Ureteric Morphogenesis Requires Fgfr1 and Fgfr2/Frs2α Signaling in the Metanephric Mesenchyme

Sunder Sims-Lucas,* Valeria Di Giovanni,* Caitlin Schaefer,* Brian Cusack,* Veraragavan P. Eswarakumar,† and Carlton M. Bates*‡

*Rangos Research Center, Children’s Hospital of Pittsburgh of UPMC, Pittsburgh, Pennsylvania; †Department of Orthopaedics and Rehabilitation, Yale University School of Medicine, New Haven, Connecticut; and ‡Division of Nephrology, Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

ABSTRACT

Conditional deletion of fibroblast growth factor receptors (Fgfrs) 1 and 2 in the metanephric mesenchyme (MM) of mice leads to a virtual absence of MM and unbranched ureteric buds that are occasionally duplex. Deletion of Fgfr2 in the MM leads to kidneys with cranially displaced ureteric buds along the Wolffian duct or duplex ureters. Mice with point mutations in Fgfr2’s binding site for the docking protein Frs2α (Fgfr2LR/LR), however, have normal kidneys; the roles of the Fgfr2/Frs2α signaling axis in MM development and regulating the ureteric bud induction site are incompletely understood. Here, we generated mice with both Fgfr1 deleted in the MM and Fgfr2LR/LR point mutations (Fgfr1Mes−/−/Fgfr2LR/LR). Unlike mice lacking both Fgfr1 and Fgfr2 in the MM, these mice had no obvious MM defects but had cranially displaced or duplex ureteric buds, probably as a result of decreased Bmp4 expression. Fgfr1Mes−/−/Fgfr2LR/LR mice also had subsequent defects in ureteric morphogenesis, including dilated, hyperproliferative tips and decreased branching. Ultimately, they developed progressive renal cystic dysplasia associated with abnormally oriented cell division. Furthermore, mutants had increased and ectopic expression of Ret and its downstream targets in ureteric trunks, and exhibited upregulation of Ret/Etv4/5 signaling effectors, including Met, Myb, Cxcr4, and Crifl1. These defects were associated with reduced expression of Bmp4 in mesenchymal cells near mutant ureteric bud tips. Taken together, these results demonstrate that Fgfr2/Frs2α signaling in the MM promotes Bmp4 expression, which represses Ret levels and signaling in the ureteric bud to ensure normal ureteric morphogenesis.


Metanephric kidney development occurs via interactions between mesenchyme and epithelia. The metanephric mesenchyme (MM) induces the ureteric bud from the Wolffian duct, which elongates and undergoes branching to form the collecting ducts, pelvis, and ureter. Ureteric bud tips stimulate the MM to differentiate into nephron epithelia, progressing from renal vesicles, to comma-shaped bodies, to S-shaped bodies, to immature glomeruli. The cortical stroma envelops and interacts with the other renal lineages.

Fibroblast growth factor receptors (Fgfrs) are critical for renal development. Fgfrs consist of 4 receptor tyrosine kinases with 22 ligands. Although global deletion of Fgfr1 or Fgfr2 leads to embryonic lethality before kidney formation, our laboratory has used conditional knockout approaches that have revealed many roles for Fgfrs in developing kidneys (Supplemental Table 1). Conditional deletion of Fgfr2 in the ureteric bud causes defects in ureteric bud morphogenesis, and deletion in the MM using a Pax3cre transgenic line
often results in inappropriate ureteric bud induction (duplex or cranial displacement of the ureteric bud). These defects often result in abnormalities of the kidney and urinary tract (hypoplasia, dysplasia, obstructed ureters) and high rates of vesicoureteral reflux.12,19 Conditional deletion of Fgfr1 and Fgfr2 in the MM (Fgfr1ΔMes−/−) results in a virtual absence of MM and ureteric bud branching.13

Fgfr signal through adaptor proteins, including Fgfr substrate 2α (Frs2α). Frs2α constitutively binds Fgfrs and upon receptor stimulation becomes phosphorylated, then activating Erk, Akt, and protein kinase C (PKC) λ and ε. Deletion of Frs2α results in early embryonic lethality, probably because of the inability of Fgfrs and other receptor tyrosine kinases to signal.20 Deletion of Frs2α in the ureteric bud leads to branching defects, although not as severe as ureteric deletion of Fgfr2.21 Etv4 and Etv5, downstream targets of Frs2α signaling, are necessary for ureteric bud morphogenesis.22–24 Mice with point mutations in the Frs2α binding site of Fgfr2 (Fgfr2LR/LR) have normal kidneys,25 suggesting redundancy between Fgfr2 and other receptor tyrosine kinases that use Frs2α.

Our objective was to determine whether Fgfr2/Frs2α in the MM mediates the renal dysgenesis seen with Pax3cre deletion of Fgfr1 and 2 and ureteric bud induction abnormalities in combined mutants and Fgfr2 knockouts. We generated compound mutants with conditional deletion of Fgfr1 in the MM and with Fgfr2LR/LR alleles (Fgfr1ΔMes−/−Fgfr2LR/LR). Fgfr1ΔMes−/−Fgfr2LR/LR mice have no obvious MM defects, suggesting that Fgfr2/Frs2α signaling is dispensable for early kidney formation. Fgfr1ΔMes−/−Fgfr2LR/LR mice have ureteric induction abnormalities, including anterior displacement or duplex ureters that is probably secondary to decreased Bmp4 expression at E10.5. E13.5 Fgfr1ΔMes−/−Fgfr2LR/LR embryos developed dilated and hyperproliferative ureteric tips and decreased ureteric branching. With age, Fgfr1ΔMes−/−Fgfr2LR/LR mice developed progressive renal cystic dysplasia associated with aberrant oriented cell division. Mutants have increased ectopic expression of Ret and downstream targets, including activated Erk and Etv4 and Etv5, into ureteric trunks. Expression of other Ret/Etv4/Etv5 signaling targets, including Met, Myb, Cxcr4, and Crlf1, were also increased. These defects were associated with reduced Bmp4 expression in mesenchymal cells near mutant ureteric bud tips and reduced phospho-p38 expression in ureteric tips. Thus, Fgfr2/Frs2α signaling in the MM appears to promote Bmp4 expression that represses Ret levels and signaling in the ureteric bud, ensuring normal ureteric morphogenesis.

RESULTS

Fgfr1ΔMes−/−Fgfr2LR/LR Kidneys Have Ureteric Induction Abnormalities with Decreased Bmp4 Levels

Unlike Fgfr1ΔMes−/− mice, Fgfr1ΔMes−/−Fgfr2LR/LR mice form a robust MM and ureteric bud that elongates with normal initial branching (Supplemental Figure 1). Similar to Fgfr1ΔMes−/− and Fgfr2LR/LR mice,12,13 however, Fgfr1ΔMes−/−Fgfr2LR/LR mice had ureteric induction abnormalities by whole mount in situ hybridization for Ret at E11.0 and three-dimensional (3D) reconstruction at E13.5. As shown in Figure 1, the common nephric duct length (segment of Wolffian duct from the ureteric bud base to the cloaca) was increased in E11.0 Fgfr1ΔMes−/−Fgfr2LR/LR kidneys (406.9±75.3 μm; mean ± SD) versus controls (235.1±35.0 μm; P<0.001), in Fgfr2LR/LR kidneys (215.8±43.8 μm; P<0.001), and Fgfr1ΔMes−/− kidneys (242.9±54.8 μm; P<0.001). Thus, Fgfr1ΔMes−/−Fgfr2LR/LR mice often have cranial displacement of the ureteric induction site. Three-dimensional reconstructions of E13.5 lower urogenital regions showed that many mutants have partially or completely duplicated ureters that insert ectopically into the ductus deferens or into the urogenital sinus (future bladder) (Figure 2, Supplemental Movie 1). Thus, Fgfr1ΔMes−/−Fgfr2LR/LR mice develop a kidney but have ureteric bud induction abnormalities.

Among potential downstream targets that could explain the ureteric induction defects, we saw no changes in Slit2, Robo2, Ret, Etv4, or Etv5 expression by real-time PCR (qPCR) in E10.5 urogenital ridges (Supplemental Table 2). However, Bmp4 levels appeared to be reduced in E10.5 Fgfr1ΔMes−/−Fgfr2LR/LR

Figure 1. Common nephric duct lengths in E11.0 Fgfr1ΔMes−/−Fgfr2LR/LR and littermate embryos. (A and B) Representative images of whole-mount Ret in situ hybridization that shows no differences compared with controls (A), the common nephric ducts (CNDs) in Fgfr1ΔMes−/−Fgfr2LR/LR mice (B, white bars) appear longer, indicating cranial displacement of the mutant ureteric induction site. (C) Mean common nephric duct lengths are larger in Fgfr1ΔMes−/−Fgfr2LR/LR mice than in control, Fgfr2LR/LR, and Fgfr1ΔMes−/− kidneys. *P<0.001. Scale bars (A and B) = 200 μm.
BASIC RESEARCH

At E13.5, Fgfr1Mes−/− Fgfr2LR/LR mutant with bilateral partial duplex ureters that fuse (arrows) before entering the ductus deferens on the left (concave arrowhead) or the urogenital sinus on the right (arrowhead). (C) Fgfr1Mes−/− Fgfr2LR/LR mutant with a normal single left ureter inserting into the urogenital sinus (arrowhead) and completely duplex right ureters with one inserting into the ductus deferens (concave arrowhead) and the other into the urogenital sinus (arrowhead). L = left; R, right. Scale bars (A and B) = 100 μm.

urogenital ridges by whole-mount in situ hybridization (Supplemental Figure 2) and by qPCR (43% decrease; P=0.0002) versus controls. Prior publications show that a one-allele loss of Bmp4 leads to a 40% reduction in Bmp4 mRNA levels and ureteric induction abnormalities.25,26

Fgfr1Mes−/− Fgfr2LR/LR Kidneys Have Ureteric Branching Abnormalities and Fewer Nephrons

At E13.5, Fgfr1Mes−/− Fgfr2LR/LR kidneys appeared grossly similar to controls and 3D reconstruction (not shown); however, mutant ureteric tips appeared dilated by histologic examination and 3D reconstruction (Figure 3). Mean mutant and control kidney and ureteric volumes were not statistically different (Table 1); however, mutants had larger proportional ureteric volumes (ureteric/kidney volume; Table 1), consistent with dilated ureteric segments. Mean mutant ureteric tip diameters were 85% larger than those in controls, whereas branch diameters were unchanged (Table 1). Proliferating cell nuclear antigen (PCNA) staining revealed increased mean rates of mutant E13.5 ureteric tip proliferation (control, 60.6%; mutant, 80.7%; P=0.00012), whereas proliferation was equivalent in controls (61.7%±3.9%; mutant, 55.5%±7.1%; P=0.25; Figure 4). Ureteric apoptosis was similar (not shown). Skeletonized reconstructed E13.5 ureteric trees revealed 40% fewer branch and tip segments and a decrease in total ureteric length in mutants versus controls (Figure 4, Table 1, and Supplemental Movies 2 and 3). Thus, E13.5 Fgfr1Mes−/− Fgfr2LR/LR kidneys had dilated, hyperproliferative ureteric tips and defective ureteric branching.

We examined E13.5 Fgfr1Mes−/− Fgfr2LR/LR kidneys for mesenchymal defects. Immunostaining for Foxd1 (cortical stroma) and Six2 (nephron progenitors) appeared normal in mutants (Figure 5). Mutants had a 40% reduction in developing nephron number and volume (Table 1), correlating with fewer ureteric tips. Mutants and controls had equivalent relative developing nephron volumes (nephron volume/kidney volume), sizes, and stages of nephrogenesis (vesicles, comma-shaped bodies, S-shaped bodies, immature glomeruli) (Table 2, Figure 5, and not shown). Thus, the reduction in mutant nephron number was probably due to fewer ureteric tips.

Fgfr1Mes−/− Fgfr2LR/LR Kidneys Develop Progressive Cystic Abnormalities

By E16.5, hematoxylin and eosin staining revealed dilated tubules in Fgfr1Mes−/− Fgfr2LR/LR kidneys that appeared otherwise normal (Supplemental Figure 3). By postnatal day 21 (P21), mutant kidneys appeared dimpled, translucent, or grossly distorted with cysts (Figure 6). Mutant kidneys with minimal translucency had cysts of variable size usually near the cortico-medullary junction. Very translucent or grossly distorted kidneys contained cysts that almost replaced the renal parenchyma (Figure 6). All mutants had glomerular cysts (not shown), and images at P21 revealed abnormal glomerular lobulation and focal and segmental sclerosis, as well as interstitial fibrosis, inflammation, and proteinaceous tubular casts (Figure 7).

We assayed E18.5 mutants for abnormal collecting duct planar cell polarity/oriented cell division since this is often associated with renal cystogenesis.25 In controls, 83% of dividing collecting duct cells had mitotic angles less than 30°, nearly parallel to the tubular longitudinal axis, whereas in mutants 50% of the non-dilated dividing collecting duct cells had mitotic angles greater than 30° (P<0.001) (Figure 8). There were no differences in proliferation in nondilated mutant collecting ducts versus controls (4%±1.9% versus...
4%±2.5%, P=0.6), as measured by phospho-histone H3–positive cells over total cells. Thus, Fgfr1Mes−/−Fgfr2LR/LR kidneys have defects in oriented cell division in predilated collecting ducts without changes in proliferation.

**Fgfr1Mes−/−Fgfr2LR/LR** Kidneys Display Aberrant Ret Signaling and Reduced Bmp4 Expression

In focusing on candidate molecules that could be driving mutant ureteric defects, we examined Ret expression.28 Whereas Ret is confined to control ureteric tips at E13.5, Ret signal was expanded into ureteric trunks in E13.5 mutants (Figure 9). Whole-kidney qPCR confirmed increased Ret expression in both E13.5 and E18.5 mutants (Figure 9). Similarly, Ret targets, Etv4 and Etv5, had expanded expression into mutant ureteric trunks by *in situ* hybridization and increased expression at E13.5 and E18.5 by qPCR (Figure 9), suggesting increased Ret signaling in addition to expression. We detected increases in Ret/Etv4/Etv5 targets, Crf1, Cxc4, Myb, and Met, in E13.5 mutant ureteric bud by qPCR, further supporting increased Ret activity (Figure 9). Finally, immunostaining revealed increased phospho-Erk1/2 (effector of Ret signaling) in mutant ureteric bud tips and ureteric bud trunks in E13.5 and E18.5 mutants, mimicking Ret expression (Figure 10). Thus, ureteric defects in Fgfr1Mes−/−Fgfr2LR/LR mice appear secondary to increases in Ret expression and activity.

We interrogated molecules expressed in the MM that could regulate ureteric bud morphogenesis and Ret expression in a noncell autonomous manner. We saw no changes in Sprouty1 and Gdnf expression at E13.5 (Supplemental Figure 4); however, Bmp4 expression was reduced in mesenchyme near ureteric bud tips in E13.5 Fgfr1Mes−/−Fgfr2LR/LR animals by *in situ* hybridization (Figure 11) and by 46% in E13.5 mutant kidneys versus controls at E13.5 by qPCR (P=0.014). To determine consequences of reduced Bmp4 expression on the adjacent ureteric bud, we immunostained E13.5 sections for activated proteins downstream of Bmp signaling. Although there were no changes in phospho-Smad 1/5/8 expression (readout of canonical Bmp signaling) (not shown), phospho-p38 (readout of noncanonical Bmp signaling) was reduced in mutant ureteric bud structures (Figure 11). Thus, it appears that reduced Bmp4 expression in Fgfr1Mes−/−Fgfr2LR/LR MM causes de-repressed Ret expression and signaling and subsequently defective Fgfr1Mes−/−Fgfr2LR/LR ureteric morphogenesis.

**DISCUSSION**

Like Fgfr1/2Mes−/− mice,13 Fgfr1Mes−/−Fgfr2LR/LR mutants have abnormal ureteric bud induction, revealing that Fgfr2/Frs2a signaling constrains ureteric bud outgrowth to the proper site, probably by promoting Bmp4 expression in stroma around the Wolfian duct (see below). The role of Fgfr1 is more vague; whereas Fgfr2Mes−/− mice have cranial displacement of the ureteric induction site and duplex ureters, Fgfr1Mes−/− mice do not.12,13 Conversely, Fgfr2LR/LR mice have normal ureteric induction unless combined with Fgfr1Mes−/− mutations. We have also observed that combining Fgfr2LR/LR alleles with other conditional Fgfr1 mutations results in abnormalities

---

**Table 1. Mean kidney measurements in E13.5 control and Fgfr1Mes−/−Fgfr2LR/LR mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (n=4)</th>
<th>Mutant (n=4)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>volume (μm³)</td>
<td>5.16×10^7 ± 1.48×10^7</td>
<td>3.59×10^7 ± 0.95×10^7</td>
<td>0.13</td>
</tr>
<tr>
<td>Ureteric tree</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>volume (μm³)</td>
<td>6.53×10^6 ± 2.03×10^6</td>
<td>5.23×10^6 ± 1.81×10^6</td>
<td>0.37</td>
</tr>
<tr>
<td>relative volume (%)</td>
<td>12.6±0.626</td>
<td>15.6±2.19</td>
<td>0.04</td>
</tr>
<tr>
<td>total length (μm)</td>
<td>9.69×10^3 ± 1.34×10^3</td>
<td>5.79×10^3 ± 1.12×10^3</td>
<td>0.004</td>
</tr>
<tr>
<td>tip diameter (μm)</td>
<td>32.3±8.23</td>
<td>59.8±18.8</td>
<td>4.90×10^-11</td>
</tr>
<tr>
<td>branch diameter (μm)</td>
<td>30.8±7.86</td>
<td>30.9±4.41</td>
<td>0.96</td>
</tr>
<tr>
<td>tip number</td>
<td>60.3±7.54</td>
<td>35.0±6.00</td>
<td>0.002</td>
</tr>
<tr>
<td>branch number</td>
<td>55.5±5.74</td>
<td>31.8±6.29</td>
<td>0.001</td>
</tr>
<tr>
<td>Nephron</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>volume (μm³)</td>
<td>5.21×10^6 ± 1.53×10^6</td>
<td>3.13×10^6 ± 6.25×10^5</td>
<td>0.02</td>
</tr>
<tr>
<td>relative volume (%)</td>
<td>10.1±0.968</td>
<td>9.0±2.37</td>
<td>0.41</td>
</tr>
<tr>
<td>tip number</td>
<td>61.3±14.4</td>
<td>35.0±13.3</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD.
Figure 5. Stromal and nephrogenic tissues in E13.5 Fgf1<sup>1Mes–/−/Fgf2<sup>2LR/LR</sup> (mutant) and control embryos. (A and B) Whole-mount immunofluorescence shows that in outer cortical regions, control (A) and mutant (B) have similar appearance of Foxd1-positive stroma (green) and Six2-positive nephrogenic cap mesenchyme (red), surrounding unstained ureteric epithelium (arrowheads). (C and D) Hematoxylin and eosin-stained sections deeper within the cortex show similar appearance of control (C) and mutant (D) developing nephrons (arrows) at the S-shaped stage adjacent to ureteric epithelium (arrowheads). Scale bars (A and B) = 100 μm. Scale bars (C and D) = 50 μm.

Table 2. Primers used for genotyping

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Pairs</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax3cre&lt;sup&gt;Tg&lt;/sup&gt;</td>
<td>5′- AATCTTATGGCTACCTCGAGTGTAAATGCTCAATTGC-3′ 5′- CATCTTCAGGTCTGCGGG-3′</td>
<td>230</td>
</tr>
<tr>
<td>Fgf1&lt;sup&gt;1lox&lt;/sup&gt;</td>
<td>5′- TGACACGCTGATACCACACACACCAC-3′ 5′- AAGGCCACCATACCTGAGGAA-3′</td>
<td>602 (wt)</td>
</tr>
<tr>
<td></td>
<td>5′- AAGCCCACTACCTGAGGAA-3′</td>
<td>402 (Lox)</td>
</tr>
<tr>
<td>Fgf2&lt;sup&gt;2LR&lt;/sup&gt;</td>
<td>5′- GAGTACATCTGCTGAATGTACG-3′ 5′- GGAGAGGCATCTCTGTTTCAAGACC-3′</td>
<td>225 (wt)</td>
</tr>
<tr>
<td></td>
<td>5′- GGAGAGGCATCTCTGTTTCAAGACC-3′</td>
<td>315 (LR)</td>
</tr>
</tbody>
</table>

2 domain-containing adapter protein B, are probably critical for early MM patterning. There also may be overlapping and additive actions of different downstream signaling pathways. For example, an allelic series of point mutations in downstream adapter sites in platelet-derived growth factor-β receptor revealed progressively more severe defects in vascular smooth muscle development with an increasing number of point mutations per receptor. Thus, mutations in other adapter sites in addition to Fgf2<sup>2LR</sup> mutations could lead to defective MM development.

A new role for Fgfr/Frs2α signaling in the MM shown in Fgf1<sup>1Mes–/−/Fgf2<sup>2LR/LR</sup> mice is to regulate collecting duct planar cell polarity/oriented cell division, which is defective in other genetic models of renal cystic disease. The defects in Fgf1<sup>1Mes–/−/Fgf2<sup>2LR/LR</sup> collecting duct oriented cell division appear to occur before any change in proliferation. In lens explants, Fgf-induced fiber cell differentiation appears to be driven in part by alterations in expression of planar cell polarity signaling molecules. Perhaps in a similar manner, Fgf signaling could be required to regulate these planar cell polarity molecules in the developing kidney. Alternatively, a recent paper described that in developing lung airway, increases in Erk activity led to alterations in mitotic spindle orientation; thus, the alterations in Erk activation in Fgf1<sup>1Mes–/−/Fgf2<sup>2LR/LR</sup> ureteric epithelium may be driving the defects in oriented cell division.

Another new role for Fgfr/Frs2α signaling revealed in Fgf1<sup>1Mes–/−/Fgf2<sup>2LR/LR</sup> mice is to regulate ureteric bud morphogenesis by controlling molecular pathways that non-cell autonomously limit Ret. Although E13.5 stromal and nephrogenic mesenchymal patterning is normal, ureteric morphogenesis is perturbed, including dilated, hyperproliferative ureteric tips leading to decreased branching and fewer developing nephrons. Although the Fgf2<sup>2LR</sup> mutant is global (in both ureteric bud and MM), the effects seen in Fgf1<sup>1Mes–/−/Fgf2<sup>2LR/LR</sup> mice are probably due to Fgfr2 MM signaling in the MM shown in Fgf1<sup>1Mes–/−/Fgf2<sup>2LR/LR</sup> mice is to regulate ureteric bud morphogenesis of an Erk inhibitor restricted cyst growth of progressive cystogenesis; furthermore, in the inv and pcy mouse model of juvenile nephronophthisis, administration of an Erk inhibitor restricted cyst growth.

The increased/ectopic expression and activity of Ret and its downstream targets seen in Fgf1<sup>1Mes–/−/Fgf2<sup>2LR/LR</sup> mice are probably a major driving force behind the ureteric branching defects and cystic dysplasia. Excessive Erk activation has been identified in patients with and in models of progressive cystogenesis; furthermore, in the inv and pcy mouse model of juvenile nephronophthisis, administration of an Erk inhibitor restricted cyst growth.

The increased/ectopic expression and activity of Ret and its downstream targets seen in Fgf1<sup>1Mes–/−/Fgf2<sup>2LR/LR</sup> mice are probably a major driving force behind the ureteric branching defects and cystic dysplasia. Excessive Erk activation has been identified in patients with and in models of progressive cystogenesis; furthermore, in the inv and pcy mouse model of juvenile nephronophthisis, administration of an Erk inhibitor restricted cyst growth.
and improved kidney function. Transgenic mice with the Hoxb7 promoter driving ectopic expression of Ret throughout the ureteric trunk display variable degrees of renal cystic dysplasia, as well as decreased branching. Although the renal phenotype is more severe in these transgenic mice than in Fgfr1Mes−/− Fgfr2LR/LR mutants, the former have ectopic Ret expression throughout the entire ureteric bud, whereas the latter have ectopic Ret expression in more peripheral trunk regions. The ectopic and increased expression of the Etv4/5 target genes Cxcr4 and Met in our Fgfr1Mes−/− Fgfr2LR/LR mutants could also be affecting ureteric bud morphogenesis because they are expressed in ureteric bud tips. In addition, Fgfr1Mes−/− Fgfr2LR/LR mutants have increased expression of the Etv4/5 target, Myh, which promotes proliferation and could be exacerbating the cystogenesis. Thus, Ret overexpression in Fgfr1Mes−/− Fgfr2LR/LR mice may be driving increased ureteric bud proliferation, ureteric branching defects, and cystogenesis.

Bmp4 appears to be a target of Fgfr/Frs2α signaling in kidney mesenchyme leading to both the ureteric induction and ureteric branching defects, the latter through nonautonomous actions on Ret. Fgfr2 promotes expression of Bmp4 in developing eyelids, and Bmp4 represses Ret expression in melanocytes. In the current study, we detected decreased Bmp4 expression in Fgfr1Mes−/− Fgfr2LR/LR E10.5 urogenital ridges and E13.5 kidneys to levels equivalent to those reported in Bmp+/− mice. Moreover, Miyazaki and colleagues showed that Bmp4+/− mice develop ureteric induction defects (including duplex ureters), ureteric branching defects, and cystogenesis, similar to Fgfr1Mes−/− Fgfr2LR/LR mice. Whereas in two later papers Bertram and colleagues’ group reported that Bmp4+/− mice had no significant ureteric branching defects or reduced nephron numbers, they excluded mice with macroscopically abnormal kidneys in their analysis (meaning that at least a subset of mice had abnormal kidneys). Differences in mouse strain may explain discrepancies between what Bertram and associates’ group found and what Miyazaki and our laboratory observed. It is also possible, however, that other genetic alterations in Fgfr1Mes−/− Fgfr2LR/LR mice contribute to the abnormalities. Gdnf and Sprouty1, candidate molecules that have known effects on ureteric morphogenesis and Ret levels, were normal. The reduction of Bmp4 expression in Fgfr1Mes−/− Fgfr2LR/LR MM appears to act on the ureteric epithelium through noncanonical pathways. Although no changes in phospho-Smad 1/5/8 levels were seen on immunostaining, we did note a reduction in phospho-p38, a readout of noncanonical Bmp signaling (Figure 11). Moreover, Bmp-signaling through phospho-p38 is required for normal kidney development, including regulation of ureteric bud morphogenesis and for restricting proliferation in ureteric bud cells. Taken together, we propose that Fgfr/Frs2α signaling in MM promotes Bmp4 expression that represses Ret expression nonautonomously through noncanonical pathways, thus ensuring proper Ret levels to prevent hyperproliferative ureteric tips, ureteric branching defects, and cystogenesis.
In summary, Fgfr1<sup>Mes</sup><sup>−/−</sup> Fgfr2<sup>L/LR</sup> mice have ureteric induction abnormalities similar to those in Fgfr1<sup>−/−</sup> Mes<sup>−/−</sup> mice, revealing critical roles of Fgfr2/Frs2<sup>a</sup> signaling in this process. Fgfr1<sup>Mes</sup><sup>−/−</sup> Fgfr2<sup>L/LR</sup> mice have no early MM defects, showing that Fgfr2/Frs2<sup>a</sup> signaling is dispensable for early kidney formation. The defects in ureteric bud branching in Fgfr1<sup>Mes</sup><sup>−/−</sup> Fgfr2<sup>L/LR</sup> mice are probably due to increased and ectopic Ret expression/activity from reduced Bmp4 expression. This in turn leads to renal cystic dysplasia associated with increased ureteric bud tip proliferation, abnormal ureteric bud branching, and...
aberrant oriented division of collecting duct cells. Therefore, the MM Fgfr/Frs2α signaling axis appears to oppose Fgfr and Frs2α in the ureteric bud to ensure proper Ret expression and gene dosage in the ureteric epithelium.

CONCISE METHODS

Mouse Model
The transgenic Pax3creTg/+ mouse line49 has been shown to drive deletion of Fgfr1 in the MM.13 Initially, Fgfr1Lox/Lox mice46 were bred with Fgfr2LR/LR mice, which have point mutations converting amino acids Leu-424 (L) and amino acid Arg-426 (R) to Ala residues, which prevents Frs2α association with Fgfr2.21,51 Fgfr1Lox/LoxFgfr22R/LR mice were then bred with Pax3creTg/+ mice to generate Pax3creTg/+Fgfr1Lox/+Fgfr22R/LR progeny. These mice were bred back with Fgfr1Lox/LoxFgfr22R/2R to produce Pax3creTg/+Fgfr1Lox/+Fgfr22R/2R (Fgfr2Mm−/−Fgfr22R/2R) mice. Cre-negative littermates were used as controls. All animals were housed in the vivarium at the Rangos Research Center at the Children’s Hospital of Pittsburgh of UPMC, Pittsburgh, Pennsylvania.

Genotyping
Genotyping was performed via PCR. Briefly, tail clippings or embryonic tissues were collected and digested and genomic DNA was isolated. PCR amplification was used to identify various mutant and control alleles. Table 2 lists the primers and fragment sizes for the various genes.

Tissue Collection
For histologic assays in tissue sections, embryos or kidneys were removed, placed into 4% paraformaldehyde (PFA) in PBS overnight, transferred into 70% ethanol, and embedded in paraffin for sectioning. Tissue sections were then subjected to hematoxylin and eosin staining or in situ hybridization. For whole-mount in situ hybridization or immunofluorescence, embryos were removed and placed into 4% PFA in PBS overnight, dehydrated through to 100% methanol, and kept at −20°C. For frozen sections, tissue was fixed in 4% PFA and then dehydrated in sucrose and embedded in optimal cutting temperature medium. Thirty-micrometer sections were cut on a cryostat and stored at −20°C. For qPCR, kidneys were snap-frozen for RNA extraction.

3D Reconstruction and Analysis
3D reconstructions of E13.5 kidney capsules, developing nephrons, ureteric trees, ureters, ductus deferens, and urogenital sinuses (future bladders) were performed as described elsewhere, with only slight modifications for the ureteric skeletonization.19,52 Briefly, image layers were generated from serial hematoxylin and eosin–stained sections by tracing around capsules, developing nephrons by stage (vesicles, comma-shaped bodies, S-shaped bodies, and immature glomeruli), ureteric tissues, ureters, ductus deferens, and urogenital sinuses (Stereoinvestigator, Microbrightfield, VT). The traced layers were then aligned into a stack, rendering a 3D image that could be manipulated to show any of the traced structures (either separately or simultaneously). Nephron numbers were determined from 3D images. Movies and still images of the 3D structures were generated.

Using Neurolucida Explorer (MBF), volume and surface area were calculated for the entire kidney, the nephrons, and the ureteric epithelium. From this, relative volumes of ureteric epithelium (ureteric volume/kidney volume) were determined. Cross-sectional diameters were determined in 10 different branches (ureteric segments between two branch points) and 10 different tips (ureteric segments from the last branch point to the outer cortex) from each traced kidney. In addition, relative volume, surface, and a shape index (inverse of volume versus inverse of surface area) were calculated for the developing nephrons.

To skeletonize the ureteric tree, individual layers from Stereo-investigator were exported into Microsoft Paint (Redmond, WA), and tracings were filled in. Images were then imported into Imaris software (Bitplane, Zurich, Switzerland) and a 3D skeleton of the ureteric tree was generated. The total ureteric length and the number of branch points and tips were calculated. Movies and still images of the skeletonized ureteric trees were also generated.

In Situ Hybridization
Section in situ hybridization was performed on E10.5 urogenital ridges or E13.5 Fgfr1Mm−/−Fgfr22R/2R and control embryos (sectioned at 7–8 μm) as described elsewhere.13,21 Digoxigenin-labeled antisense and sense RNA probes were created for Ret, Bmp4, Erv4, Erv5, Cxcr4, Met, and Mysf from templates as described elsewhere.72

To determine the length of the common nephric duct (hence the position of the induction site), and to determine whether there was a delay in ureteric branching, Ret whole-mount in situ hybridization was performed on E11.0 and E11.5, respectively, on urogenital blocks of Fgfr1Mm−/−Fgfr22R/2R and control embryos as described elsewhere.12

Immunohistochemistry
Kisneous mice were fixed in 4% PFA at 4°C and paraffin embedded. Immunohistochemistry was performed using 8-μm sections, which were dewaxed and sequentially treated with 3% H2O2, heated in a pressure cooker in 0.1 M sodium citrate/citric acid buffer (pH, 6.0), and blocked in a 30% BSA/Donkey serum solution and incubated with primary antibodies overnight at 4°C. Sections were incubated with antirabbit secondary (Vectastain, Vector Labs, Burlingame, CA), followed by avidin-biotin complex (Vector Labs), and visualized with 3-amino-9-ethylcarbazole (Invitrogen, Carlsbad, CA). Primary antibodies included anti–phospho-Smad1/5/8 (1:150, Cell Signaling, Danvers, MA), anti–phospho-Erk1/2 (1:200, Cell Signaling), and anti–phospho-p38 mitogen-activated protein kinase (1:200, Cell Signaling).

Assessment of Oriented Cell Division
Thirty-micrometer frozen sections from E18.5 kidneys were fixed at room temperature for 1 hour and stained with anti–phospho-histone H3 (Ser10) antibody (1:500; Abcam, Cambridge, MA) to identify separating chromosomes in mitotic cells and fluorescently conjugated Dolichos biflorus (DBA) lectin (1:250; Vector Labs) to identify collecting duct cells. A goat antirabbit Alexa Fluor-594 (Invitrogen) secondary antibody was used to label the anti–phospho-histone H3.
Sections were then visualized with an Olympus confocal microscope (Center Valley, PA), and Imaris software was used to measure the mitotic angles by calculating the angle between the separating chromosomes and the longitudinal axis of the collecting duct. Care was taken to assess mitotic angles in mutants where the collecting ducts had no significant dilatation. One hundred mitotic cells were counted among three kidneys in both the control and the mutant groups.

**Whole-Mount Immunofluorescence**

E13.5 kidneys were rehydrated through graded methanol to 0.1% Tween in PBS. After blocking in 10% donkey serum for 1 hour at room temperature, kidneys were incubated with anti-Foxd1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Six2 (Proteintech, Chicago, IL) primary antibodies at 4°C overnight. The kidneys were then washed extensively in 0.1% Tween in PBS and subsequently incubated with donkey antigoat Alexa Fluor-488 (Invitrogen, Carlsbad, CA) and goat antirabbit Alexa Fluor-594 (Invitrogen). The kidneys were then extensively washed, mounted, and visualized with an Olympus confocal microscope.

To determine whether there was a delay in early ureteric branching, E11.25 urogenital blocks were dissected and subjected to whole-mount immunofluorescence with anti-CalbindinD28k antisera (Sigma, St. Louis, MO) as above. The early ureteric bud branch pattern was then observed and photographed under a fluorescence microscope.

**qPCR**

We performed qPCR as described elsewhere. Briefly, mRNA was extracted from snap-frozen E13.5 Fgfr1Mes−/− Fgfr2LR/LR and control kidneys (n=3 per group) (Qiagen, Valencia, CA). Primers for Ret, Bmp4, Gdnf, Sprouty1, Etv4, Cxcr4, Myb, and Met were used, with Gapdh as an endogenous control (Invitrogen, Carlsbad, CA). Applied Biosystems ABI 7900 HT (Foster City, CA) was used for qPCR.

**Apoptosis Assays**

Terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick-end labeling assays on E13.5 Fgfr1Mes−/− Fgfr2LR/LR and control tissue sections (n=3 per group), using a Fluorescent FragEl DNA Fragmentation Detection kit (Oncogene, Cambridge, MA) as described elsewhere.

**Proliferation Assays**

A PCNA kit was used (Invitrogen) at E13.5. In brief, paraffin sections from E13.5 control and Fgfr1Mes−/− Fgfr2LR/LR (n=3 per group) kidneys were dewaxed and endogenous peroxidases were blocked using 3% H2O2; heat-induced antigen retrieval was then carried out using a citrate-based buffer. After cooling to room temperature, tissues were washed and incubated with blocking solution. Tissues were then incubated with biotinylated monoclonal anti-PCNA primary antibody for 1 hour at room temperature. After extensive washing, sections were incubated with streptavidin peroxidases. After washing, tissues were incubated with 3,3′ diaminobenzidine chromogen to form a brown precipitate; slides were counterstained with hematoxylin. In three separate tissue levels (approximately 100 μm apart), numbers of PCNA-positive (brown) and -negative (blue) cells were tabulated in all tips and branches from each mutant and control embryonic kidney. From this, proliferation rate was determined by dividing the number of PCNA-positive cells by the total number of cells.

To assess proliferation rates in E18.5 nondilating collecting ducts in Fgfr1Mes−/− Fgfr2LR/LR (and controls), we counted the number of phospho-histone H3 cells over the total number of DBA-positive cells per 20× fields (see section on Assessment of oriented cell division). For Fgfr1Mes−/− Fgfr2LR/LR mice, we assessed 2907 DBA-positive cells (excluding dilated regions) over 15 fields and for controls, we assessed 4138 DBA-positive cells over 15 fields. The number of phospho-histone H3 cells was represented as a percentage of total DBA-positive cells.

**Statistical Analyses**

Statistical analyses were carried out upon all biologic replicates with a t test or a one-way ANOVA, followed by Tukey post hoc tests. All values are represented as mean ± SD. For the oriented cell division assays, statistical analysis was performed using a Mann–Whitney U test to determine whether the distribution of two sample sets was significantly different. P<0.05 was considered to represent a statistically significant difference.

**ACKNOWLEDGMENTS**

The authors thank Dr. Janet Rossant for the use of the floxed Fgfr1 mouse line.

This study was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK070030 (C.M.B.) and National Institutes of Health Program Grant P50 AR054086 (V.P.E.).

**REFERENCES**

7. Nguyen HQ, Danilenko DM, Bucay N, DeRose ML, Van Gy, Thomason A, Simonet WS: Expression of keratinocyte growth factor in embryonic liver of transgenic mice causes changes in epithelial growth and
47. Piscione TD, Phan T, Rosenblum ND: BMP7 controls collecting tu-
bule cell proliferation and apoptosis via Smad1-dependent and
-independent pathways. Am J Physiol Renal Physiol 280: F19–F33,
2001
Carroll TJ, Fässler R, Pozzi A, Rosenblum ND, Zent R: Integrin-linked
kinase regulates p38 MAPK-dependent cell cycle arrest in ureteric bud
49. Li J, Chen F, Epstein JA: Neural crest expression of Cre recombinase
directed by the proximal Pax3 promoter in transgenic mice. Genesis
26: 162–164, 2000
50. Hébert JM, Lin M, Partanen J, Rossant J, McConnell SK: FGF signal-
ing through FGFR1 is required for olfactory bulb morphogenesis. Devel-
opment 130: 1101–1111, 2003
DJ, Lax I, Schlessinger J: Attenuation of signaling pathways stimulated
by pathologically activated FGF-receptor 2 mutants prevents cranio-
52. Sims-Lucas S, Argyropoulos C, Kish K, McHugh K, Bertram JF, Quigley R,
Bates CM: Three-dimensional imaging reveals ureteric and mesenchymal
53. Sims-Lucas S, Cusack B, Baust J, Eswarakumar VP, Masatoshi H,
Takeuchi A, Bates CM: Fgfr1 and the IIIc isoform of Fgfr2 play critical
roles in the metanephric mesenchyme mediating early inductive events
54. Manns M, Sigler MH, Teehan BP: Continuous renal replacement thera-

This article contains supplemental material online at http://jasn.asnjournals. org/lookup/suppl/doi:10.1681/ASN.2011020165/-/DCSupplemental.