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High incidence of vesico-ureteral reflux in mice with deletion of fibroblast growth factor receptor 2 in kidney mesenchyme

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Abstract

Purpose—Mice with conditional deletion of fibroblast growth factor receptor 2 (*Fgfr2*) in metanephric mesenchyme (*Fgfr2^{Mes}/−*) have ureteric bud induction abnormalities. Our goal was to determine if *Fgfr2^{Mes}/−* mutants developed abnormally positioned ureters predisposing to vesico-ureteral reflux (VUR).

Materials and Methods—We measured common nephric duct (CND) lengths and assayed for apoptosis in embryonic day (E) 11.5 mice. We performed 3 dimensional (3D) reconstructions of and real time PCR and whole mount *in situ* hybridization for *Fgfr2* in urinary tracts in E15.5 embryos. We performed cystograms followed by 3D reconstructions in postnatal animals.

Results—Compared with controls, *Fgfr2^{Mes}/−* embryos had increased CND lengths (with no differences in apoptosis) indicating cranially displaced ureteric buds. 3-D reconstructions at E15.5 showed low insertions of ureters into the bladder (near bladder necks) in *Fgfr2^{Mes}/−* mice. Postnatal *Fgfr2^{Mes}/−* mutants had high rates of VUR compared with controls (47.4% vs. 4.0%, $p=0.00006$). In postnatal mutants with unilateral reflux, the refluxing ureters inserted closer to the bladder neck than non-refluxing ureters. The external ureteral insertional angles at the outer bladder wall (formed by the ureteral insertion points and bladder neck) were greater in mutant refluxing ureters compared to contralateral non-refluxing ureters and to control ureters. At E15.5, *Fgfr2* levels were decreased in *Fgfr2^{Mes}/−* kidneys compared with controls, but were not statistically different in ureters or bladders.

Conclusions—*Fgfr2^{Mes}/−* mice have ureteric induction abnormalities, associated with abnormal ureteral insertion in the bladder and subsequent VUR, consistent with the Mackie and Stephens hypothesis.

Keywords

Embryonic development; Vesico-ureteral Reflux; Receptors; Fibroblast Growth Factor

Introduction

Vesico-ureteral reflux (VUR), backflow of urine from the bladder into ureters, affects 1–2% of children.¹ Reflux nephropathy, kidney damage associated with chronic reflux is the 4th leading cause of chronic kidney disease (CKD) and the most common cause of severe hypertension in children.^{1,2} Despite aggressive medical and surgical management of VUR, the incidence of CKD from reflux nephropathy has not changed over the last few decades.¹

The bladder and ureter, tissues involved in VUR, originate from distinct lineages. The ureteric bud, arising from Wolffian duct, forms the ureter, while bladder epithelium comes from endoderm. As development proceeds, the ureter inserts in the bladder creating the ureterovesical junction (UVJ).³ Normally, this connection forms a physiologic valve preventing urine backflow into the ureter.

Sometimes anatomical abnormalities near or at the UVJ are associated with VUR. Shortened intravesicular tunnel lengths (distance of the ureter traversing through the bladder), abnormal ureteral musculature, decreased microvasculature and/or innervation, and extracellular matrix protein abnormalities have been seen in experimental models and some humans with VUR.^{1,4–8} Whether these abnormalities cause or are secondary to VUR is unknown.

One compelling hypothesis about the cause of VUR relates to where the ureter inserts into the bladder. As proposed by Drs. Mackie and Stephens in the 1970s, the ureteric bud induction site from Wolffian duct dictates where the ureter inserts into the bladder. They postulated that if the ureteric bud evaginates too low/posterior from the Wolffian duct, the ureter would insert too high and lateral in the bladder.^{9,10} Conversely, if the ureteric bud arose too high/anterior, then the ureter would insert low, near the bladder neck (or might insert ectopically in the ductus deferens). While a low ureteral insertion could result in obstruction (particularly if it is ectopic), both low as well as a high insertion increases the risk of VUR. Recent studies in experimental models have confirmed that alterations in ureteric bud induction sites lead to abnormal ureter insertion in the bladder (although incidence of VUR was not determined).³

Our laboratory has generated a mouse line with conditional deletion of fibroblast growth factor receptor 2 (Fgfr2) in kidney mesenchyme that has aberrant ureteric bud induction.^{11,12} These mice have anterior ectopic ureteric buds 67% of the time during initial ureteric induction [Embryonic days (E) 10.5 and E11.5]. We hypothesize that many single ureters in post-natal mutant mice insert improperly in the bladder, increasing risk of VUR as proposed by Drs. Mackie and Stephens.

Methods

Fgfr2^{Mes-/-} conditional knockout mice

The Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children's Hospital approved mouse handling in this study. The conditional knockout mice have been described previously.^{11, 12} Briefly, we crossed transgenic mice with the Pax3 promoter driving cre recombinase expression in metanephric mesenchyme (*Pax3cre*^{Tg}) (gift from Dr. Jon Epstein, University of Pennsylvania) with mice with lox-p sites flanking a

critical portion of *Fgfr2* (*Fgfr2^{Lox}*) (gift from Dr. David Ornitz, Washington University). Mice hemizygous for *Pax3cre^{Tg}* and homozygous for *Fgfr2^{Lox}* (*Pax3cre^{Tg}+* *Fgfr2^{Lox/Lox}*) have deletion of *Fgfr2* in metanephric mesenchyme (*Fgfr2^{Mes-/-}*). Mice with other alleles are phenotypically normal and used as controls.

Genotyping

As described, we extracted DNA from tail clippings or embryonic tissues for PCR genotyping.^{11,12} Genotyping primers are included in Table 1.

Common Nephric Duct (CND) Lengths and Apoptosis assays

E11.5 mutant and littermate control embryos were assayed for whole mount *in situ* hybridization as described utilizing digoxigenin-UTP-labeled RNA probes against *Ret*.¹² *Ret* labeled embryos were positioned in identical orientation and images were captured via an AxioCam digital camera (Zeiss, Thornwood, NY) mounted on a DM LB microscope (Leica, Wetzlar, Germany). CND length (caudal end of the nephric duct at the cloaca to the cranial edge of the ureteric bud stalk origin) was measured using ImageJ software (Wayne Rasband, NIH, USA).

To examine apoptosis in CNDs, we methanol fixed E11.5 mutant (n=10) and control littermate (n=10) embryos, followed by whole mount immunofluorescence with antibodies against pancytokeratins (Sigma, cat # C9687; to detect CNDs) and activated caspase-3 (Invitrogen, cat # G748A; to detect apoptosis), as described.¹¹ We detected pancytokeratins with anti-mouse Alex Fluor 488 (Jackson ImmunoResearch, cat# 115-485-003) and caspase with anti Rabbit Cy3 (Jackson ImmunoResearch, cat #711-165-152).

Cystograms

Cystograms were performed in postnatal day 1 (P1) mice to assess for VUR, with the operator blinded to genotypes. After a ventral midline incision, urinary tracts were isolated. The bladder was punctured with a 30-gauge needle, connected via tubing to a 60 mL syringe filled with 1% methylene blue in PBS. The syringe was raised from 30 cm above the microscope stage to 150 cm at 6 cm/sec. The height when reflux was visualized and dye passed through the urethra was noted. VUR was graded according to international classifications.¹³ The experiment was stopped after the syringe was held at 150 cm for 10 seconds and no reflux occurred. Among 21 *Fgfr2^{Mes-/-}* and 51 control mice used for cystograms, 2 mutants and 1 control were not assessed due to technical difficulties (bladder punctured completely through). Kidney long axes were measured in the first consecutive 15 *Fgfr2^{Mes-/-}* and 45 control mice with successful cystograms using ImageJ. Gravity filling mouse bladders to assess VUR has been validated.¹⁴

3-D Reconstructions

Processing tissue—After P1 mice underwent cystograms, 4 non-refluxing controls and 3 refluxing mutants were fixed in 4% paraformaldehyde overnight at 4°C and paraffin embedded. Three E15.5 *Fgfr2^{Mes-/-}* mutants and three littermate controls were similarly fixed and embedded. Postnatal mice and embryos were serially sectioned at 10 µm through portions of the bladder and ureters and stained with hematoxylin and eosin.

Rendering images—Serial sections were then traced using Stereo Investigator software (Microbrightfield, Colchester, VT). Bladder and ureter tissues were identified and traced including muscle (red), bladder luminal surface (yellow), ureteral urothelium (blue) and bladder lamina propria (white). From these serial images the 3-D solids module was utilized, and 3-dimensional images were rendered.

Measuring triangles including ureteral-bladder junctions and bladder neck—

After rendering 3D images, Neurolucida software (Microbrightfield) was used to generate and measure lengths and angles of triangles connecting posterior ureter insertion sites with the midpoint of the posterior surface of the proximal bladder neck (external triangles). Similarly triangles connecting ureteral orifices in the bladder lumen to the midpoint of the luminal surface of the proximal bladder neck (internal triangles) were generated.

Measuring intravesicular tunnel lengths—The central point of external and internal ureteral insertion sites was identified. Using Neurolucida, the ureteral urothelial tract through the bladder was mapped and exported into Neurolucida explorer to render tunnel lengths.

Assessment of *Fgfr2* in E15.5 mice—Whole-mount *in situ* hybridization was performed on kidneys, ureters, and bladders dissected out from E15.5 *Fgfr2*^{Mes^{-/-}} (n=3) and control (n=3) embryos as described.¹² Digoxigenin-UTP-labeled antisense and sense *Fgfr2* RNA probes were generated. For real time PCR, urinary tract tissues were harvested and mRNA extracted (Qiagen, Valencia, CA) from 4 mutant and 4 control E15.5 embryos. Probes for *Fgfr2* and *Gapdh* (endogenous control) were used (Invitrogen, Carlsbad, CA) for quantitative real time PCR using standard protocols on Applied Biosystems ABI 7900 HT (Foster City, CA).

Statistics—Mean triangle lengths and angles and kidney long axes in mutants and controls were compared via Student t-tests with a p-value 0.05 considered significant. Presence of VUR and mutant long kidney axes < 1 standard deviation below control means were compared by Fisher exact test with a p-value 0.05 considered significant.

Results

Embryonic *Fgfr2*^{Mes^{-/-}} mice have abnormally positioned ureteric buds

While our previous work demonstrated that some *Fgfr2*^{Mes^{-/-}} mice initially had 2 ureteric buds¹¹, it was unclear if single ureteric buds in mutants were positioned abnormally. We measured the common nephric duct, the nephric duct distal to ureteric bud stalks, by performing whole mount *in situ* hybridization on E11.5 mutants and controls for *Ret* (Figure 1). Controls had a mean ($\pm$ standard deviation) CND length of 19.9 $\pm$ 2.38 μ m (n=10 CNDs). The mean CND length of all mutant *Fgfr2*^{Mes^{-/-}} mice including those with multiple ureteric buds (measured from the most anterior bud), was 27.0 $\pm$ 5.31 μ m (n=10) (p=0.0012 compared with controls). Among *Fgfr2*^{Mes^{-/-}} mice with single ureteric buds, mean CND length was 24.4 $\pm$ 3.2 μ m (n=7) (p=0.004 versus controls).

To ensure that differences in CND length were not from apoptosis, we performed whole mount immunofluorescence in E11.5 embryos for activated caspase-3 and pancytokeratins. As shown (Figure 2), mutants and controls stain similarly for caspase 3 along CNDs, indicating similar apoptotic rates. Taken with *Ret* expression, as a group *Fgfr2*^{Mes^{-/-}} mice have longer CNDs and thus cranially shifted ureteric buds (even when only one bud is present).

E15.5 *Fgfr2*^{Mes^{-/-}} embryos have low/caudal ureteral insertions into the bladder

To determine whether high/cranial ureteric bud position results in ureteral insertion abnormalities, we performed 3D reconstructive imaging on E15.5 *Fgfr2*^{Mes^{-/-}} and controls through the UVJ (supplemental movies 1 and 2). As shown (Figure 3), still images of the external/posterior bladder surface show that mutant ureters appear to insert low/caudal compared with controls. As shown (Table 2), mutant external ureteral angles are more acute

than controls (mutant: $20.2 \pm 6.7^\circ$; control: $27.1 \pm 3.3^\circ$, $p = 0.048$). Two mutants had ureteral angles on right and left that were each > 2 standard deviations smaller than control mean angles. Smaller angles are consistent with mutant ureters inserting more caudal along the bladder.

In interrogating position of internal ureteral orifices in the bladder (Figure 3, Table 2), mutants had smaller mean lengths from the ureteral orifice to the bladder neck than controls (mutants: $162 \pm 16.2 \mu\text{m}$; controls: $247 \pm 17.3 \mu\text{m}$, $p = 0.0000048$). Mean mutant interureteral length also trended toward being smaller than control length, but did not reach statistical significance (mutants: $227 \pm 27.5 \mu\text{m}$; controls: $296 \pm 37.4 \mu\text{m}$, $p = 0.063$). Thus, it appears that *Fgfr2*^{Mes-/-} ureters empty in a more caudal position (and closer together) into the bladder versus controls.

***Fgfr2*^{Mes-/-} newborn mice have high rates of VUR**

To test whether abnormal ureteric bud position predisposed *Fgfr2*^{Mes-/-} mice to VUR, we performed cystograms on newborn P1 mice (Figure 4). Among 49 controls, only 2 (4.0%) displayed unilateral VUR. In contrast, 9 out of 19 (47.4%) *Fgfr2*^{Mes-/-} mutants had unilateral or bilateral VUR ($p=0.00006$ versus controls). There were 10 total mutant kidneys that refluxed (1 bilateral, 3 unilateral left-sided, and 5 unilateral right-sided). Thus, shortly after birth, a significant number of *Fgfr2*^{Mes-/-} mice had VUR compared with controls.

In examining gross morphology of *Fgfr2*^{Mes-/-} newborn kidneys after cystograms, none had obvious ureteral obstruction, duplex collecting systems, or renal agenesis. Mean newborn mutant kidney long axis (including those with and without VUR) was 10.7% smaller than mean control long axis (mutant: $0.29 \pm 0.044 \text{ cm}$; control: $0.33 \pm 0.044 \text{ cm}$, $p = 0.00008$). *Fgfr2*^{Mes-/-} kidneys with VUR ($n = 7$) and without VUR ($n = 23$), however, had no statistical differences in mean long axis (VUR: $0.29 \pm 0.27 \text{ cm}$; no VUR: $0.29 \pm 0.31 \text{ cm}$, $p = 0.60$). Furthermore, while 4 out of 10 (40%) kidneys with reflux were one standard deviation or smaller than mean control length compared with 7 out of 30 (23.3%) mutant kidneys without VUR, the differences did not reach statistical significance ($p = 0.42$). Thus, while newborn *Fgfr2*^{Mes-/-} kidneys are sometimes smaller than controls, there do not appear to be differences between mutants with or without reflux.

***Fgfr2*^{Mes-/-} mutants with VUR have abnormal ureteral insertions in the bladder**

To examine ureteral insertion into the bladder, we performed 3-D reconstruction of the UVJ in a subset of P1 *Fgfr2*^{Mes-/-} ($n=3$) with VUR and controls without VUR ($n=3$). As shown (Figure 5, supplemental movies 3,4), control mice without VUR have ureteral insertions that are distant from the bladder neck and that insert parallel to the transverse plane through the bladder dome (Figure 5A, supplemental movie 3). In contrast, a mutant mouse with right sided, unilateral VUR, has a right ureter that inserts low/caudal on the external bladder wall, near the bladder neck (Figure 5B, supplemental movie 4). Furthermore the interureteral plane in the *Fgfr2*^{Mes-/-} mouse deviates significantly from the transverse plane of the bladder dome.

To quantify differences between mutants with VUR and controls, we generated triangles formed by the external ureteral insertion points and bladder neck (Figure 5 C, D) and internal triangles from ureter orifices and bladder neck and intravesicular tunnel lengths (Table 3). *Fgfr2*^{Mes-/-} mice had aberrant increases in external angles formed at the ureteral insertion on the side that had reflux (in all cases, mutants had right sided VUR). Mutants had a right sided mean angle of $56.0 \pm 6.1^\circ$ versus $40.8 \pm 3.2^\circ$ in the controls ($p=0.0075$). In addition, the mean of the right (refluxing) angles were statistically different than the means of the left (non-refluxing) angles ($56.0 \pm 6.1^\circ$ versus $32.1 \pm 7.5^\circ$; $p=0.013$) in mutants.

In contrast control exterior right and left sided angles were not statistically different ($p=0.354$). Angles formed at internal ureteral orifices were not different between mutants and controls or between mutant right and left sides. There were also no differences between intravesicular tunnel lengths of refluxing mutant ureters and controls or non-refluxing mutant ureters. Thus *Fgfr2*^{Mes^{-/-}} mutants appear to have refluxing ureters that insert abnormally into the bladder.

***Fgfr2*^{Mes^{-/-}} mutants have decreased *Fgfr2* expression in kidneys**

We then determined whether differences in *Fgfr2* expression might contribute to the high incidence of VUR in *Fgfr2*^{Mes^{-/-}} mice versus controls. As shown (Figure 6), *Fgfr2* is expressed in ureteric tips of both mutant and control kidneys, but relatively absent in mesenchymal tissues between mutant ureteric tips compared with controls. Relative *Fgfr2* signal in ureters and bladders was comparable in mutants and controls by *in situ* hybridization (not shown). Quantitative real time PCR confirmed less *Fgfr2* mRNA in mutant kidneys (88.5% compared with controls, $p = 0.0017$). In contrast there were no statistical differences between mutant ureters (100.4% compared with controls, $p = 0.93$) and although bladders trended toward a difference (90.5% compared with controls, $p = 0.066$). Thus, there is less *Fgfr2* in mutant kidneys, but similar levels in ureters and bladders compared with controls.

Discussion

Previous reports have demonstrated varied roles of fibroblast growth factors and their receptors in renal development.^{11,12,15-21} Recently we demonstrated that deletion of *Fgfr2* from metanephric mesenchyme often results in 2 ureteric buds on one side.¹¹ Presently, we find that *Fgfr2*^{Mes^{-/-}} embryonic mice have cranially malpositioned ureteric buds even when only one ureteric bud is induced, leading to low/caudal ureteral insertions in bladders later in embryogenesis. *Fgfr2*^{Mes^{-/-}} newborn mice have significantly high rates of VUR compared with littermate controls. While newborn *Fgfr2*^{Mes^{-/-}} kidneys are smaller than controls, there are not size differences between mutants with versus without VUR. 3-D reconstructive imaging shows that mutant mice with unilateral reflux have ureters inserting into the bladder at abnormally low positions on the side of reflux. Aberrant increases in angles (formed by triangles connecting the ureters and bladder neck) on the side of VUR, confirms that insertion sites of the refluxing ureters are malpositioned. It is possible that if higher numbers of animals were assessed, other anatomic defects (ureteral tunnel length for example) would correlate with VUR in mutants. We did not see any statistical differences in *Fgfr2* expression between *Fgfr2*^{Mes^{-/-}} and control mice that might contribute to high rates of VUR in mutants. The combination of cranially shifted ureteric buds, low insertion sites of ureters, and high rates of VUR in *Fgfr2*^{Mes^{-/-}} mice fit nicely with Mackie and Stephens models (see Figure 7). The reason we did not see more developmental anomalies in postnatal *Fgfr2*^{Mes^{-/-}} mice was that there was likely bilateral disease (aplasia, severe dysplasia, obstruction, etc.) incompatible with postnatal life.

The *Fgfr2*^{Mes^{-/-}} mouse model of VUR complements 2 other mouse models, *Pax2* *1Neu*^{+/-} and *HoxB7/Ret*^{+/-} mutants. The latter mouse models demonstrate caudally-shifted ureteric bud induction sites off the Wolffian duct and high rates of VUR.^{1,14} As in **cranially** shifted ureteric buds (as seen in *Fgfr2*^{Mes^{-/-}} mice), the Mackie and Stephens model predicts that caudally-positioned ureteric induction sites cause abnormal ureteral insertion into the bladder and high rates of VUR. It would be interesting to determine if *Pax2* *1Neu*^{+/-} and *HoxB7/Ret*^{+/-} mice had lateral and high ureteral insertion as predicted. Another difference between *Fgfr2*^{Mes^{-/-}} mice and the other 2 models is that the latter had shortened intravesicular tunnel lengths (on the side of VUR) while *Fgfr2* mutants did not. Perhaps the

site of aberrant ureteral insertion (low versus high and lateral on the bladder) affects ureteral tunnel length. The different models may represent a spectrum of different reasons why VUR may occur, as predicted by Mackie and Stephens.

Gene mutations that lead to VUR in mice have not been shown found in humans with VUR and genes associated with VUR in one species have not been tested in the other. Although deletion of Uroplakin III in mice results in VUR, there were no significant uroplakin gene polymorphisms in humans with VUR.²² Another mouse mutant with VUR is the angiotensin type II receptor knockout (*Agtr2*^{-/-}) model that also presents with renal hypoplasia.²³ While the authors found a high incidence of an *AGTR2* polymorphism in humans with multicystic dysplastic kidneys and/or ureteropelvic junction obstruction, they did not test for association of the mutations with human VUR.²³ Also, a family with a high incidence of duplex kidneys and VUR was found to have a *ROBO2* mutation in affected members.²⁴ While mutations in *Robo2* and its ligand *Slit2* in mice lead to multiple ureteric buds, the mice have not been tested for VUR.²⁵ Thus, there have been few correlates between genetics of non-syndromic VUR in mice versus humans.

Some human *FGFR2* mutations are linked with urinary tract abnormalities, including VUR. Activating *FGFR2* mutations resulting in Pfeiffer syndrome lead to high grade VUR.²⁶ Other *FGFR2* activating mutations, associated with Apert's syndrome, Antley-Bixler syndrome, and Beare-Stevenson syndrome, sometimes lead to urogenital anomalies such as hydroureter and solitary kidney.^{27,28} While it is unclear if these patients have risks of VUR, congenital anomalies of the kidney and urinary tract do predispose to high VUR rates.^{29, 30} Taken together, *FGFR2* mutations could increase risks of human VUR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Murawski IJ, Gupta IR. Gene discovery and vesicoureteric reflux. *Pediatr Nephrol.* 2008; 23:1021. [PubMed: 18253765]
2. North American pediatric renal transplant cooperative study (NAPRTCS). 2006 Annual report. Rockville; 2006. p. 1-270.
3. Batourina E, Tsai S, Lambert S, et al. Apoptosis induced by vitamin A signaling is crucial for connecting the ureters to the bladder. *Nat Genet.* 2005; 37:1082. [PubMed: 16186816]
4. King LR, Kazmi SO, Belman AB. Natural history of vesicoureteral reflux. Outcome of a trial of nonoperative therapy. *Urol Clin North Am.* 1974; 1:441. [PubMed: 4610948]
5. Oswald J, Brenner E, Schwentner C, et al. The intravesical ureter in children with vesicoureteral reflux: a morphological and immunohistochemical characterization. *J Urol.* 2003; 170:2423. [PubMed: 14634444]
6. Oswald J, Schwentner C, Brenner E, et al. Extracellular matrix degradation and reduced nerve supply in refluxing ureteral endings. *J Urol.* 2004; 172:1099. [PubMed: 15311048]
7. Schwentner C, Oswald J, Lunacek A, et al. Loss of interstitial cells of Cajal and gap junction protein connexin 43 at the vesicoureteral junction in children with vesicoureteral reflux. *J Urol.* 2005; 174:1981. [PubMed: 16217373]

8. Schwentner C, Oswald J, Lunacek A, et al. Structural changes of the intravesical ureter in children with vesicoureteral reflux-does ischemia have a role? *J Urol.* 2006; 176:2212. [PubMed: 17070295]
9. Mackie GG, Awang H, Stephens FD. The ureteric orifice: the embryologic key to radiologic status of duplex kidneys. *J Pediatr Surg.* 1975; 10:473. [PubMed: 1151582]
10. Mackie GG, Stephens FD. Duplex kidneys: a correlation of renal dysplasia with position of the ureteral orifice. *J Urol.* 1975; 114:274. [PubMed: 1171997]
11. Hains D, Sims-Lucas S, Kish K, et al. Role of fibroblast growth factor receptor 2 in kidney mesenchyme. *Pediatr Res.* 2008; 64:592. [PubMed: 18670373]
12. Poladia DP, Kish K, Kutay B, et al. Role of fibroblast growth factor receptors 1 and 2 in the metanephric mesenchyme. *Dev Biol.* 2006; 291:325. [PubMed: 16442091]
13. Lebowitz RL, Olbing H, Parkkulainen KV, et al. International system of radiographic grading of vesicoureteric reflux. International Reflux Study in Children. *Pediatr Radiol.* 1985; 15:105. [PubMed: 3975102]
14. Yu OH, Murawski IJ, Myburgh DB, et al. Overexpression of RET leads to vesicoureteric reflux in mice. *Am J Physiol Renal Physiol.* 2004
15. Barasch J, Qiao J, McWilliams G, et al. Ureteric bud cells secrete multiple factors, including bFGF, which rescue renal progenitors from apoptosis. *Am J Physiol.* 1997; 273:F757. [PubMed: 9374839]
16. Celli G, LaRochelle WJ, Mackem S, et al. Soluble dominant-negative receptor uncovers essential roles for fibroblast growth factors in multi-organ induction and patterning. *Embo J.* 1998; 17:1642. [PubMed: 9501086]
17. Grieshammer U, Cebrian C, Ilagan R, et al. FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. *Development.* 2005; 132:3847. [PubMed: 16049112]
18. Ohuchi H, Hori Y, Yamasaki M, et al. FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. *Biochem Biophys Res Commun.* 2000; 277:643. [PubMed: 11062007]
19. Perantoni AO, Dove LF, Karavanova I. Basic fibroblast growth factor can mediate the early inductive events in renal development. *Proc Natl Acad Sci U S A.* 1995; 92:4696. [PubMed: 7753867]
20. Qiao J, Uzzo R, Obara-Ishihara T, et al. FGF-7 modulates ureteric bud growth and nephron number in the developing kidney. *Development.* 1999; 126:547. [PubMed: 9876183]
21. Revest JM, Spencer-Dene B, Kerr K, et al. Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4. *Dev Biol.* 2001; 231:47. [PubMed: 11180951]
22. Jiang S, Gitlin J, Deng FM, et al. Lack of major involvement of human uroplakin genes in vesicoureteral reflux: implications for disease heterogeneity. *Kidney Int.* 2004; 66:10. [PubMed: 15200408]
23. Nishimura H, Yerkes E, Hohenfellner K, et al. Role of the angiotensin type 2 receptor gene in congenital anomalies of the kidney and urinary tract, CAKUT, of mice and men. *Mol Cell.* 1999; 3:1. [PubMed: 10024874]
24. Lu W, van Eerde AM, Fan X, et al. Disruption of ROBO2 Is Associated with Urinary Tract Anomalies and Confers Risk of Vesicoureteral Reflux. *Am J Hum Genet.* 2007; 80:616. [PubMed: 17357069]
25. Grieshammer U, Le M, Plump AS, et al. SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site. *Dev Cell.* 2004; 6:709. [PubMed: 15130495]
26. Seyedzadeh A, Kompani F, Esmailie E, et al. High-grade vesicoureteral reflux in Pfeiffer syndrome. *Urol J.* 2008; 5:200. [PubMed: 18825630]
27. Passos-Bueno MR, Wilcox WR, Jabs EW, et al. Clinical spectrum of fibroblast growth factor receptor mutations. *Hum Mutat.* 1999; 14:115. [PubMed: 10425034]
28. Cohen MM Jr. Kreiborg S. Visceral anomalies in the Apert syndrome. *Am J Med Genet.* 1993; 45:758. [PubMed: 8456856]
29. Atiyeh B, Husmann D, Baum M. Contralateral renal abnormalities in multicystic-dysplastic kidney disease [see comments]. *J Pediatr.* 1992; 121:65. [PubMed: 1625095]

30. Guarino N, Casamassima MG, Tadini B, et al. Natural history of vesicoureteral reflux associated with kidney anomalies. *Urology*. 2005; 65:1208. [PubMed: 15913724]

Figure 1. Representative images demonstrating common nephric duct lengths in E11.5 embryos Whole mount *in situ* hybridization for *Ret* demonstrate that common nephric duct lengths (brackets) of mutants (B) are longer compared with controls (A). nd = nephric duct; ub = ureteric bud (**Mag 40x**)

Figure 2. Representative images demonstrating apoptosis along common nephric ducts in E11.5 embryos

In controls (A–C), whole mount immunofluorescence for pancytokeratins (A, green) and activated caspase-3 (B, red, arrowhead) shows apoptosis (arrowhead) along the ventral surface of the common nephric duct (between arrows) on merged images (C). The most intense signal is at the caudal end of the common nephric duct. In *Fgfr2*^{Mes^{-/-}} mutants (D–F), pancytokeratins staining (D) and caspase-3 signal (E) shows a similar pattern of apoptosis (arrowhead) along the common nephric duct (between arrows) on merged images (F). Scale bars = 100 μ m.

Figure 3. Representative images of 3-D reconstructions through the UVJ of E15.5 mice (posterior views)

Red tissues denote bladder serosa and muscularis. Blue tissues represent ureteral urothelium. Yellow tissues denote bladder urothelial luminal surface. **A–C** = external bladder surface. Compared with control mouse (**A**), *Fgfr2^{Mes-/-}* mouse (**B**) has ureters inserting low/caudal, closer to the bladder neck. Overlay of external triangles (**C**) confirms that mutant ureters (yellow) insert closer to the bladder neck (arrowheads) and have more acute external ureteral angles than controls (blue, asterisks). **D–F** = bladder urothelial luminal surface. Compared with control mouse (**D**), *Fgfr2^{Mes-/-}* mouse (**E**) has ureters inserting low/caudal, closer to the bladder neck and likely more near each other. Overlay of internal triangles (**F**) confirms the low malposition of the mutant ureteral insertion (yellow, arrowheads) into the bladder lumen than controls (blue, asterisks). BN = bladder neck. Scale bars = 100 μm.

Figure 4. Representative images of cystograms in P1 mice

Arrowheads outline course of ureters associated with the Right (R) and left (L) kidneys. **A.** Control mouse has no dye in either ureter. **B.** *Fgf2^{Mes}/-* mouse has right sided VUR with dye present in both the ureter and the renal pelvis (arrowhead). The left ureter and renal pelvis are devoid of dye (the dye present on the left is overlying the urinary tract). (**Mag 12x**)

Figure 5. Representative images of 3-D reconstructions through the UVJ of newborn mice following cystograms (posterior views)

Red tissues denote serosa and muscularis. Blue tissue denotes ureter urothelium (intravesicular portion). Yellow tissue is bladder urothelium. Compared with control mouse (A), *Fgfr2^{Mes-/-}* mouse (B) demonstrates grossly asymmetrically positioned ureter on the right (side of reflux). With the bladder muscularis removed, control mouse shows symmetric external ureteral insertional angles on the right (asterisk) and left. In contrast, *Fgfr2^{Mes-/-}* mouse (D) has an increase in the insertional angle on the right (asterisk) compared with the left side and with both control angles. R = right ureter, L = left ureter. BN = bladder neck. Scale bars = 100 μ m.

Figure 6. Representative images of whole mount *in situ* hybridization for *Fgfr2* in E15.5 mice In both controls (A) and *Fgfr2*^{Mes-/-} mutants (B), there is signal in the tips of the ureteric tree (white arrowheads); however in comparison with controls, there appears to be a relative lack of signal in the background mesenchymal tissues between the ureteric tips in mutants, particularly at the periphery of the kidneys (black arrowheads). This gives the mutants much lighter overall staining. Scale bars = 300 μ m.

Figure 7. Diagram illustrating the Mackie and Stephens Hypothesis

The letters A–H along the lower right demonstrate how ureteric bud induction position off the Wolffian duct correlates with ureteral insertion sites on the left. The box around ureteric induction positions F and G denotes the cranially displaced ureteric buds in the *Fgfr2*^{Mes^{-/-}} embryo. The asterisk (*) demonstrates where the cranially displaced *Fgfr2*^{Mes^{-/-}} ureteric bud is predicted to insert as a ureter.

This figure is modified from a previously published article with permission: “Duplex Kidneys: A Correlation of Renal Dysplasia with Position of the Ureteral Orifice. Mackie GG, Stephens FD. *Journal of Urology*; 144: 274–280, Copyright Elsevier (1975).”