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Fibroblast growth factor receptor signaling in kidney and lower urinary tract development

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Abstract

Fibroblast growth factor receptors (FGFRs) and FGF ligands are highly expressed in the developing kidney and lower urinary tract. Several classic studies showed many effects of exogenous FGF ligands on embryonic renal tissues *in vitro* and *in vivo*. Another older landmark publication showed that mice with a dominant negative Fgfr fragment had severe renal dysplasia. Together these studies revealed the importance of FGFR signaling in kidney and lower urinary tract development. With the advent of modern gene targeting techniques, including conditional knockout approaches, several publications have revealed critical roles for FGFR signaling in many lineages of the kidney and lower urinary tract at different stages of development. FGFR signaling has been shown to be critical for early metanephric mesenchymal patterning, Wolffian duct patterning including induction of the ureteric bud, ureteric bud branching morphogenesis, nephron progenitor survival and nephrogenesis, and bladder mesenchyme patterning. FGFRs pattern these tissues by interacting with many other growth factor signaling pathways. Moreover, the many genetic Fgfr and Fgf animal models have structural defects mimicking numerous congenital anomalies of the kidney and urinary tract seen in humans. Finally, many studies have shown how FGFR signaling is critical for kidney and lower urinary tract patterning in humans.

Keywords

Fibroblast growth factors; Metanephric mesenchyme; Ureteric bud; Renal organogenesis; Bladder organogenesis; Conditional knockout

Introduction

Since our most recent review for *Pediatric Nephrology* on roles of fibroblast growth factor receptors (FGFRs) in the developing kidney [1], several additional studies have identified critical roles for Fgfr signaling in the embryonic kidney and the lower urinary tract. This review will first briefly discuss the FGF/FGFR pathway and then present a synopsis of kidney and lower urinary tract development to orient the reader. The review will then focus on the vast number of tissue and animal models describing Fgfr activity in the developing

kidney and lower urinary tract (see Table 1 for mouse knockout publications in the order presented and Figure 1 for key receptor and ligands that regulate mouse kidney development). The review will then highlight the few, but growing numbers of studies showing roles in humans.

Overview of fibroblast growth factor receptor signaling

FGFRs are receptor tyrosine kinases (RTKs) consisting of four signaling family members (FGFR1-4) and a non-signaling receptor [2] lacking most of the normal C-terminus, including the tyrosine domain (see a review on FGFRs in [3]). FGFRs bind to FGF ligands, of which 22 have been identified in mammals to date. FGFR signaling is thought to occur principally via a constitutively associated docking protein, fibroblast growth factor receptor substrate 2 alpha (FRS2 α), which activates MAP kinase pathways, AKT, and various protein kinase C isoforms; signaling also occurs via other docking proteins/adapters including phospholipase C gamma (PLC γ), CRK2, and as yet unidentified adapters. FGFR activation leads to pro-proliferative, anti-apoptotic, and pro-survival responses in tissues. FGFRs1-3 have two major isoforms (IIIb or IIIc) differing in the C-terminal portion of the third Ig-like domain on the external portion of the receptor, whereas FGFR4 consists of a IIIc form only. IIIb isoforms are generally present and functional in epithelium, whereas IIIc isoforms are found in mesenchyme. While all receptors share downstream signaling docking proteins and pathways, different receptor family members and receptor isoforms within a family have different ligand binding characteristics. Also, most ligands bind multiple receptors and vice versa, with few exceptions (e.g. Fgf7 and Fgf10 are specific for the Fgfr2IIIb isoform).

Multiple ligands and FGF receptors are expressed in the mammalian kidney and lower urinary tract [4-7], and mice with a dominant negative Fgfr fragment have severe renal dysplasia [8], together suggesting the importance of FGF signaling in these tissues. Global targeted deletion of *Fgfr3* or *Fgfr4* in mice results in no obvious renal or bladder structural defects [9, 10], making *Fgfr1* and/or *Fgfr2* the likely candidates to be necessary for kidney and lower urinary tract development. A challenge has been that global deletion of *Fgfr1* or *Fgfr2* leads to early embryonic lethality, prior to the onset of kidney or lower urinary tract development [11-14]. Thus, studies using conditional knockouts, receptor isoform knockouts, and isolated tissues with agonists or antagonists have been critical for clarifying roles for Fgfrs in the developing kidney and bladder. Similarly, many global ligand knockout mice are early embryonic lethal necessitating similar approaches to clarify the roles of these genes in developing renal and lower urinary tract tissues.

Synopsis of kidney and lower urinary tract development

Metanephric kidney development starts during the 5th week of gestation in humans and the 10th day of gestation (E10.5) in mice (of a 19 day gestational cycle) as the ureteric bud evaginates from the Wolffian (nephric) duct to penetrate the nearby metanephric mesenchyme (see a recent review of kidney development in [15]) (Figure 1). Positioned between the metanephric mesenchyme and Wolffian duct is stromal mesenchyme (marked by Tbx18) [16], which acts to constrain the ureteric bud to a single and properly positioned induction site, and ultimately contributes to the mesenchyme surrounding the ureter and bladder. As development proceeds, ureteric bud ampullary tips (source of ureteric

progenitors) receive signals from the surrounding metanephric mesenchyme to proliferate and iteratively branch, ultimately forming the collecting ducts, renal pelvis and ureter. Early metanephric mesenchyme consists of self-renewing nephron progenitors (marked by *Six2*) that form caps around the ureteric tips and renal cortical stroma (marked by *Foxd1*) that surrounds the cap mesenchyme and ureteric epithelia. Signals from the ureteric tips drive surrounding cap mesenchyme/nephron progenitors to differentiate into nephrons, progressing from renal vesicles to comma-shaped bodies to S-shaped bodies, and finally to immature glomerular epithelia and nephron tubules that attach to the nascent collecting ducts. The renal cortical stroma (that also bi-directionally signals with cap mesenchyme and ureteric epithelia) ultimately forms glomerular mesangial cells, vascular smooth muscle cells, renin cells, pericytes, perivascular fibroblasts and a subset of peritubular endothelium [17].

Ureter and bladder development occur in an analogous manner with a reciprocal signaling between a central epithelium (urothelium) lining a lumen and an outer mesenchymal layer (see a recent review in [18]). Given that there are no published data on roles of *Fgfr* signaling in ureter morphogenesis, bladder development will be described. Bladder development begins at about the 5th week of gestation in humans and at E11.5-12.5 in mice as the anterior portion of the urogenital sinus. By E13.5, there is a clearly identifiable embryonic bladder with a urothelium surrounded by undifferentiated mesenchyme derived at least in part from the *Tbx18*-marked stroma that at earlier stages was present around the Wolffian duct [19]. During development, the urothelium converts from a simple to a stratified epithelium, which ultimately consists of three different cell types and is very water impermeable by virtue of apically-expressed uroplakins. Secreted factors from the urothelium, predominantly Sonic hedgehog (SHH), spread outward to and pattern the surrounding mesenchyme by binding to Patched-1 receptors. A high concentration of SHH in mesenchyme near the urothelium leads to formation of the collagen-containing lamina propria, whereas a low concentration of SHH at the mesenchyme periphery drives development of smooth muscle (the detrusor).

Role of FGFR signaling in the Wolffian duct and ureteric epithelium

Many studies have shown roles for *Fgfr* signaling in ureteric epithelium. Early studies showed that addition of *Fgf1*, *Fgf2*, *Fgf7* and/or *Fgf10* to rat embryonic kidney explants and/or isolated ureteric bud cultures drove growth and had variable effects on branching [20, 21]. Other studies showed that overexpression of human recombinant *FGF7* or *FGF10* in developing rats *in vivo* led to cyst formation in ureteric-derived collecting ducts [22, 23]. Some clarity about the endogenous ligand(s) and receptor modulating ureteric morphogenesis came from some global gene knockout studies in mice. Targeted deletion of *Fgf7*, expressed in renal cortical stroma, led to a marked reduction in ureteric branching with secondary loss of nephrons [20]. A study one year later revealed that loss of *Fgf10*, also expressed in cortical stroma, resulted in smaller kidneys with fewer collecting ducts (in addition to severe dysgenesis of lungs and limbs) [24]. More recent publications have shown that *Fgf10* can also (partially) substitute for glial cell line-derived neurotrophic factor in early ureteric induction and elongation in the context of *Sprouty1* and *Fras1* mutations [25, 26]. As *Fgf7* and *Fgf10* have a high degree of specificity for the IIIb isoform of *Fgfr2* (the

presumed major isoform in the ureteric epithelium), it was not surprising that *Fgfr2IIIb* null mice had very small kidneys and fewer nephrons (in addition to severe lung and limb defects) versus controls [27]. Given the severe extrarenal defects of the *Fgf10* and *Fgfr2IIIb* knockouts, the mice were all perinatal lethal, preventing the ability to assess postnatal kidneys in these mice.

To circumvent the early lethality of the global *Fgfr1* and *Fgfr2* knockouts and to drive deletion in kidneys but not lungs or limbs, our laboratory used a cre-lox conditional targeting approach. Breeding floxed *Fgfr1* and/or *Fgfr2* mice to our *Hoxb7creEGFP* line (expressing cre and enhanced green fluorescent protein in the Wolffian duct and its derivatives including the ureteric bud), we observed that loss of *Fgfr2* in the ureteric lineage led to fewer and smaller ampullary tips with reduced proliferation and increased apoptosis versus controls; these defects in the ureteric progenitors led to many fewer ureteric branches with longer and markedly thinner trunks compared with their control littermates [28, 29] (Figure 2). Real time PCR and *in situ* hybridization revealed that *Hoxb7cre; Fgfr2^{fl/fl}* mutants have a marked reduction in expression of *Ret* (Figure 2 and C.M.B. unpublished data), another receptor tyrosine kinase localized to ureteric tips that is critical for their growth and survival [30]. The loss of ureteric tip number was accompanied by secondary non-autonomous defects in the surrounding mesenchyme, including thickened renal cortical stroma and reduced numbers of cap mesenchymal cells, resulting in many fewer mature nephrons. Postnatally, the renal hypoplasia in the *Fgfr2* mutants led to chronic kidney disease, hypertension and left ventricular hypertrophy [31]. *Hoxb7cre*-mediated loss of *Fgfr1* led to no defects and combined loss of *Fgfr1* and *Fgfr2* had no additive effects beyond the loss of *Fgfr2* alone [28].

Our lab then sought to determine how the loss of *Frs2α*, the purported major signaling docking protein for Fgf receptors, affected ureteric morphogenesis. Given that global deletion of *Frs2α* leads to early embryonic lethality, we used our *Hoxb7cre* line to conditionally delete it from the ureteric lineage [32]. *Hoxb7cre; Frs2α^{fl/fl}* mice had a reduction in ureteric tip and branch number, associated with reduced *Ret* expression, and as a secondary consequence, fewer nephrons versus controls; however, the defects were more mild than in *Fgfr2* mutants, suggesting that *Fgfr2* was likely utilizing other docking proteins/adapters in addition to or independently of *Frs2α* in the ureteric lineage. To resolve this, we examined how combinations of *Fgfr2* and/or *Frs2α* alleles affected ureteric development [33]. To our surprise, mice with point mutations in the *Frs2α* binding site of *Fgfr2* (*Fgfr2^{LR/LR}* mice) had no ureteric defects and developed normal kidneys. Even breeding *Fgfr2^{LR/LR}* mice with *Hoxb7cre; Fgfr1^{fl/fl}* mice (to avoid potential rescue of *Fgfr1* monomers forming heterodimers with *Fgfr2^{LR}* monomers to allow recruitment of *Frs2α*) resulted in no kidney anomalies. Moreover, combined *Hoxb7cre*-mediated loss of *Fgfr2* and *Frs2α* resulted in E13.5 ureteric defects indistinguishable from *Fgfr2* alone, including a dramatic decrease of *Ret* expression in ureteric tips (Figure 2); however, starting at E17.5 and continuing through P30 *Hoxb7cre; Fgfr2^{fl/fl}; Frs2α^{fl/fl}* mice developed severe renal cystic hypoplasia/dysplasia, defects that were never observed in either *Hoxb7cre*-driven *Fgfr2* or *Frs2α* mutants. Thus, we concluded that in ureteric epithelium, *Frs2α* acts independently of and additively with *Fgfr2* (challenging the previously held dogma that *Frs2α* always acts as the major signaling docking protein downstream of Fgf receptors).

Finally, a recent publication using a different *Hoxb7cre* line (with apparently less mosaic cre expression in the Wolffian duct than our *Hoxb7cre* line), revealed how loss of *Fgfr2* in the Wolffian duct led to variable regression of a segment of the duct cranial to the ureteric bud induction site [34]. These partially penetrant defects had no effect on ureteric bud induction, but led to variable dilatation of epididymis and the vas deferens (other Wolffian duct derivatives) in males. As was the case with our *Hoxb7cre* studies focused on ureteric morphogenesis, these Wolffian duct defects were never observed when *Fgfr1* was deleted, and combined loss of *Fgfr1* and *Fgfr2* had no additive effects beyond the loss of *Fgfr2* alone. Taken with our studies, *Fgfr2* appears to be the major Fgf receptor mediating Wolffian duct and ureteric bud morphogenesis.

Role of FGFR signaling in metanephric mesenchyme

Several studies from 1995-2001 revealed how Fgf2 treatment of rodent or *Xenopus* embryonic kidney explants (alone or with other growth factors) promoted survival of kidney mesenchyme and sometimes even induced formation of nephrons [35-39]. While Fgf2 is secreted by ureteric tips, it is unlikely to be the endogenous factor driving nephron progenitor survival as *Fgf2* knockout mice have no kidney defects [40]. Two landmark papers established a role for Fgf8 in the nephron lineage, using mouse cre lines that were expressed throughout metanephric mesenchyme (nephron progenitors and stroma). Conditional deletion of *Fgf8*, expressed in early nephron progenitor-derived pretubular aggregates, with either a transgenic *Pax3cre* line [41] or a T (brachyury) cre line [42], led to small kidneys with a complete block in nephrogenesis past the renal vesicle stage and some evidence of nephron progenitor depletion at later stages of renal development. Global deletion of Fgfr like-1 receptors, which have a small intracellular domain lacking a tyrosine kinase domain, leads to a complete block in nephrogenesis similar to the conditional *Fgf8* knockouts [2]. As no other Fgf receptor mutants have a complete block in nephrogenesis (see below), it is likely that Fgf8 is the ligand for Fgfr1.

To search for the signaling tyrosine domain-containing receptors that promote survival of kidney mesenchyme, our laboratory used the transgenic *Pax3cre* line to drive deletion of *Fgfr1* and/or *Fgfr2* [43]. Loss of any three *Fgfr1* and *Fgfr2* alleles generally resulted in well-formed kidneys, whereas mice missing all four alleles (*Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{fl/fl}*) had a nearly complete loss of metanephric mesenchyme (Figure 3). While an early ureteric bud (and on occasion multiple buds) did form at E10.5, there was only a thin rim of mesenchyme around the bud that expressed early markers of metanephric mesenchyme including *Eya1* and *Six1*; other downstream mesenchymal markers such as *Six2*, *Sal1*, and *Pax2* were absent. Finally, glial cell line-derived neurotrophic factor (*Gdnf*), the ligand for Ret expressed on ureteric tips, was present in the thin remnant metanephric mesenchyme tissues at E10.5, but absent at E11.5, leading to arrest of ureteric bud outgrowth and no branching. A follow up study in which mice with *Pax3cre*-mediated deletion of *Fgfr1* and a global deletion of the IIIc isoform of *Fgfr2* (*Pax3cre; Fgfr1^{fl/fl}; Fgfr2IIIc-/-*) had a similar, albeit clearly less severe, defect in metanephric mesenchyme formation at E10.5 compared to *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{fl/fl}* mice; moreover, we noted low levels of Fgfr2IIIb isoform mRNA expression in cap mesenchyme of both *Pax3cre; Fgfr1^{fl/fl}; Fgfr2IIIc-/-* and control mice, suggesting that IIIb isoforms of Fgfrs may signal in mesenchymal tissues (and not just

epithelium as has been long believed) [44]. Taken together, these studies revealed redundant and critical roles of Fgfr1 and Fgfr2 in establishing and sustaining early metanephric mesenchyme.

Our group then sought to determine whether Fgfr1 and Fgfr2 signaling in the metanephric mesenchyme was mediated by Frs2α. To that end, we generated compound mutant mice with Pax3cre-mediated deletion of Fgfr1 and with point mutations in the Frs2α binding site of Fgfr2 (*Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}*) [45]. Unlike mice with Pax3cre deletion of both Fgfr1 and Fgfr2, *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}* mice made kidneys, suggesting that other docking proteins must be necessary for full signaling of the receptors in the metanephric mesenchyme. Surprisingly, while early metanephric mesenchymal tissues (nephron progenitors and renal cortical stroma) appeared unaffected in the *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}* mice, ureteric tips were hyperproliferative and dilated and ureteric trunks were shortened starting at E13.5 (Figure 2); this ultimately led to a reduction in overall ureteric branching and in collecting duct number as well as progressive cyst formation in older embryos and postnatal mutants. Mechanistically, the reduction in mesenchymal Fgfr/Frs2α signaling led to a decrease in *Bmp4* expression in metanephric mesenchyme near ureteric tips, and subsequent de-repression of *Ret* signal (including *Ret* targets *Etv4* and *Etv5*) in ureteric tips and misexpression of *Ret* (and its targets) into early ureteric trunks (Figure 2 and not shown). Thus, Fgfr/Frs2α signaling in the ureteric epithelium and Fgfr/Frs2α signaling in metanephric mesenchyme appear to have opposing effects on ureteric *Ret* expression and ureteric tip (and subsequent overall ureteric tree) morphogenesis (Figure 2). Finally, *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}* did have ureteric bud induction defects (multiple buds and displaced buds), similar to *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{fl/fl}* mice (see below).

Studies performed up to this point had given little clarity about which endogenous Fgf ligands and receptors had roles in nephron progenitor survival (a role that was suggested by the early Fgf2 explant studies noted earlier). An important study published by Dr. Leif Oxburgh's group in 2011 examined the effects of 30 different recombinant growth factors known to be expressed in embryonic kidney on isolated mouse embryonic nephrogenic zone cultures, mostly comprised of nephron progenitors [46]. Among the factors tested, only recombinant FGF ligands (1, 2, 9 and 20) and epidermal growth factor (EGF) receptor ligands (EGF and transforming growth factor alpha) were able to drive proliferation of nephron progenitors expressing *Cited1* (marker of stemness) and only the FGFs were able to drive expression of *Six2* and *Meox1*, other specific markers for nephron progenitors. This study established that Fgf signaling was a critical (if not the most important) growth factor pathway in maintaining survival and promoting proliferation of nephron progenitors, and gave some insights as to what the endogenous ligands might be. As noted, *Fgf2* null mice have no renal defects [40] and *Fgf1* knockout mice (alone and in combination with *Fgf2* knockouts) have no nephron progenitor defects [47], making Fgf9 and Fgf20 attractive candidates.

A recent study by Dr. Raphael Kopan's group firmly established that global deletion of *Fgf9* (mostly expressed in the ureteric bud) or *Fgf20* (present in nephron progenitors) led to nephron progenitor apoptosis, loss of nephron progenitor stemness, ectopic renal vesicle formation and subsequent renal dysplasia [48]. Moreover, simultaneous deletion of *Fgf9* and

Fgf20 produced severe renal dysgenesis reminiscent of *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{fl/fl}* mice. Another recent publication reported that *Wt1*, a transcription factor that is required for nephron progenitor survival, directly and indirectly drives expression of *Fgf20* (and other *Fgf* ligands) [49]. A separate group reported how the scaffolding proteins, *DLG1* and *CASK*, act synergistically to promote nephron progenitor survival, likely by enhancing *Fgf* signaling [50]. Our lab recently published a study examining the role of *Fgf* receptor and *Frs2α* signaling in nephron progenitors using a *Six2cre* line that deletes specifically in this cell population [51]. An allelic series of mice, including *Six2cre; Fgfr1^{fl/fl}; Fgfr2^{fl/fl}*, *Six2cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}*, and *Six2cre; Frs2α^{fl/fl}* all developed severe depletion of *Six2*-positive nephron progenitors (see Figure 3), associated with increased apoptosis, loss of *Cited1* expression (marker of stemness) and ultimately renal cystic dysplasia. The *Fgfr1*/*Fgfr2* full receptor knockouts had the earliest onset and most severe renal dysplasia, followed by the *Fgfr1*/*Fgfr2^{LR}* mice, and then the *Frs2α* mutants, suggesting that *Frs2α* was the main (but not exclusive) driver of *Fgfr* signaling in nephron progenitors (in stark contrast to the ureteric bud where *Fgfr2* and *Frs2α* act independently). Re-examination of *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}* mice also revealed previously unrecognized nephron progenitor depletion at later stages of development. Mechanistically, the *Six2cre; Frs2α^{fl/fl}* mutants appeared to have ectopic Notch activation, which is likely driving many of the progenitor defects in all of these receptor and *Frs2α* mutants. Given the similarity of the *Fgf9*/*Fgf20* and the (*Six2cre*) *Fgfr*/*Frs2α* mutants, the former are the likely ligands for the latter in nephron progenitors.

Role of FGFR signaling in *Tbx18*-positive stroma

One defect that our laboratory has consistently noted in multiple *Pax3cre*-mediated *Fgfr* deletion mouse lines has been early ureteric bud induction abnormalities. As noted earlier, while *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{fl/fl}* mice had aborted ureteric buds, they often had two ureteric buds per Wolffian duct [43]. Similarly, *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{IIIc-/-}* and *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}* mice had multiple and/or displaced ureteric buds [44, 45]. These defects raised two questions: first, was loss of either *Fgfr1* or *Fgfr2* alone sufficient to drive the ureteric induction defects (and what were the consequences of the induction defects); and second, which mesenchymal tissue(s) was(were) the receptor(s) acting in to constrain the ureteric induction site (given that the *Pax3cre* line expresses *cre* in renal cortical stroma, nephron progenitors and *Tbx18*-positive peri-Wolffian duct stroma). To answer the first question, we examined *Pax3cre; Fgfr1^{fl/fl}* and *Pax3cre; Fgfr2^{fl/fl}* mice more carefully. While *Pax3cre*-deletion of *Fgfr1* did not affect ureteric induction, *Pax3cre; Fgfr2^{fl/fl}* mice had a high incidence of early ureteric bud induction defects including duplex ureteric buds, aborted ureteric buds and abnormally-positioned single ureteric buds [52]. These early ureteric induction defects often led to a host of partially penetrant later anomalies, including renal aplasia, hypoplastic kidneys, partially duplicated kidneys, duplicated ureters and obstructed hydroureter.

Given that abnormal ureteric bud induction sites have been hypothesized to lead to abnormal insertion into the bladder and predispose to vesicoureteral reflux (as proposed by Drs. Mackie and Stephens in the 1970s), we ascertained whether *Pax3cre; Fgfr2^{fl/fl}* mice had these defects [53]. Excluding those mice with multiple ureteric buds per Wolffian duct, we

observed a virtual randomization of the ureteric bud induction site along the Wolffian duct (1/3 too high and 1/3 too low in relation to the cloaca) at E11.5. Moreover, 3D reconstructive imaging revealed abnormal positions of ureter insertion in a large number of *Pax3cre; Fgfr2^{fl/fl}* newborn mice compared to controls (consistent with the Mackie and Stephen hypothesis). Cystograms performed by gravity-driven infusion of methylene blue dye into dissected newborn bladders revealed 47% rates of reflux in *Pax3cre; Fgfr2^{fl/fl}* mutants versus 4% rates in littermate controls ($p < 0.001$). Thus, loss of *Fgfr2* led to malpositioned ureteric buds causing aberrant bladder insertion and high rates of reflux.

While *Fgfr2* appeared critical for constraining the ureteric bud to a single and properly positioned induction site, it was not clear which mesenchymal tissue(s) it was acting in. In fact, *in situ* hybridization confirmed that *Fgfr2* is normally expressed in renal cortical stroma, nephron progenitors and *Tbx18*-positive stroma at early stages of renal development, and that *Pax3cre; Fgfr2^{fl/fl}* mice had no discernable expression in any of these tissues [53]. Moreover, none of the *Six2cre* mutants (driving deletion in nephron progenitors) have ever had any ureteric induction defects [51], making this tissue the unlikely site. We then generated several *Foxd1cre*-mediated *Fgfr* mutants (driving deletion in renal cortical stroma) and saw no renal or ureteric induction defects, even in *Foxd1cre; Fgfr1^{fl/fl}; Fgfr2^{fl/fl}* lines (C.M.B. unpublished data). Thus, the *Tbx18*-expressing peri-Wolffian duct stroma was the likely mesenchymal tissue in which *Fgfr2* was acting on ureteric induction. In support of this, we observed that *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}* mice had a 43% decrease in *Bmp4* mRNA expression in E10.5 urogenital ridges (containing peri-Wolffian duct stroma) by real time PCR versus littermate controls ($p < 0.001$) [45]; moreover, studies from other groups have shown that *Bmp4* heterozygous mice have an approximately 40% decrease in *Bmp4* mRNA levels versus controls and have ureteric induction defects similar to our *Pax3cre* mutants [54, 55].

To definitively determine whether the peri-Wolffian duct stroma was the site at which *Fgfr2* was acting to constrain the ureteric induction site, we generated *Tbx18cre; Fgfr2^{fl/fl}* mice [56]. Importantly, these mutants recapitulated the aberrant ureteric induction phenotypes (i.e. randomization of the induction site) observed in *Pax3cre; Fgfr2^{fl/fl}* (and other *Pax3cre* mutant) mice. Furthermore, *Tbx18cre; Fgfr2^{fl/fl}* mice exhibited impaired ureteral insertion into the bladder and even higher rates of vesicoureteral reflux than in *Pax3cre; Fgfr2^{fl/fl}* mice (75% vs. 47%, respectively), presumably due to greater efficiency of *Tbx18cre*-mediated *Fgfr2* deletion in the peri-Wolffian duct stroma than in *Pax3cre* mutants (Figure 4). Mechanistically, *Tbx18cre; Fgfr2^{fl/fl}* mice also exhibited a ~40% reduction in urogenital ridge *Bmp4* mRNA similar to the *Pax3cre* mutants. These data together confirm a definitive role for *Fgfr2* signaling in the peri-Wolffian duct stroma for constraining the site of ureteric bud induction by driving *Bmp4* expression.

Knowing that the *Tbx18*-positive stroma subsequently gives rise to developing bladder mesenchyme, we utilized the *Tbx18cre* line to determine whether *Fgfrs* had a role in patterning of these tissues [57]. Although Euxpress (www.euxpress.org) and GUDMAP (www.gudmap.org) databases reveal expression of both *Fgfr1* and *Fgfr2* in developing bladder mesenchyme, *Tbx18cre; Fgfr1^{fl/fl}* mice have no bladder defects. In contrast, E16.5 *Tbx18cre; Fgfr2^{fl/fl}* mice have an approximately 15% increase in inner, collagen-containing

lamina propria volume and a reciprocal decrease in outer smooth muscle volume with no changes in overall bladder volumes (Figure 4). The volume alterations appear secondary to a cell fate switch from some of the muscle to lamina propria, as well as decreases in proliferation and increases in apoptosis of developing muscle relative to controls. From P1 to P30, the mutants exhibit progressive muscle loss and increases in total bladder collagen, including collagen deposition within the muscle relative to controls (Figure 4). *In vivo* cystometry and isolated bladder sheet assays show that the postnatal *Tbx18cre; Fgfr2^{fl/fl}* bladders are very poorly compliant (stiff) with high intravesicular pressures, shortened intercontractile intervals, and markedly reduced agonist-induced contractility versus littermate controls. Mechanistically, while urothelial sonic hedgehog mRNA levels are unchanged, *Tbx18cre; Fgfr2^{fl/fl}* embryonic bladders have multiple readouts indicating augmented sonic hedgehog activity; this likely drives the muscle cell fate switch to lamina propria as well as the changes in muscle layer apoptosis and proliferation. The enhanced mutant bladder mesenchyme responsiveness to Shh (with no changes in ligand levels) appears to be secondary to increased mesenchymal expression of Boc and Cdo, Patched-1 co-receptors that augment Shh activity. Thus, Fgfr2 appears to normally dampen Cdo and Boc expression and Shh activity in bladder mesenchyme for proper patterning of the muscle and lamina propria. This is the first study to demonstrate a significant role for Fgfr signaling in patterning bladder mesenchyme, and also reveals a very novel mechanism whereby Fgfr signaling can modulate hedgehog signaling (which may be relevant in other developing tissues). Finally, it shows how subtle patterning changes in bladder muscle and lamina propria may have profound effects on bladder structure and function, particularly with age.

FGFR signaling in human disease

There are examples of structural kidney and lower urinary tract diseases associated with FGF or FGFR mutations in humans. Activating mutations of *FGFR2* (or rarely *FGFR1*) can lead to Apert, Pfeiffer, Antley-Bixler or Beare-Stevenson Syndromes, which can have associated hydroureter, unilateral renal aplasia and/or vesicoureteral reflux [58-61]. These rare syndromes share significant phenotypic overlap (and in some cases the same gene mutations), including craniosynostosis, other craniofacial anomalies, proximal skeletal defects, distal defects (i.e., fingers and toes) and frequently developmental delay. Beare-Stevenson syndrome is also characterized by cutis gyrata, or furrowing of the skin on the face and feet. Kallmann syndrome, characterized by delayed or absent puberty, hypo- or anosmia and often renal aplasia (mostly unilateral), includes loss of *FGFR1* activity (and has disease variants with direct *FGFR1* gene mutations) [62]. Occasionally, activating mutations in *FGFR3*, causing thanatophoric dysplasia, are associated with renal hypoplasia or cystic dysplasia [63, 64]. In addition, as part of their study on *Fgf9* and *Fgf20* deletion in mice, the Kopan group identified homozygous inactivating mutations in *FGF20* causing bilateral renal aplasia in fetuses from a family with consanguinity [48]. Another recent paper identified that several related and non-related patients with congenital anomalies of the kidney and urinary tract had mutations in the dual serine-threonine and tyrosine protein kinase (DSTYK) gene [65]; this gene is co-expressed with FGFRs in the developing kidney and is thought to block receptor phosphorylation (activation) of extracellular signal-regulated kinase (ERK). Finally, many of the genetic determinants for structural kidney disease and bladder dysfunction (e.g. age-related) remain unknown. Given the wide array of actions of the FGF/FGFR signaling

family in animal models, mutations in these genes are strong candidates as causative for many human cases of structural kidney and/or lower urinary tract disease.

Given the growing number of animal and human studies showing the relevance of FGFR signaling in kidney and bladder development, there may be future potential treatment strategies manipulating FGFR expression. Based on studies noted earlier, recombinant FGF10 may be useful in rescuing ureteric branching defects as seen in Fraser syndrome (FRAS1 mutations) or other diseases in which there is deficient GDNF/RET signaling [25, 26]. Given that the FGFR signaling pathway appears so critical for nephron progenitor survival and proliferation [46, 48, 51], there could be future therapies using FGF ligands to drive kidney regeneration, either directly *in vivo* or *in vitro* (e.g. to generate progenitors from induced pluripotent stem cells for infusion into patients). The requirement for Fgfr2 signaling in lower urinary tract mesenchyme [57] may lead to FGF-driven therapies for repair of smooth muscle defects in the ureter or bladder. There are also animal studies showing that Fgfr signaling may have critical roles in bladder urothelial injury and repair [66]; moreover, intravenous administration of recombinant human FGF7 largely prevented cyclophosphamide injury in rats [67], making this a potential therapy for patients subjected to drugs with potential for bladder urothelial injury.

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Figure 1. Diagram outlining key Fgf receptors and ligands that regulate renal development in the mouse

Fgfr2 in the Wolffian duct (light blue) is critical for Wolffian duct morphogenesis cranial to the ureteric bud induction site [34]. Fgfr2 in Tbx18-positive (Tbx18+ve) stroma near the Wolffian duct (black) represses ectopic ureteric bud induction from the Wolffian duct (WD) [43-45, 51-53, 56, 57]. Fgf7 and Fgf10 (blue) in the Foxd1-positive (Foxd1+ve) renal stroma bind to Fgfr2 (blue) in the ureteric bud to regulate ureteric morphogenesis [20, 24, 27-29]. Fgf9 in ureteric tips (green) and Fgf10 in Six2 positive (Six2+ve) cap metanephric mesenchyme/nephron progenitors (green) likely bind to Fgfr1 and Fgfr2 (green) to pattern early metanephric mesenchyme and for nephron progenitor survival [43-45, 48, 51]. Fgf8 and Fgfr11 in renal vesicles (purple) are necessary for nephrogenesis past the vesicle stage (it is unclear if the ligand and receptor interact) [2, 41, 42]. Please also note that while cap mesenchyme and renal vesicles normally associate with each ureteric tip, the diagram only shows those around the dorsal ureteric tips. (Adapted from Fig. 2 of [68] and re-printed with permission from the American Physiological Society).

Figure 2. Differential effects of ureteric and mesenchymal Fgfr/Frs2α signaling on ureteric morphogenesis at E13.5

A. Real time PCR graph reveals marked depression of *Ret* expression in *Hoxb7cre* (*Hcre*); *Fgfr2^{fl/fl}* and *Hoxb7cre; Fgfr2^{fl/fl}; Frs2α^{fl/fl}* kidneys vs. controls. B, C. Whole mount *in situ* hybridization confirms vast reduction in *Ret* levels in *Hoxb7cre; Fgfr2^{fl/fl}; Frs2α^{fl/fl}* ureteric tips (C, arrowhead) vs. the control (B). 3D reconstructions reveal fewer and smaller ureteric tips (arrowhead) and longer trunks in *Hoxb7cre; Fgfr2^{fl/fl}; Frs2α^{fl/fl}* kidney (E) vs. the control (D). F. Real time PCR graph reveals increased *Ret* expression in *Pax3cre* (*Pcre*); *Fgfr1^{fl/fl}; Fgfr2^{LR/LR}* kidneys vs. controls. G, H. Section *in situ* hybridization reveals expression of *Ret* in ureteric tips in both control (G, arrowheads) and *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}* (H) kidneys, but *Ret* misexpression in mutant ureteric trunks (H, arrows). I, J. 3D reconstructions reveal markedly larger and dilated ureteric tips in the *Pax3cre; Fgfr1^{fl/fl}; Fgfr2^{LR/LR}* kidney (J, arrowheads) vs. the control (I). B-E, I-J: scale bar = 100μm; G-H: scale bar = 50 μm. (Panels D and E are reprinted from Figure 2, panels A and D from [33]; Panels F, G, and H are reprinted from Figure 9, panels A, D, and G from [45] with permission from the American Society of Nephrology; Panels I and J are reprinted from Figure 3, panels C and D from [45] with permission from the American Society of Nephrology).

Figure 3. Effects of mesenchymal Fgfr/Frs2 α signaling on metanephric mesenchymal morphogenesis

A, B. Hematoxylin and Eosin (H&E) stained images from E10.5 embryos reveal compacted metanephric mesenchyme (arrows) around the Wolffian duct and ureteric bud (arrowhead) in the control (A), but no identifiable metanephric mesenchyme around the ureteric bud (arrowhead) in the *Pax3cre* (*Pcre*); *Fgfr1*^{fl/fl}; *Fgfr2*^{fl/fl} mutant (B). C, D. H&E stained images from E14.5 kidneys reveal robust cap mesenchyme/nephron progenitors (arrows) around ureteric bud tips (arrowheads) in the control (C), but a marked depletion of cap mesenchyme (arrows) around ureteric tips (arrowheads) in the *Six2cre* (*Scrc*); *Fgfr1*^{fl/fl}; *Fgfr2*^{LR/LR} mutant (D). E, F. Six2 immunofluorescence in E14.5 kidneys confirms robust cap mesenchyme (arrows) in the control (E), but depletion of nephron progenitors in a *Six2cre*; *Fgfr1*^{fl/fl}; *Fgfr2*^{LR/LR} embryo (F). A-B: scale bar = 200 μ m; C-D: scale bar = 25 μ m; E-F: scale bar = 50 μ m. (Panels A and B are reprinted from Figure 7, panels C and D from [43] with permission of Elsevier; Panels E and F are reprinted from Figure 3, panels C and D from [51] with permission of Elsevier).

Figure 4. Effects of Fgfr2 signaling in Tbx18-positive stromal mesenchyme

A, B. Cystograms performed on newborn urinary tracts reveal no methylene blue dye moving from the bladder (Bl) up the ureters (arrowheads) of the control (A), whereas the *Tbx18cre (Tcre); Fgfr2^{fl/fl}* mouse has dye tracking from the bladder (Bl) up the right ureter (arrowheads) to the level of the renal pelvis (asterisk). C, D. H&E stained sections of bladder cross sections at E16.5 show that compared to the control (C), the *Tbx18cre (Tcre); Fgfr2^{fl/fl}* embryo (D) has a thinner muscle (Mus) layer and an expanded lamina propria (LP) layer, while the urothelium (U) appears unaltered. E, F. H&E stained sections of bladder cross sections at P30 reveal that compared to the control (E), the *Tbx18cre (Tcre); Fgfr2^{fl/fl}* mouse has dramatically attenuated muscle (Mus) that is less compact and has infiltrating connective tissue. A-B: scale bar = 3 mM; C-D: scale bar = 300 μm; E-F: scale bar = 500 μm. (Panels A and B are reprinted from Figure 5, panels A and B from [56]; Panels C and D are reprinted from Figure 3, panels A and B from [57] with permission from the American Physiological Society; Panels E and F are reprinted from Figure 8, panels B and B' from [57] with permission from the American Physiological Society).

Table 1

Targeted deletions of Fgf ligands, receptors, and signaling docking proteins resulting in kidney and/or urinary tract developmental anomalies

Targeted gene	If conditional, cre line used/ lineage targeted	Reported Results	References
<i>Fgf7</i> [*]		Small kidneys with fewer ureteric buds and nephrons	[20]
<i>Fgf10</i> [*]		Small kidneys with fewer ureteric buds	[24]
<i>Fgfr2IIb</i> [*]		Small kidneys with fewer nephrons	[27]
<i>Fgfr2</i>	Hoxb7cre/ureteric bud	Small kidneys with thin ureteric tips, aberrant ureteric bud branching, fewer nephrons and stromal mesenchymal patterning defects	[28, 29]
<i>Frs2a</i>	Hoxb7cre/ureteric bud	Mild renal hypoplasia with less ureteric branching and fewer nephrons	[32]
<i>Fgfr2/Frs2a</i>	Hoxb7cre/ureteric bud	Embryonic kidneys with thin ureteric tips and branching defects and fewer nephrons leading to severe postnatal renal cystic hypoplasia/dysplasia	[33]
<i>Fgfr2</i>	Hoxb7cre/Wolffian duct	Variable regression of Wolffian duct cranial to ureteric induction site leading to dilated epididymis and vas deferens	[34]
<i>Fgf8</i>	Pax3cre and T (brachyury) cre/ metanephric mesenchyme	Small kidneys with interrupted nephron formation	[41, 42]
<i>Fgfr11</i>		Small kidneys with interrupted nephron formation	[2]
<i>Fgfr1/Fgfr2</i>	Pax3cre/metanephric and peri- Wolffian duct mesenchyme	Aplasia with poorly formed metanephric mesenchyme and unbranched (occasionally duplex) ureteric buds	[43]
<i>Fgfr1/Fgfr2IIb</i> [†]	Pax3cre/metanephric and peri- Wolffian duct mesenchyme	Severe dysgenesis with poorly formed metanephric mesenchyme and unbranched (occasionally duplex) ureteric buds	[44]
<i>Fgfr1/Fgfr2LR</i> [†]	Pax3cre/metanephric and peri- Wolffian duct mesenchyme	Embryonic kidneys with dilated ureteric tips and branching defects, nephron progenitor loss, leading to severe postnatal renal cystic disease and ureteric bud induction defects	[45, 51]
<i>Fgf9 and/or 20</i>		Nephron progenitor depletion (increased apoptosis, loss of stemness), ectopic renal vesicles, renal dysplasia	[48]
<i>Fgfr1/Fgfr2 Fgfr1/Fgfr2LR</i> [†] <i>Frs2a</i>	Six2cre/nephron progenitors	Nephron progenitor depletion (increased apoptosis, loss of stemness), renal dysplasia	[51]
<i>Fgfr2</i>	Pax3cre/metanephric and peri- Wolffian duct mesenchyme	Partially penetrant defects including duplex ureters, duplex kidneys, obstructive hydronephrosis, renal aplasia, abnormal ureteric bud induction site, and vesicoureteral reflux	[52, 53]
<i>Fgfr2</i>	Tbx18cre/peri-Wolffian duct and bladder mesenchyme	Aberrant ureteric bud induction with abnormal ureter insertion into bladder and vesicoureteral reflux; abnormal bladder mesenchyme patterning leading to poorly compliant and poorly contractile postnatal bladders	[56, 57]

* Note that Fgf7 and Fgf10 are the ligands for Fgfr2IIb

† Note that these Fgfr2 mutant alleles are global