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Role of fibroblast growth factor receptor signaling in kidney development

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Abstract

Fibroblast growth factor receptors (Fgfrs) are expressed throughout the developing kidney. Several early studies have shown that exogenous fibroblast growth factors (Fgfs) affect growth and maturation of the metanephric mesenchyme (MM) and ureteric bud (UB). Transgenic mice that overexpress a dominant negative receptor isoform develop renal aplasia/severe dysplasia, confirming the importance of Fgfrs in renal development. Furthermore, global deletion of *Fgf7*, *Fgf10*, and *Fgfr2IIIb* (isoform that binds Fgf7 and Fgf10) in mice leads to small kidneys with fewer collecting ducts and nephrons. Deletion of *Fgfrl1*, a receptor lacking intracellular signaling domains, causes severe renal dysgenesis. Conditional targeting of *Fgf8* from the MM interrupts nephron formation. Deletion of *Fgfr2* from the UB results in severe ureteric branching and stromal mesenchymal defects, although loss of *Frs2α* (major signaling adapter for Fgfrs) in the UB causes only mild renal hypoplasia. Deletion of both *Fgfr1* and *Fgfr2* in the MM results in renal aplasia with defects in MM formation and initial UB elongation and branching. Loss of *Fgfr2* in the MM leads to many renal and urinary tract anomalies as well as vesicoureteral reflux. Thus, Fgfr signaling is critical for patterning of virtually all renal lineages at early and later stages of development.

Keywords

fibroblast growth factors; fibroblast growth factor receptors; metanephric mesenchyme; ureteric bud; renal organogenesis; conditional knockout

Introduction

Congenital kidney diseases are the leading causes of chronic kidney disease in children [1]. Although the underlying genetic defects leading to structural kidney disease are largely unknown, there are syndromes in which mutations in fibroblast growth factor receptors (FGFRs) lead to renal anomalies. For example activating mutations of *FGFR1* and/or *2* cause syndromes such as Apert's syndrome, Antley-Bixler syndrome, Pfeiffer syndrome, and Beare-Stevenson syndrome, which are sometimes associated with urogenital anomalies

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such as hydroureter, solitary kidney, and VUR [2–5]. Loss of function mutations in *FGFR1* have also been associated with some variants of Kallman syndrome that can be associated with unilateral renal aplasia. Rarely, activating mutations in *FGFR3* leading to thanatophoric dysplasia are associated with renal hypoplasia or cystic dysplasia [6, 7]. Given recent technological advances in genome and exome sequencing, it is possible that many of the sporadic cases of congenital renal anomalies will be associated with polymorphisms or copy number variations in FGFRs. The remainder of this review will focus on tissue and animal studies that are beginning to clarify the role of Fgfr signaling in kidney development.

Background on kidney development and fibroblast growth factor receptors

The metanephric kidney develops from tissues primarily arising from the intermediate mesoderm, the Wolffian (nephric) duct and the nephrogenic cord [8]. Between embryonic days (E) 10.5–11.0 in the mouse, the nephrogenic cord gives rise to the metanephric mesenchyme (MM), that then induces the formation of the ureteric bud (UB) from the Wolffian duct in the region of the hindlimb [8]. Stromal mesenchyme lying between the Wolffian duct and the MM restricts the UB to its proper position and prevents ectopic budding [9]. As the ureteric bud elongates, the metanephric mesenchyme divides into a nephrogenic lineage lying adjacent to the bud, and a surrounding renal cortical stromal lineage (that may also have elements of paraxial mesoderm) [8, 10]. The kidney continues to develop due to reciprocal inductive interactions, with the ureteric bud receiving signals to branch dichotomously, ultimately to form the collecting ducts and ureters [8]. At each terminal tip, the ureteric bud induces local areas of nephrogenic mesenchyme to differentiate into nephron epithelia, progressing from renal vesicles, to comma-shaped bodies, to S-shaped bodies, and finally to mature nephrons [8].

Fibroblast growth factor receptors (Fgfrs) are receptor tyrosine kinases with four signaling family members that are activated by binding to one of 22 known Fgf ligands in mammals [11]. Fgfr proteins consist of up to three external immunoglobulin (Ig)-like domains, a transmembrane domain, and an intracellular kinase domain [11]. Fgfrs1-3 exist as either a IIIb or IIIc isoform, through alternate splicing of the C-terminal portion of the third Ig-like domain, whereas Fgfr4 only has a IIIc isoform [11]. These splice variants have different ligand binding specificities; for instance, Fgf7 and Fgf10 both bind to Fgfr2IIIb but not to Fgfr2IIIc [11]. In addition, IIIb isoforms are usually expressed in epithelium, while IIIc isoforms are present in mesenchyme [11]. All of the receptor isoforms utilize molecules, such as fibroblast growth factor receptor substrate 2α (Frs2α), and phospholipase Cγ (PLCγ), to transmit intra-cellular signaling [11]. Frs2α constitutively binds Fgfrs in the juxtamembrane region and upon receptor activation becomes phosphorylated, then activating Erk, Akt, and protein kinase C (PKC) λ and ξ. PLCγ is recruited to a phosphorylated tyrosine on activated receptors, leading to activation of PKC (and possibly Src and Grb2). Given that all of the receptor isoforms use the same intracellular signaling molecules, much of the specificity of the pathway is likely dictated by the ligands. In addition to the four signaling Fgfrs, fibroblast growth factor receptor-like 1 (Fgfrl1, formerly Fgfr5) is a molecule with an extracellular domain highly homologous to Fgfrs that can bind Fgf ligands, but it lacks a tyrosine kinase domain for intracellular signaling [12].

Although Fgfr3 and Fgfr4 have been detected in embryonic kidneys [13–15], Fgfr1, Fgfr2, and Fgfrl1 appear to be the most relevant receptors regarding renal development (see below). Fgfr1 is expressed most prominently in rodent mesenchymal tissues (early metanephric mesenchyme, condensing mesenchyme, and developing nephrons), but is present at lower levels in the ureteric lineage and in renal cortical stroma [16–20]. Fgfr2 is strongly expressed in the Wolffian duct and the ureteric bud tree (tips and trunks) and differentiating nephrons, but is present at lower levels in early MM and stromal mesenchyme adjacent to the Wolffian duct and main ureteric trunk [13, 16–20]. Fgfrl1 is present in renal vesicles [12].

Roles of Fgfs in regulating renal development

The first studies suggesting relevance of Fgfr signaling in renal development focused on addition of Fgf ligands to isolated embryonic kidney tissues or on overexpression of ligands *in vivo*. In rodent and Xenopus explants, exogenous Fgf2 (alone or in combination with other growth factors) was able to sustain mesenchymal tissue growth and in some cases to induce formation of mature nephrons [21–25]. In addition, Fgf1, Fgf2, Fgf7, and Fgf10 stimulated growth and differentially affected branching morphogenesis in isolated rat ureteric bud cultures and/or in intact rat embryonic kidney explants [26, 27]. Finally, overexpression of *FGF2* (basic fibroblast growth factor) or *FGF7* (keratinocyte growth factor) in developing rodent kidneys *in vivo* leads to cystic dilation of collecting ducts [28, 29]. Thus, addition or overexpression of fibroblast growth factors *in vitro* and *in vivo* affects growth and maturation of both mesenchymal and ureteric bud lineages in the developing kidney.

Mouse knockout studies have verified the relevance of Fgf signaling in the embryonic kidney (see Table). Targeted deletion of *Fgf7* (produced by cortical stromal cells) leads to viable mice with a reduction in ureteric branch number and fewer nephrons, likely due to decreased signaling through Fgfr2IIIb receptors on the ureteric epithelium [27]. Deletion *Fgf10*, another ligand expressed in metanephric mesenchyme with specificity for Fgfr2IIIb, resulted in perinatal lethality likely from severe dysgenesis/agenesis of lungs and limbs; similar to *Fgf7*−/− mice, *Fgf10*−/− mice had smaller kidneys and fewer collecting ducts [30]. Fgf8, expressed in early MM condensates, is an attractive candidate ligand mediating nephron development; however, *Fgf8*−/− mice are early embryonic lethal [31]. To circumvent the early lethality of *Fgf8* null mice two groups of investigators used a conditional knockout approach with $Pax3cre^{Tg/+}$ transgenic mice (that express cre recombinase in the MM) or $Tcre^{Tg/+}$ transgenic mice (that express cre in all mesodermal tissues) [32, 33]. The resulting mice that had no functional *Fgf8* in the metanephric mesenchyme ($Fgf8^{Mes-/-}$) survived until birth, but had small kidneys with an interruption in nephron formation after the epithelial vesicle stage. Thus, Fgfr signaling appears crucial for ureteric branching morphogenesis and nephron maturation.

Effects of genetic blockade of Fgfr signaling on the developing kidney

The first mouse with altered *Fgfr* expression leading to renal abnormalities was a transgenic line carrying a solubilized Fgfr2IIIb fragment, which acted as a dominant negative by

binding many different Fgfs (and thus preventing much of the signaling through endogenous Fgfrs) [34]. The mutant embryos developed severe renal dysgenesis or agenesis (as well as severe lung, exocrine and endocrine gland, cutaneous, and limb anomalies) [34]. Regarding specific receptors, mice with deletion of *Fgfr3* or *Fgfr4* alone or in combination are viable and have no obvious kidney abnormalities [35, 36] (and unpublished data), making these receptors unlikely candidates to mediate signaling in the developing kidney. Global targeting of *Fgfr2IIIb* (the receptor isoform for both Fgf7 and Fgf10) results in a renal phenotype similar to the *Fgf7* and *Fgf10* knockouts, including smaller kidneys with fewer nephrons than normal [37] (see Table). Deletion of *Fgfrl1* resulted in severe renal dysgenesis secondary to interruption of nephron differentiation and ureteric branching [12] (see Table). More recently, Fgfrl1 was shown to act in part as a decoy receptor, competing for ligands with signaling Fgfrs [38]; thus, a proper balance of Fgfr signaling may be crucial for normal metanephric development.

Roles of Fgfr1 and Fgfr2 (outside of Fgfr2IIIb) in the kidney have been difficult to ascertain with global knockout techniques due to early embryonic lethality of *Fgfr1* and *Fgfr2* null mice [39–42]. To circumvent the early lethality of *Fgfr1* and *Fgfr2* knockout mice, our laboratory has used conditional knockout strategies in different renal lineages (see Table). We first bred *Hoxb7creEGFP*$^{Tg/+}$ transgenic mice (expressing cre recombinase and a green fluorescent protein in the Wolffian duct and ureteric lineage) to floxed *Fgfr* mice to delete the receptors in the ureteric lineage (*Fgfr*$^{UB-/-}$) [18]. While all mice were viable, *Fgfr2*$^{UB-/-}$ mice had small kidneys with ureteric branching defects, including longer branches and fewer tips (total and corrected for surface area) (Figure 1). *Fgfr1*$^{UB-/-}$ mice had no defects and *Fgfr1/2*$^{UB-/-}$ were indistinguishable from *Fgfr2*$^{UB-/-}$. *Fgfr2*$^{UB-/-}$ mice also had thickened cortical stroma with no interdigitations and many fewer nephrons than controls, likely from fewer ureteric tips.

We recently characterized *Fgfr2*$^{UB-/-}$ kidneys by three dimensional (3D) reconstructive imaging that revealed more striking defects and allowed for better quantification of abnormalities than in explants [43] (Figure 1). In the ureteric lineage, mutants had more dramatic decreases in tip number versus controls (59% 3D vs. 42% in explants). 3D reconstructions also allowed quantification of a reduction in *Fgfr2*$^{UB-/-}$ ureteric tissue per kidney volume and increases in mutant median ureteric segment lengths versus than controls. In addition to revealing striking decreases in *Fgfr2*$^{UB-/-}$ nephron number (vs. controls) 3D reconstructions also revealed that mean *Fgfr2*$^{UB-/-}$ developing nephron sizes were larger than controls; mutant vesicles are the same size as controls but gradually increase in volume and surface area, reaching statistical differences as mature glomeruli. Since murine nephrons are not yet filtering urine at E13.5, the increase in glomerular size is not likely the result of “compensatory hypertrophy.” Thus *Fgfr2* in the ureteric lineage is critical for normal ureteric branching and as a secondary consequence, nephron development.

To determine the role of Frs2α, a major intracellular signaling adapter for Fgfrs, in the ureteric lineage, we generated conditional knockout mice using the Hoxb7cre line (*Frs2a*$^{UB-/-}$) [44]. Surprisingly, *Frs2a*$^{UB-/-}$ mice developed only mild renal hypoplasia characterized by decreased ureteric branching events, but with normal overall branching

architecture and normal stromal mesenchymal development. The alterations in ureteric morphogenesis were likely secondary to decreased *Ret* and *Wnt11* expression characterized by *in situ* hybridization and real time PCR. There were also minor reductions in nephron endowment secondary to the decrease in ureteric tip number. Thus $Frs2a^{UB-/-}$ mice had a much more mild renal phenotype than $Fgfr2^{UB-/-}$ mice, suggesting that Fgfr2 likely signals through other adapter molecules in the ureteric epithelium. In addition to Fgfrs, Frs2α transmits intracellular signaling for other receptor tyrosine kinases such as Ret, neurotrophin receptors, and anaplastic lymphoma kinase [45].

To determine whether Fgfr2 signals through Frs2α at all in the ureteric epithelium, we generated mice with point mutations in the Frs2α binding site on Fgfr2 (termed $Fgfr2^{LR}$ based on the amino acids that were mutated to alanine- such point mutations have been shown to abrogate Frs2α-Fgfr2 binding, while not affected signaling through other adapter proteins) [46]. $Fgfr2^{LR/LR}$ mice had phenotypically normal kidneys based on histology, explant analysis, and size measurements [44]. To avoid any potential rescue by intact Fgfr1 forming heterodimers with $Fgfr2^{LR}$, we are currently examining mice that are $Hoxb7cre^{Tg/+}Fgfr1^{Lox/Lox}Fgfr2^{LR/LR}$ compound mutant mice. If the compound mutant mice are phenotypically normal, it would suggest that Frs2α serves primarily as an adapter protein for other receptor tyrosine kinases in the ureteric epithelium, such as Ret.

To investigate the role(s) of Fgfrs in MM we used conditional knockout approaches with the $Pax3cre^{Tg/+}$ transgenic mouse line ($Fgfr^{Mes-/-}$) [19]. While deletion of any three out of four *Fgfr1* and *Fgfr2* alleles generally resulted in robust kidney formation, deletion of all four alleles ($Fgfr1/2^{Mes-/-}$) resulted in severe renal dysgenesis with no recognizable MM at E10.5 (Figure 2). MM markers, *Eya1* and *Six1*, were actually present in a very restricted domain, but downstream markers *Six2*, *Sall1*, and *Pax2* were absent in mutant kidney mesenchyme. In the ureteric lineage, E10.5 $Fgfr1/2^{Mes-/-}$ embryos had ureteric outgrowth (and occasionally multiple buds) secondary to glial cell line derived neurotrophic factor (*Gdnf)* expression in the MM; however, by E11.5 *Gdnf* was no longer present leading to no ureteric elongation or branching. Thus, Fgfr1 and Fgfr2 signaling (together) in the metanephric mesenchyme are critical for formation of the metanephric mesenchyme and early development of the ureteric bud. To determine whether the early MM patterning defects seen in $Fgfr1/2^{Mes-/-}$ are dependent on Frs2α mediated signaling, we are currently examining compound mutant mice with Pax3cre mediated deletion of Fgfr1 and with $Fgfr2^{LR}$ point mutations.

Since $Fgfr1/2^{Mes-/-}$ mice sometimes had multiple UBs per Wolffian duct, we explored whether mice with single Pax3cre-driven *Fgfr* deletions had ureteric induction defects [47]. While $Fgfr1^{Mes-/-}$ were normal, 67% of E10.5–11.5 $Fgfr2^{Mes-/-}$ mice had two UBs per nephric duct (that often later fused into one UB). The UB induction defects resulted in structural defects in ~25% of older embryos including duplex kidneys/collecting systems (partial and complete), aplasia, and obstructive hydroureter [47]. Key molecules known to regulate the UB induction site, including *Gdnf*, *Robo2*, Bone morphogenetic protein 4, and Sprouty1 were expressed normally by *in situ* hybridization; thus, mechanisms causing UB induction defects were unclear. Knowing that stromal mesenchyme around the nephric duct constrains the UB to its proper site, we examined expression of *Fgfr2* in this region in

mutants. Whole mount and section *in situ* hybridization revealed that *Fgfr2* was deleted in these cells, possibly meaning that Fgfr2 acts in stromal tissue to constrain the UB induction site. To test this further, we have begun studies to conditionally delete *Fgfr2* using a *Tbx18cre*^{Tg} mouse line (gift from Fen Chen) [48] that drives cre expression in the stromal tissues but not the kidney mesenchyme.

Given that abnormally positioned ureteric buds are postulated to lead to vesicoureteral reflux (VUR) post-natally, we assessed whether Pax3cre-driven *Fgfr2*$^{Mes-/-}$ mutants (including those with single ureteric buds per nephric duct) had displaced ureteric buds and were predisposed to VUR. Compared with controls, *Fgfr2*$^{Mes-/-}$ embryos with single UBs per nephric duct had increased common nephric duct lengths indicating cranial displacement of the UBs along the Wolffian duct. Young *Fgfr2* mutants also had high rates of VUR compared with controls (40% vs. 3.8%, p = 0.0003) (Figure 3). 3D reconstructive imaging of mutants with unilateral reflux revealed that the refluxing ureters inserted much closer to the bladder neck than non-refluxing ureters (Figure 3). Moreover, the external insertional angles of the ureter at the outer wall of the bladder (formed by the ureteral insertion points and bladder neck) were much greater in the mutant refluxing ureters compared to contralateral non-refluxing ureters as well as control ureters. Thus, Pax3cre mediated deletion of *Fgfr2* causes abnormal positioning of the ureteric bud, which is associated with abnormal ureteral insertion in the bladder and subsequent VUR. If *Tbx18cre* mediated deletion of Fgfr2 results in ureteric induction abnormalities (see above), our laboratory will assess these mice for the presence of VUR as well.

Conclusion

In conclusion, many fibroblast growth factors and all of their receptors are expressed in the developing kidney. *In vitro* and *in vivo* studies have shown that exogenous fibroblast growth factors affect both renal mesenchymal and ureteric bud development. Follow up transgenic and gene knockout studies have shown that fibroblast growth factor receptor signaling is required for patterning of all renal lineages, including the ureteric bud, nephrogenic mesenchyme, and renal stromal mesenchyme. Furthermore, Fgfr activity is critical at early stages as well as later stages of kidney development. Also, there is clear redundancy between *Fgfr1* and *Fgfr2* in kidney mesenchyme. In humans, activating and loss of function mutations in *FGFR*s cause syndromes that are sometimes associated with urogenital anomalies [2–7]. Perturbations in fibroblast growth factor receptor signaling may also be responsible for sporadic cases of human renal congenital malformations and VUR.

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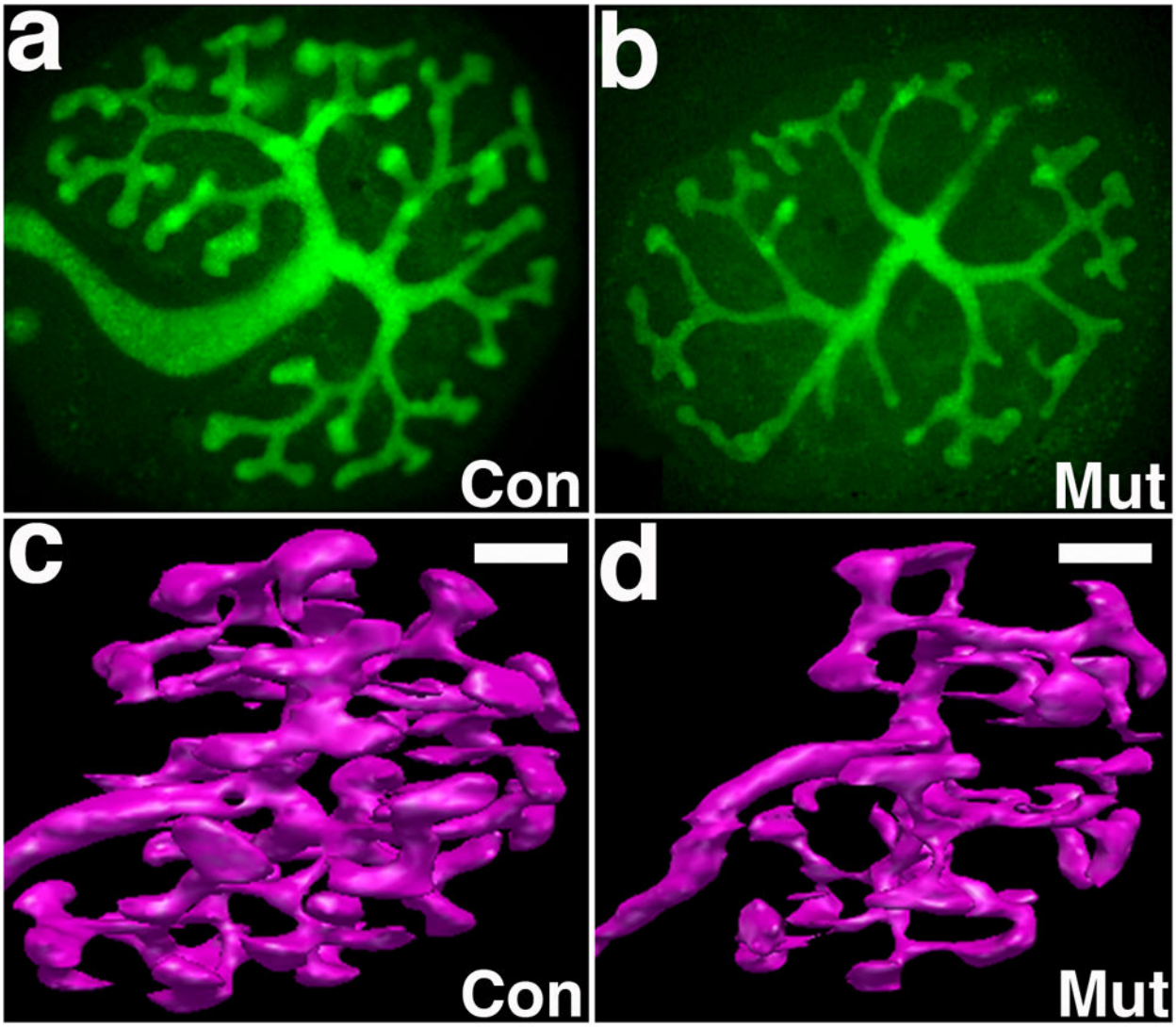

Figure 1. Comparison of control and *Fgfr2*$^{UB-/-}$ ureteric bud branching in explants and by 3D reconstructive imaging

a, b. Fluorescent micrographs of E11.5 explants after three days growth demonstrating that compared with controls (a) *Fgfr2*$^{UB-/-}$ explants (b) have thin, long ureteric trunks and fewer ureteric bud tips with thin ampullae (40x mag.**) c, d.** 3D reconstructive imaging of E13.5 kidneys confirm that compared with controls (c), *Fgfr2*$^{UB-/-}$ mutant (d) ureteric epithelium occupies less relative volume and has fewer but longer ureteric segments. Scale bar = 100μm. (Panels a, b reprinted from Figure 5 in [18] with permission from Elsevier. Panels c, d reprinted from Figure 2 in [43] with permission from American Society of Nephrology).

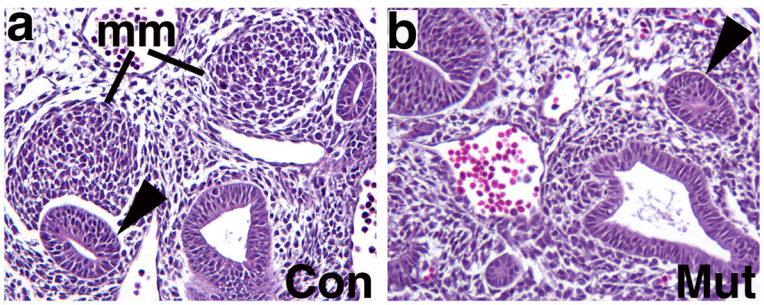

Figure 2. Comparison of the E10.5 metanephric mesenchyme and ureteric bud in control and *Fgfr1/2*$^{Mes-/-}$ mice

a, b. Hematoxylin & Eosin stains of transverse sections through caudal intermediate mesoderm demonstrate that control embryos (a) possess initial ureteric buds (arrowheads) and compacted metanephric mesenchymal (mm) tissues; *Fgfr1/2*$^{Mes-/-}$ mice (b) appear to possess initial ureteric buds (arrowheads) but no obvious metanephric mesenchyme (200x mag.) (Reprinted from Figure 7 in [19] with permission from Elsevier).

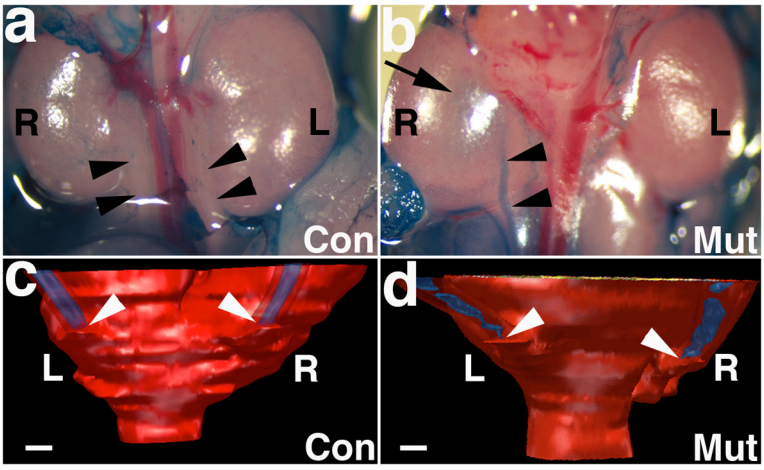

Figure 3. Representative images cystograms and 3D reconstructions through the ureterovesical junction (UVJ) of newborn *Fgfr2*$^{Mes-/-}$ and control mice

a, b. Gravity-driven methylene blue cystograms show a control mouse (a) with no dye in either ureter (arrowheads) and a mutant mouse (b) with dye in the right ureter (arrowheads) and the pelvis of the right kidney (arrow). (12x mag.) **c, d.** Posterior views of 3D reconstructions of the UVJ following cystograms show that insertion of the ureters (arrowheads) is at the same level in controls with no reflux (c); in the mutant (d) the right ureter (side of reflux, asterisk) inserts lower than the left (non-refluxing) ureter. Red tissues represent serosa and muscularis. Blue tissues represent ureteral urothelium. Scale bars = 100 μm.

Table

Targeted deletions of Fgf ligands, receptors, and signaling adapter proteins resulting in renal developmental anomalies

Targeted gene	If conditional, lineage targeted	Reported Results	References:
Fgf7[*]		Small kidneys with fewer ureteric buds and nephrons	[27]
Fgf10[*]		Small kidneys with fewer ureteric buds	[30]
Fgfr2IIIb[*]		Small kidneys with fewer nephrons	[37]
Fgfrl1		Severe renal dysgenesis	[12]
Fgfr2	ureteric bud	Small kidneys with aberrant ureteric bud branching, fewer nephrons and stromal mesenchymal patterning defects	[18]
Frs2α	ureteric bud	Mild renal hypoplasia with less ureteric branching and fewer nephrons	[44]
Fgf8	metanephric mesenchyme	Small kidneys with interrupted nephron formation	[32, 33]
Fgfr1/Fgfr2	metanephric mesenchyme	Aplasia with poorly formed metanephric mesenchyme and unbranched ureteric buds	[19]
Fgfr2	metanephric mesenchyme	Partially penetrant abnormalities including duplex ureters, duplex kidneys, obstructive hydroureter, renal aplasia, anterior shift of ureteric bud, vesicoureteral reflux	[47, 49]

[*] Note that Fgf7 and Fgf10 are the ligands for Fgfr2IIIb