/journal.pone.0056062.g001

Figure 2. Expression of Fgfr2 in E10.5 in control and Fgfr2ST−/− embryos. A,C. Lower power (A) and higher power (C) images show that control embryos have a wide band of Fgfr2 signal (between arrowheads) encompassing the Wolffian duct and surrounding stroma. B,D. Lower power (B) and higher power (D) images show that Fgfr2ST−/− embryos have a linear band of Fgfr2 expression in the Wolffian duct epithelium (arrows) and not in the surrounding stroma. E. Quantitative real-time PCR of FAC-sorted E10.5 Cag-Tbx18cre+/+ (Control) and Cag-Fgfr2ST−/− (Fgfr2ST−/−) urogenital ridges confirms a dramatic decrease in Fgfr2 mRNA expression in mutant Tbx18cre expressing cells. Scale bars = 100 μm. ***p<0.001 vs. control embryos. doi:10.1371/journal.pone.0056062.g002
Aberrant ureteric bud induction following deletion of Fgfr2 from ST

Previously, deletion of Fgfr2 expression with a transgenic Pax3cre line in the MM and peri-Wolffian duct stroma was associated with aberrant UB induction [10]. To determine if deletion of Fgfr2 in ST is sufficient to induce abnormal ureteric budding, we measured the common nephric duct length in E11.5 urogenital ridges after performing whole mount cytokeratin staining (Figure 3A–C). Mean CND lengths were similar between controls and mutants (Control: 170.75±25.34 μm; Fgfr2st−/−: 102.74±53.92 μm; p = 0.5, Figure 3D). However, it appeared that the mutants had a tri-modal distribution of CND lengths as opposed to control CND lengths that appeared to have a Gaussian distribution (Figure 3D). In Fgfr2st−/− mice, 69% of the CND lengths were >1 control SD away from the mean (both shorter and longer), whereas only 20% of the control CND lengths were >1 control SD from the mean (p = 0.01). Mutants also had a greater intra-embryo variability in CND length between right and left sides than controls; 75% (6/8) of Fgfr2st−/− mice had intra-embryonic CND lengths that were >1 control SD different between the right and left side, whereas only 29% (2/7) of controls had intra-embryonic CND lengths that were >1 SD different between each side (p = 0.04) (Figure 3E). Despite the differences in CND length at E11.5, whole-mount activated caspases-3 staining illustrates similar patterns of ventral apoptosis of the distal CND in both control and Fgfr2st−/− urogenital ridges at E12.5 (Figure S1).

Similarly, phospho-histone H3 staining of cells in the CND of control and Fgfr2st−/− embryos demonstrated a similar percentage of proliferating cells in both genotypes at E12.5 (Figure S2). Together indicating that aberrant ureteric bud induction is not associated with changes in CND apoptosis or proliferation (either decreases or increases) at later stages. Thus, deletion of Fgfr2 from the peri-Wolffian duct stroma induces aberrant UB induction relative to controls.

Fgfr2st−/− embryos have improper ureteral insertion in bladders at E15.5

To determine whether the ureteric induction defects led to abnormal ureteral insertion in the bladder during embryogenesis, we performed H&E staining and 3D reconstructions of E15.5 control and Fgfr2st−/− lower urinary tracts. Histologically, the E15.5 ureters and bladders appeared normal (Figure S3). After performing 3D reconstructions (Figure 4), we determined the position of the ureter insertions into the bladder in two ways: First, we generated triangles connecting the two external ureteral insertion sites and the bladder neck (external bladder trigone) from which we could determine whether ureters were displaced. Second, we noted the ureteral insertion site in relation to the plane of the pubic symphysis, which we used as an anatomic reference point.

In control embryos, ureteral insertion occurs just superior to the pubic symphysis, with both ureters inserting into the bladder at a...
similar anatomical level (Figure 4A). Furthermore, the control external trigones formed nearly perfect inverted isosceles triangles with similar-appearing angles formed at the insertion sites (Figure 4D). In contrast, Fgfr2ST−/− embryos usually had one displaced ureter including those with low bladder insertions (Figure 4B) and others with high bladder insertions (Figure 4C) relative to the pubic symphysis. The external trigones formed triangles that were distorted from the shape of an isosceles triangle with significant differences between angles formed at the insertion sites (Figure 4E-F). Quantitatively, controls had right and left external angles that were generally within 10° whereas mutants had average differences of approximately 30° (Figure 4G). Thus, E15.5 Fgfr2ST−/− embryos had evidence of improper unilateral ureteral insertion consistent with the ureteric induction defects noted earlier.

Fgfr2ST−/− mice have high incidence of VUR and occasionally other urinary tract anomalies at birth

Given the evidence of ureteric induction defects and improper ureteral insertion in the bladder in embryos, we assessed P1 Fgfr2ST−/− mice for VUR with cystograms by gravity filling bladders with methylene blue dye. As shown, P1 Fgfr2ST−/− mice (excluding those with duplex ureters) had high rates of vesicoureteral reflux relative to control littermates (Figure 5, Table 1). The VUR in Fgfr2ST−/− mice had no gender bias (Table 1). In addition, mutants usually had unilateral VUR with only one Fgfr2ST−/− mouse exhibiting bilateral reflux (Table 1). The mutants most frequently had grade II VUR followed by grade III VUR while the few control mice with VUR had grade I reflux (Tables 1 and 2). No VUR greater than grade III was observed in mutants most frequently had grade II VUR followed by grade III VUR while the few control mice with VUR had grade I reflux. Thus, E15.5 Fgfr2ST−/− embryos had average differences of approximately 30° (Figure 4G). Thus, E15.5 Fgfr2ST−/− embryos had evidence of improper unilateral ureteral insertion consistent with the ureteric induction defects noted earlier.

Refluxing P1 Fgfr2ST−/− mice have improper ureteral insertion and shortened intravesicular tunnel lengths

We then sought to determine whether there were defects in either urinary tract morphology and/or in ureteral insertion that could explain the high rates of VUR in Fgfr2ST−/− mice. Thus, we performed histological assays and 3D reconstructions of the lower urinary tracts of P1 mice after cystograms. Morphologically, P1 mutant bladders and ureters appeared normal by H&E staining and by immunostaining with markers for muscle and urothelium (Figure 6). However, as in E15.5 mutants, 3D reconstructions revealed abnormal unilateral ureteral insertion sites into the bladders of P1 Fgfr2ST−/− mice with VUR (including high and low insertions) but normal-appearing insertion sites in mutants without VUR (Figure 7 and not shown). Quantitatively, P1 controls and Fgfr2ST−/− mice without VUR had right and left external angles that were generally within 10° whereas mutants with unilateral reflux had average differences of approximately 35° (Figure 7E). Furthermore, ureters from Fgfr2ST−/− mice that refluxed had significantly shortened intravesicular tunnel lengths compared with both control and non-refluxing Fgfr2ST−/− ureters (Control: 286.1±47.92 μm; Fgfr2ST−/− with no reflux: 302.18±44.41 μm; Fgfr2ST−/− with reflux: 186.23±59.76 μm; p<0.001) (Figure 7F). Thus the presence of VUR correlated with displaced ureteral insertion sites into the bladder and shorter intravesicular tunnel lengths.

Fgfr2 deletion results in decreased Bmp4 expression in urogenital regions

Bmp4, which is secreted by the peri-Wolffian duct stroma, is known to constrain the ureteric bud to its proper induction site along the Wolffian duct [6]. Given that deletion of Fgfr2 in this tissue leads to defects in ureteric induction, we examined Fgfr2ST−/− mutants at E10.5 for changes in Bmp4 and Ret expression. Whole mount in situ hybridization at E10.5 illustrated that Fgfr2ST−/− embryos have significantly less Bmp4 mRNA expression in the peri-Wolffian duct stroma relative to controls (Figure 8). In contrast, ureteric bud and Wolffian duct expression of Ret mRNA appeared similar between both genotypes. Quantitative real-time PCR of dissected urogenital ridges confirmed ~40% reductions in Bmp4 mRNA expression in Fgfr2ST−/− embryos relative to controls while Ret expression was equivalent (Figure 8). Thus, the decrease in Bmp4 expression in Fgfr2ST−/− embryos correlates with the ureteric induction defects that likely lead to VUR and other phenotypes observed in the mutant mice [6,7].

Table 1. Incidence and severity of VUR at P1 in control and Fgfr2ST−/− mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Incidence of Reflux</th>
<th>Unilateral Reflux Observed</th>
<th>Grade of Reflux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Control</td>
<td>3% 2/76</td>
<td>4% 2/45</td>
<td>0% 0/31</td>
</tr>
<tr>
<td>Fgfr2ST−/−</td>
<td>75%*** 23/31</td>
<td>72% 13/18</td>
<td>77% 10/13</td>
</tr>
</tbody>
</table>

***p<0.001 vs. control, analysis via a Fisher’s Exact Test.

doi:10.1371/journal.pone.0056062.t001
Discussion

Fibroblast growth factor receptors, their ligands and their downstream signaling adapters have previously been shown to be integral for the development of the metanephric kidney, often by conditional deletion in the Wolffian duct/ureteric bud and/or metanephric mesenchyme [9–17]. Using a Pax3cre transgenic line, we determined that deletion of Fgf2 in both the peri-Wolffian duct stroma and the metanephric mesenchyme led to ureteric induction defects and subsequent VUR and other anomalies such as duplex systems and renal hypoplasia; however, it was unclear as to the contribution of each of these tissues to those defects [9,10]. By utilizing the Tbx18cre mouse line that drives cre expression in the peri-Wolffian duct stroma and not in the metanephric mesenchyme, we now conclude that Fgf2 expression in the peri-Wolffian duct stroma is most critical for regulating the ureteric bud induction site and preventing VUR and other urinary tract anomalies.

We did observe some differences between Fgf2ST/−/− mice and Pax3creTg/+ Fgf2Lox/Lox mice [10]. First, we detected higher rates of VUR in Fgf2ST/−/− mice compared to the Pax3cre mice; this discrepancy could be due to more complete deletion of Fgf2 in the peri-Wolffian duct stroma with the Tbx18cre line versus the Pax3cre line, as cre expression in the MM has been shown to be variable with the Pax3cre line when bred with reporter mice (Drs. Valeria Di Giovanni and Norman Rosenblum, unpublished observations). In addition, we noted shortened intravesicular tunnel lengths and both cranial and caudal displacement of ureteric bud induction sites in Fgf2ST/−/−, whereas the Pax3cre mutant mice had normal tunnel lengths and only cranially displaced ureteric buds. We suspect that we had more power in the current study (larger numbers of mutant mice and higher VUR rates) than the previous study and were therefore able to detect these defects in the Fgf2ST/−/− mice.

The reason for the VUR in the Fgf2ST/−/− mice appears to be secondary to ureteric bud induction defects, leading to abnormal ureteric induction sites and incompetent anti-reflux mechanisms, as described by Drs. Mackie and Stephens in humans with VUR [24,25]. The reflux observed does not appear to be related to a persistence or premature loss of the CND, as apoptosis and proliferation were unaltered in the mutant mice at E12.5. Fgf2ST/−/− mutants did have evidence of abnormal ureteral insertion sites in the bladder by E15.5; moreover, abnormal position of the ureteral insertion site correlated directly with the presence of VUR in P1 mutants. The P1 mutant ureters with VUR also had shortened intravesicular tunnel lengths, which is known to further increase the risks of VUR in humans [5]. Most of the P1 Fgf2ST/−/− mice had unilateral VUR, which likely reflects that many of the ureteric bud induction sites at E11.5 were at or near the normal position. We did not detect any histological defects in the ureters or bladders during development or in adult mice to explain the VUR. Finally, we noted other urinary tract anomalies in the mutants including duplex systems and renal hypoplasia, which likely reflect the ureteric induction defects. The high incidence of VUR and the presence of all the aforementioned anatomical features in Fgf2ST/−/− mice are very consistent with the Mackie-Stephens hypothesis for the developmental origin of VUR in our mice [24,25].

Table 2. Grades of VUR at P1 in control and Fgf2ST/−/− mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Grade I</th>
<th></th>
<th></th>
<th>Grade II</th>
<th></th>
<th></th>
<th>Grade III</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Control</td>
<td>100% (2/2)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fgf2ST/−/−</td>
<td>9% (2/23)*</td>
<td>1</td>
<td>1</td>
<td>69% (16/23)</td>
<td>9</td>
<td>7</td>
<td>22% (5/23)</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*p<0.05 vs. control, analysis via a Fisher’s Exact Test.
doi:10.1371/journal.pone.0056062.t002

Figure 6. Bladder and ureter morphology in P1 Fgf2ST/−/− and control mice following cystograms. A–B. H&E staining shows similar bladder histology between controls (A) and mutants (B). C–D. Immunofluorescence (IF) also shows normal urothelium (green) and alpha smooth muscle actin staining (red) in control (C) and Fgf2ST/−/− mice (D). E–H. H&E staining and IF shows normal urothelium and muscle layers in control ureters (E, G) and mutant ureters (F, H). Arrows indicate muscle layer; Arrowheads indicate urothelium. Scale bar = A–D: 300 μm, E–H: 500 μm.
doi:10.1371/journal.pone.0056062.g006
An important finding of the current study is a likely molecular mechanism responsible for the ureteric induction defects (and associated CAKUTs and VUR) in Fgfr2ST2/2 mice. While we previously found that reduction of Fgfr expression in kidney mesenchyme led to increases in Ret expression in the ureteric bud [16], we found no evidence of altered Ret expression in the UB or Wolffian duct in Fgfr2ST2/2 embryos. Moreover, signaling through Fgfrs has been shown to directly and indirectly regulate expression of secreted growth factors, such as Bmp4 [16,26]. In the current study, Fgfr2ST2/2 mice exhibit a 40% reduction in Bmp4 mRNA expression prior at E10.5, at the onset of ureteric bud induction. Interestingly, in situ hybridization indicated that the entire field of Bmp4 expression seen in control embryos at this stage was significantly reduced in Fgfr2ST2/2 mice. Furthermore, it is possible that this wide area of decreased Bmp4 expression in the peri-Wolffian duct stroma adjacent to the metanephric mesenchyme may account for the variability in the site of ureteric bud induction observed in Fgfr2ST2/2 mice. Moreover, Bmp4 heterozygous mice have a 40% reduction in Bmp4 mRNA levels and have a high incidence of ureteric induction abnormalities [6,7,27]. Reduced Bmp4 mRNA expression as seen in Bmp4 heterozygous mice or following disruption of upstream regulators of Bmp4 is strongly associated with urinary tract anomalies such as duplex systems and renal hypoplasia (they have not to our knowledge been tested for VUR) [6,7,27,28]. Findings from our study strongly suggest that Fgfr acts upstream of Bmp4 in the peri-Wolffian duct stroma to maintain the proper ureteric induction site.

In humans, dysregulation of both FGFRs and BMP4 have been associated with a wide range of urinary tract abnormalities. Non-syndromic kidney and urinary tract anomalies have been associated with mutations in BMP4 in humans [29,30]. In addition mutations in FGFRs lead to syndromes including Apert’s syndrome, Antley-Bixler syndrome, Pfeiffer syndrome, and Beare-Stevenson syndrome, which are sometimes associated with urogenital anomalies such as hydroureter, solitary kidney, and VUR [31]. Thus it is possible that unidentified mutations in FGFs or FGFRs could affect BMP4 levels in humans leading to VUR and other urinary tract anomalies; thus, FGF receptors and their

Figure 7. Three-dimensional reconstructions of ureteral insertion sites into the bladders of P1 control and Fgfr2ST2/2 embryos. A–B. Control mouse without VUR (A) has ureters (blue) that insert into bladders (yellow) at the same level, whereas the Fgfr2ST2/2 mouse with left sided VUR (B) has a high and laterally displaced ureteral insertion site on that side (*). C–D. External trigone drawn from the control (from A) shows similar right and left sided external angles whereas the mutant (from B) has a marked differences in the left and right external angles. E. Graph demonstrating that the differences in external trigonal angles are much greater in mutants with VUR than controls or mutants without VUR. Values in E–F are mean±SD; ***p<0.001 vs. control and vs. Fgfr2ST / mutants without VUR).

doi:10.1371/journal.pone.0056062.g007

Figure 8. Expression of Bmp4 and Ret mRNA at E10.5 in control and Fgfr2ST2/2 urogenital ridges. A–B. In situ hybridization for Bmp4 shows apparent reduced expression in the peri-Wolffian duct stroma in mutants (B, white arrowheads) versus controls (A, white arrowheads). C–D. Graphs of real time PCR confirm that Fgfr2ST2/2 urogenital ridges have an ~40% reduction in Bmp4 mRNA (C), whereas Ret mRNA levels are equivalent (D). Values are means normalized to control expression levels; *p<0.05, **p<0.01 vs. control; n = 3 urogenital ridges per genotype per analysis. Dotted lines indicate the dorsal aspect of the Wolffian duct in each image. Scale bar = 100 μm.

doi:10.1371/journal.pone.0056062.g008
ligands are potential candidates for future genetic screening studies in patients with VUR and other urinary tract defects.

Supporting Information

Figure S1 Representative images of apoptosis of the CND at E12.5 in control and Fgfr2ST−/− embryos. A-B. Co-immunofluorescent labeling of pancytokeratin (red) and activated caspase-3 (green) highlighted a similar pattern of apoptosis (arrowhead) of the distal CND in both control and Fgfr2ST−/− embryos (the entire CND is between the arrows). Scale bar = 100 μm. (TIF)

Figure S2 Cell proliferation in the CND at E12.5 in control and Fgfr2ST−/−. A-B. Co-immunofluorescent labeling of phosphor-histone H3 (red; proliferating cells) and E-Cadherin (green; urothelium) illustrates cell proliferation in the CND (arrowhead) in both control and Fgfr2ST−/− embryos. C. Quantification of proliferation in the CND indicates no differences between control and Fgfr2ST−/− embryos at E12.5. Dotted line indicates CND; Cl – Cloaca. Scale bar = 50 μm. (TIF)

Figure S3 E15.5 bladder and ureter morphology in control and Fgfr2ST−/− embryos. A-B. H&E stains show similar bladder morphology between control (A) and Fgfr2ST−/− embryos (B). C–D. H&E stains also shows similar ureter morphology between controls (C) and mutants (D). Arrowheads indicate mesenchymal (developing muscular) layer; Arrows indicate urothelium. Scale bars = 300 μm. (TIF)

Figure S4 P1 Fgfr2ST−/− mice with duplex collecting systems/kidneys and renal hypoplasia. A. Control kidney shows normal gross morphology. B. Fgfr2ST−/− mouse has a duplex collecting system filled with dye (arrows) and a duplex kidney (* = two kidney moieties). C. Fgfr2ST−/− mouse has a hypoplastic kidney. Scale bar = 500 μm. (TIF)

Acknowledgments

The authors thank Dr. David Ornitz for the use of the floxed Fgfr2 mouse line and Dr. Indra Gupta who taught us how to perform cystograms in mice.

Author Contributions

Conceived and designed the experiments: KAW SSL VED CMB TN. Performed the experiments: KAW SSL VED CMS WS TN MPdC FC CMB. Analyzed the data: KAW SSL CMB. Contributed reagents/materials/analysis tools: KAW VED CMS TN. Wrote the paper: KAW SSL CMB. Revised and improved the paper: All authors.

References