Deletion of Frs2α from the ureteric epithelium causes renal hypoplasia

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Deletion of Frs2α from the ureteric epithelium causes renal hypoplasia. Am J Physiol Renal Physiol 297: F1208–F1219, 2009. First published September 9, 2009; doi:10.1152/ajprenal.00262.2009.—Fibroblast growth factor receptor 2 (Fgfr2) signaling is critical in maintaining ureteric branching architecture and mesenchymal stromal morphogenesis in the kidney. Fibroblast growth factor receptor substrate 2α (Frs2α) is a major docking protein for Fgfr2 with downstream targets including Ets variant (Etv) 4 and Etv5 in other systems. Furthermore, global deletion of Frs2α causes early embryonic lethality. The purpose of the study was to determine the role of Frs2α in mediating Fgfr2 signaling in the ureteric epithelium. To that end, we generated mice with conditional deletion of Frs2α in the ureteric epithelium (Frs2αUB−/−) and mice with point mutations in the Frs2α binding site of Fgfr2 (Fgfr22αβLR). Frs2αUB−/− mice developed mild renal hypoplasia characterized by decreased ureteric branching morphogenesis but maintained normal overall branching architecture and had normal mesenchymal stromal development. Reduced nephron endowment in postnatal mutant mice was observed, corresponding with the reduction in branching morphogenesis. Furthermore, there were no apparent renal abnormalities in Fgfr22αβLR mice. Interestingly, Etv4 and Etv5 expression was unaltered in Frs2αUB−/− mice, as was Sprouty1, an antagonist of Frs2α signaling. However, Ret and Wnt11 (molecules critical for ureteric branching morphogenesis) mRNA levels were lower in mutants vs. controls. Taken together, these findings suggest that Fgfr2 signals through adapter molecules other than Frs2α in the ureteric epithelium. Furthermore, Frs2α may transmit signals through other receptor kinases present in ureteric epithelium. Finally, the renal hypoplasia observed in Frs2αUB−/− mice is likely secondary to decreased Ret and Wnt11 expression.

fibroblast growth factor receptor substrate 2; fibroblast growth factor receptor 2; kidney development; branching morphogenesis; conditional knockout

METANEPHRIC KIDNEY DEVELOPMENT occurs via reciprocal signaling between two tissues derived from intermediate mesoderm, the ureteric bud and the metanephric mesenchyme. The metanephric mesenchyme, paired densities of tissue in the region of the hindlimb, sends signals causing the ureteric bud to evacuate from the Wolffian (nephric) duct. After the ureteric bud invades the metanephric mesenchyme, the mesenchyme stimulates the ureteric epithelium to branch and elongate, forming the collecting duct system of the kidney (6, 7, 31, 39). Simultaneously, ureteric tips send signals causing the mesenchyme to condense and then convert into epithelial nephrons. In addition to the nephrogenic mesenchyme, a separate mesenchymal lineage, called the stromal mesenchyme, appears early in kidney development, ultimately forming interstitial tissues and macrophages.

While there are many molecular pathways important to kidney development, signaling through fibroblast growth factor receptors (Fgfrs) is critical for many different steps of kidney development. Fibroblast growth factor receptors are receptor tyrosine kinases, with 4 known signaling family members and 23 ligands in mammals. Many studies, including genetic manipulation of Fgf and/or Fgfr expression, have shown the importance of this signaling family in ureteric bud induction and branching, nephrogenesis, and stromal development (2, 3, 15, 25, 29, 30, 32, 33, 36, 37, 47). Due to early embryonic lethality of global deletion in some ligands and receptors, conditional knockout approaches are often necessary to interrogate the roles of these genes in kidney development. Our laboratory used this approach in revealing the importance of Fgfr2 signaling in the ureteric bud. We found that Fgfr2 deletion in the ureteric epithelium (Fgfr2UB−/−) leads to profound defects in ureteric branching morphogenesis, including long, thin trunks and a dramatic reduction in ureteric bud tips (leading to a decrease in nephron number). In addition, Fgfr2UB−/− mice had stromal mispattering, including thickened cortical stroma (as evidenced by expanded FoxD1 expression) and an absence of interdigitating fingers between developing nephron units. Ultimately, these defects resulted in a dramatic reduction in kidney size and abnormally shaped kidneys (47).

Fgfrs transmit intracellular signals by phosphorylating and activating docking proteins, including fibroblast growth factor receptor substrate 2α (Frs2α). Frs2α activation leads to a wide array of downstream signaling cascades including phosphatidylinositol-3 (PI-3), mitogen activated protein (MAP) kinase Erk, and alternative forms of protein kinase C (PKC) (11, 12, 14, 16, 26, 27). Two farther downstream targets of Frs2α via Erk activation include two members of the Ets transcription factor family, Ets variant (Etv) 4 and Etv5 (42).

Although Frs2α was identified through its interactions with Fgfrs, Frs2α also acts as a docking protein for several other tyrosine kinases, including Ret, multiple neurotrophin recep-
tors (Trks), and anaplastic lymphoma kinase (Alk) (20, 21, 24). Not surprisingly, given its actions as an adapter molecule for so many receptors and its links to many downstream signaling cascades, global deletion of Frs2α in mice is lethal early in embryogenesis, before kidney development (16). To circumvent the early lethality of the global knockout, we generated a floxed Frs2α mouse line that in the presence of cre recombinase deletes the largest of the coding exons (exon 5) (22). The floxed line produced effective deletion of Frs2α and its downstream targets in both the heart (22) and prostate (46).

Based on the critical role of Frs2α in Fgfr signaling (which is essential in the ureteric bud lineage), we sought to clarify the role of this molecule in the ureteric bud of the developing kidney. To that end, we generated conditional knockouts of Frs2α in the ureteric epithelium using our Hoxb7cre line (47). To complement this approach, we examined potential renal defects in mice with point mutations in the Frs2α binding site of Fgfr2.

METHODS

Mouse Strains

All experiments involving mice were approved by the local Institutional Animal Care and Use Committee.

Frs2αUB+/− conditional knockout mice. The mice used to generate the conditional knockout line have been described previously. Briefly, transgenic mice with a portion of the Hoxb7 promoter driving cre recombinase and enhanced green fluorescent protein expression (Hoxb7creEgfpTaq1) (47) were crossed with Frs2αlox/lox mice (22). Progeny hemizygous for cre and with 2 lox-p sites had functional deletion of Frs2α (Hoxb7creEgfpTaq1/Frs2αUB+/−) from the ureteric epithelium (Frs2αUB+/−). Timed overnight matings were set up between the various genotypes; when a vaginal plug was identified, noon of that day would be deemed embryonic day 0.5 (E0.5).

Fgfr2LR/LR mice. The insertion of two point mutations into the Frs2α binding site of Fgfr2 has been previously described (9). In brief, amino acid Leu-424 and amino acid Arg-426 were replaced with Ala residues. These point mutations were shown to block the affinity of Frs2α to bind to Fgfr2 (9).

Genotyping

Genotyping was performed via PCR. Briefly, Tail clippings and or embryonic tissues were collected and digested and genomic DNA was isolated. PCR amplification was used to identify the various mutant and control alleles. To determine whether mice were carrying the HoxCreEgfp cassette, the forward primer 5′-AGCGCGATCACATGAGG-3′ and reverse primer 5′-GGCACGAGTGTCTGCAGACACATG-3′ were utilized, with a wild-type band that was evident at 224 and a mutant band at 230 bp. To determine the genotype of the Frs2α floxed mice, the forward primer 5′-GGAGAGGCATCTCTGTTTCAAGACC-3′ and reverse primer 5′-ACGTGTTCTGTGATTGGGAAGGCAG-3′ were utilized. In both cases, a single band at 230 bp was expected. To determine the genotype of the Frs2αUB+/− mice, the forward primer 5′-GGACACAGGTGTCTCAGACACTG-3′ and reverse primer 5′-GGACACAGGTGTCTCAGACACTG-3′ were utilized, with a wild-type band that was evident at 224 and a mutant band at 319 bp. To determine the genotype of the Fgfr2LR/LR mice, the forward primer 5′-GGAGAGGCATCTCTGTTTCAAGACC-3′ and the reverse primer 5′-GGAGAGGCATCTCTGTTTCAAGACC-3′ were utilized.

Fig. 1. Fibroblast growth factor receptor substrate 2α (Frs2α) expression by in situ hybridization (ISH) in the developing kidney. A and B: at embryonic day 11.5 (E11.5), whole-mount ISH with sense (A) and antisense (B) probes reveal Frs2α staining in the nephric duct (arrowhead) and the ureteric bud (arrow). Scale bar = 100 μm. C and D: following whole-mount staining in E11.5 tissue sections, the antisense probe shows Frs2α expression in the nephric duct (C, arrowhead) and ureteric trunk (C, arrow) and a lower level signal in ureteric tips (D, arrowheads); labeling is also seen in mesenchyme surrounding the tips (D, arrow). Scale bar = 100 μm. E: at E13.5, whole-mount ISH in control (left) and Frs2αUB+/− (right) kidneys reveals reduced level of expression in the mutant. Scale bar = 200 μm. F and G: following whole-mount staining, E13.5 tissue sections from control (F) and Frs2αUB+/− kidneys (G) demonstrate strong Frs2α expression in control ureteric tips (F, arrowheads) and trunk (arrow), but background levels of signal in Frs2αUB+/− ureteric tips (G, arrowheads) and trunk (arrow). Some mesenchymal Frs2α expression is present in both controls and mutants (concave arrowheads). Scale bars = 25 μm.
used, with a wild-type band that was evident at 225 and a mutant band at 315 bp.

Organ Culture

At E11.5, pregnant mice that would generate Frs2α^L/H9251 and Fgfr2^L/H11002^L/H11002 progeny (and littermate controls) were killed and embryos were quickly removed. Kidneys were dissected from embryos and placed onto nucleopore filters floating on Dulbecco’s modified Eagle medium supplemented with 10% FBS, penicillin/streptomycin, and l-glutamine at 37°C. Following a 3-day culture period, kidneys were submerged in ice-cold methanol for a minimum of 15 min before whole-mount immunohistochemistry for calbindin D28K as previously described (40). Briefly, kidneys were washed in PBS, permeabilized with 0.3% Triton X-100 in PBS, and then washed again before being blocked in 5% FBS. Kidneys were then incubated with 1:100 monoclonal mouse anti-calbindin-D28k antisera in 5% FBS for 3 h at 37°C before being extensively washed in PBS. Kidneys were treated with 1:500 goat anti-mouse Alexa Fluor 488 for 2 h at 37°C. Kidneys were

Fig. 2. Frs2α is deleted from the ureteric epithelium by dual-labeling immunofluorescence. A–C: in E13.5 control kidney sections, anti-TP3 (showing cell nuclei; A) and anti-Frs2α (B) reveal Frs2α staining in ureteric tips on the merged image (C, arrowheads). D–F: in E13.5 Frs2α^L/H9251 renal sections, anti-TP3 (D) and anti-Frs2α (E) demonstrate a lack of Frs2α expression in ureteric tips on the merged image (F, arrowheads). In both controls and mutants, Frs2α is expressed in surrounding mesenchymal tissues including condensing mesenchyme (CM). Scale bars = 50 μm.

Fig. 3. E17.5 Frs2α^L/H9251 kidneys are smaller than controls. A and B: images of cre-negative (A) and Frs2α^L/H9251 (B) kidneys reveal that the mutants appear smaller. Scale bars = 500 μm. C–E: bar graphs showing that Frs2α^L/H9251 kidneys have reduced weight (C), volume (D), and surface area (E) compared with littermate cre-negative and Frs2α^L/H9251 kidneys. *P < 0.01. **P < 0.001. †P < 0.0001.
washed in PBS and mounted. The ureteric bud branch pattern and numbers of ureteric tips were then observed under a fluorescent microscope (Leica, Bannockburn, IL).

Frs2/H9251 Immunohistochemistry

Frs2α immunostaining was performed as previously described (45). Briefly, 7-μm paraffin sections in Frs2αUB−/− and control littermates were cut, and antigen retrieval was performed using citrate buffer. Sections were incubated with anti-Frs2/H9251 antisera (Santa Cruz, CA), the primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA), and the nuclei were visualized using TP3.

In Situ Hybridization

Whole-mount nonradioactive in situ hybridization (ISH) was performed on E11.5 on E13.5 Frs2αUB−/− and control embryos and/or dissected kidneys as described previously (17). Digoxigenin UTP-labeled antisense and sense RNA probes were generated against multiple targets, including Frs2α. [GenBank accession no. BC055334, Etv4 (NM008815), Env5 (NM023794), FoxD1 (L38607), Gdnf (NM010275), Ret (BC059012), Sprouty1 (NM011896), and Wnt11 (NM009519)]. Select whole-mount in situ hybridization-stained tissues were reembedded in paraffin and sectioned at 10 μm. In addition, radioactive section ISH was performed on E13.5 Frs2αUB−/− and control embryos with probes against Frs2α as described (34, 47).

Real-Time PCR

Four mutant and four control E13.5 embryos were collected, and the kidneys were harvested. RNA was then extracted using a kit (Qiagen, Valencia, CA). Probes for Etv4, Etv5, Gdnf, Ret, Sprouty1, and Wnt11 were utilized, and Gapdh was used as an endogenous control (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed.

Table 1. Measurements of Frs2αUB−/− embryos and kidneys at E17.5

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body Wt, g</th>
<th>Crown Rump Length, cm</th>
<th>Kidney Wt, mg</th>
<th>Long Axis, cm</th>
<th>Surface Area, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre negative (n = 46)</td>
<td>1.11±0.10</td>
<td>2.17±0.08</td>
<td>5.10±0.67</td>
<td>0.281±0.019</td>
<td>0.042±0.005</td>
</tr>
<tr>
<td>Frs2αUB−/− (n = 12)</td>
<td>1.09±0.05</td>
<td>2.18±0.04</td>
<td>4.97±0.63</td>
<td>0.283±0.017</td>
<td>0.043±0.005</td>
</tr>
<tr>
<td>Frs2αUB−/− (n = 14)</td>
<td>1.09±0.08</td>
<td>2.16±0.09</td>
<td>3.77±0.40†</td>
<td>0.261±0.013†</td>
<td>0.038±0.004‡§</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of animals. Frs2α, fibroblast growth factor receptor substrate 2α; E17.5, embryonic day 17.5. *P < 0.01 vs. cre negative. †P < 0.01 vs. Frs2αUB−/−. §P < 0.001 vs. Frs2αUB−/−.
performed using standard protocols and procedures (43) on an Applied Biosystems ABI 7900 HT (Foster City, CA).

Apoptosis Assay

We conducted terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays on E13.5 wild-type and Frs2αUB−/− kidney sections, using a Fluorescent FragEl DNA Fragmentation Detection kit (Oncogene, Cambridge, MA) as previously described (34).

Morphometric Measurements

E17.5 or postnatal day 30 (P30) Frs2αUB−/− and control mice were killed and weighed. Kidneys were removed, weighed, and photographed. The surface area and kidney long axis were measured using Image J analysis software (version 1.32j from Wayne Rasband, National Institutes of Health). In addition, P30 Frs2αUB−/− and littermate control kidneys (both n = 6) as well as Ffrs2UB+/+ and littermate wild-type controls (both n = 6) were harvested for cortical-medullary ratios. Briefly the kidneys were cut in the transverse midplane, processed through to paraffin, and embedded on the cut midplane, processed through to glycol-methacrylate. Kidney volume, glomerular number, and various glomerular volumes were then collected and tabulated using the physical dissector/fractionator method.

Statistical Analysis

Statistical analysis were carried out on all biological replicates with a Student’s t-test or a one-way ANOVA followed by Fisher’s post hoc tests. All values are represented as means ± SD.

RESULTS

Frs2α Expression in Control and Frs2αUB−/− Developing Kidneys

To determine the role of Frs2α in the ureteric bud, we first characterized expression in controls and then wanted to confirm its deletion from the ureteric epithelium in Frs2αUB−/− mice. At E11.5, Frs2α is expressed in the nephric duct and the ureteric bud trunk with a low-level signal at the ureteric tips; expression is also seen in the metanephric mesenchyme (Fig. 1, A–D). At E13.5, Frs2α appears strongly expressed throughout control kidneys by whole-mount staining, compared with a much lighter signal in Frs2αUB−/− kidneys (Fig. 1E). Sections of the E13.5 tissues reveal strong expression in control ureteric tips and trunk (Fig. 1F) and the nephrogenic zone (not shown). In contrast, there was no signal in the ureteric epithelium above background in Frs2αUB−/− E13.5 sections while expression was maintained in the nephrogenic zone (Fig. 1G). Similar expression patterns were observed when the protein was visualized by immunostaining in E13.5 mutants and controls (Fig. 2). While controls had expression in both early nephron structures (condensing mesenchyme) and ureteric tips (Fig. 2, A–C), Frs2αUB−/− kidneys showed Frs2α labeling in the nephrogenic zone only and no expression in the ureteric epithelium (Fig. 2, D–F). Thus at early stages of renal development, Frs2α is expressed in both metanephric mesenchyme and ureteric lineages. Furthermore, Frs2α is deleted in ureteric epithelium in Frs2αUB−/− kidneys.

Frs2αUB−/− Kidneys are Hypoplastic

We next characterized whether deletion of Frs2α from the ureteric bud led to any renal abnormalities. While cre-negative, heterozygous, and homozygous embryos appear similar at E17.5 (not shown), Frs2αUB−/− kidneys are smaller than cre-negative and Frs2αUB+/− kidneys (Fig. 3, A and B, and not shown). Image J measurements confirmed that

![Table 2. Measurements of Frs2αUB−/− embryos and kidneys at P30](http://ajprenal.physiology.org/)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Body Wt, g</th>
<th>Kidney Wt, g</th>
<th>Long Axis, cm</th>
<th>Surface Area, cm²</th>
<th>Cortex-to-Medulla Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>Female</td>
<td>16.2±1.6</td>
<td>0.11±0.01</td>
<td>0.83±0.03</td>
<td>0.37±0.03</td>
<td>1.85±0.24:1</td>
</tr>
<tr>
<td>Control (n = 6)</td>
<td>Male</td>
<td>19.5±1.3</td>
<td>0.14±0.02</td>
<td>0.91±0.05</td>
<td>0.44±0.04</td>
<td>1.93±0.19:1</td>
</tr>
<tr>
<td>Frs2αUB−/− (n = 7)</td>
<td>Male</td>
<td>15.3±1.1</td>
<td>0.09±0.01</td>
<td>0.78±0.03</td>
<td>0.32±0.02</td>
<td>1.77±0.35:1</td>
</tr>
<tr>
<td>Frs2αUB−/− (n = 9)</td>
<td>Female</td>
<td>16.1±1.5</td>
<td>0.10±0.01</td>
<td>0.82±0.05</td>
<td>0.34±0.04</td>
<td>1.81±0.22:1</td>
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</tbody>
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<tr>
<th>Genotype</th>
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<th>Body Wt, g</th>
<th>Kidney Wt, g</th>
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<td>Female</td>
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<td>1.93±0.19:1</td>
</tr>
<tr>
<td>Frs2αUB−/− (n = 7)</td>
<td>Female</td>
<td>15.3±1.1</td>
<td>0.09±0.01</td>
<td>0.78±0.03</td>
<td>0.32±0.02</td>
<td>1.77±0.35:1</td>
</tr>
<tr>
<td>Frs2αUB−/− (n = 9)</td>
<td>Male</td>
<td>16.1±1.5</td>
<td>0.10±0.01</td>
<td>0.82±0.05</td>
<td>0.34±0.04</td>
<td>1.81±0.22:1</td>
</tr>
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- **Body Weight**
  - Male control vs. male Frs2αUB−/−, P < 0.01
  - Female control vs. female Frs2αUB−/−, NS
  - Female Frs2αUB−/− vs. male Frs2αUB−/−, NS

- **Kidney Weight**
  - Male control vs. male Frs2αUB−/−, P < 0.001
  - Female control vs. female Frs2αUB−/−, NS
  - Female Frs2αUB−/− vs. male Frs2αUB−/−, NS

- **Long Axis**
  - Male control vs. male Frs2αUB−/−, P < 0.001
  - Female control vs. female Frs2αUB−/−, P < 0.001
  - Female Frs2αUB−/− vs. male Frs2αUB−/−, NS

- **Surface Area**
  - Male control vs. male Frs2αUB−/−, P < 0.001
  - Female control vs. female Frs2αUB−/−, P < 0.001
  - Female Frs2αUB−/− vs. male Frs2αUB−/−, NS

- **Cortex-to-Medulla Ratio**
  - All NS

Values are means ± SD for body wt, kidney wt, long axis, and surface area; n = no. of animals. P30, postnatal day 30; NS, not significant.
Table 3. Characterization of glomerular number and volume in P30 kidneys

<table>
<thead>
<tr>
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<th>Control Males (n = 6)</th>
<th>Frs2αUB−/− Males (n = 6)</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>18.53 ± 2.00</td>
<td>15.13 ± 0.92</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.133 ± 0.018</td>
<td>0.102 ± 0.013</td>
</tr>
<tr>
<td>Kidney volume, mm³</td>
<td>109.0 ± 8.0</td>
<td>87.7 ± 15.4*</td>
</tr>
<tr>
<td>Total glomerular number</td>
<td>1.0823 ± 1.094</td>
<td>8.387 ± 1.265†</td>
</tr>
<tr>
<td>Mean glomerular volume, ( \times 10^{-4} ) mm³</td>
<td>2.39 ± 0.33</td>
<td>2.50 ± 0.27</td>
</tr>
<tr>
<td>Total glomerular volume, mm³</td>
<td>2.58 ± 0.38</td>
<td>2.10 ± 0.38</td>
</tr>
<tr>
<td>Mean renal corpuscle volume, ( \times 10^{-4} ) mm³</td>
<td>2.91 ± 0.34</td>
<td>3.08 ± 0.25</td>
</tr>
<tr>
<td>Total corpuscle volume, mm³</td>
<td>3.14 ± 0.33</td>
<td>2.59 ± 0.46*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of mice. *P < 0.05, †P < 0.01.

E17.5 Frs2αUB−/− kidneys were significantly smaller than cre-negative and Frs2αUB+/− kidneys by weight, long axis, and surface area (Fig. 3, C–E). In contrast, there were no significant differences between embryo body weight or crown rump length (Table 1). Histological analysis of the mutant kidneys also revealed that the general architecture of the kidneys was unchanged across all three genotypes (Fig. 4 and not shown). Thus Frs2αUB−/− mice appear to have hypoplastic kidneys compared with both cre-negative and heterozygous mice. Given that the Frs2αUB−/+ and cre-negative kidneys were not different, we used them together as controls for the remaining studies.

Similar to the findings at E17.5, gender-matched P30 mutants and controls have comparable body weights, while Frs2αUB−/− kidneys were smaller as measured by weight, long axis, and surface area than gender-matched controls (Table 2 and not shown). Also, gross histological evaluation of these mutant kidneys revealed no phenotypic abnormalities (not shown), and there were no differences between mutant and control cortex-to-medulla ratios (Table 2 and not shown). Nephron number, as determined by the physical dissector fractionator method in a separate set of P30 mice, was reduced by 22% in mutants compared with controls (Table 3); however, the total and individual glomerular and glomerular capillary (corpuscle) volumes were not different between the groups. Thus, as in the embryos, Frs2αUB−/− kidneys appear hypoplastic. Interestingly, the reductions in kidney size of the Frs2αUB−/− mutants was less dramatic than in Fgfr2UB−/− mice (47).

To further determine whether the effects of deleting Frs2α from the ureteric epithelium were different than in Fgfr2UB−/− kidneys, we analyzed ureteric branching morphogenesis and stromal patterning in Frs2αUB−/− and controls. To determine whether there were any differences between mutants and controls at the early stages of ureteric development, we performed whole-mount in situ hybridization for Ret (to visualize the Wolfian duct and ureteric epithelium) at E11.5. As shown (Fig. 5, A and B), initial ureteric invasion and branching appear similar. To examine subsequent ureteric development, we cultured E11.5 mutants and controls for 3 days and performed whole-mount immunostaining against calbindin. As shown (Fig. 5, C–E) the mutant explants were smaller and had significantly fewer tips than controls. Interestingly, however, the overall pattern of ureteric branching in Frs2αUB−/− kidneys was very similar to controls. This is in contrast to Fgfr2UB−/− mice that had alterations in branch patterning, including long, think trunks and a more severe reduction in ureteric tip number (47).

We also assessed stromal mesenchymal patterning first by expression of Foxd1 in Frs2αUB−/− mutants and controls. As shown, at E13.5, Foxd1 has a comparable expression pattern in cortical stroma of control (Fig. 6, A and A’)

![Figure 5](http://ajprenal.physiology.org/)

*Fig. 5.* Ureteric branching morphogenesis is inhibited in E11.5 Frs2αUB−/− kidney explants. A and B: images of E11.5 whole-mount ISH for Ret show similar initial ureteric elongation and branching controls (A) and mutants (B). Scale bar = 100 μm; n = 6 in each group. C and D: images of whole-mount immunohistochemistry for calbindin-D28k show that controls (C) have more branching than mutants (D) after 3 days of growth, although the overall branch pattern in the mutants appear normal. Scale bar = 100 μm. E: bar graph revealing that mutants have a significant reduction in ureteric tips compared with controls (Frs2αUB−/− 47.6 ± 2.0, control 30.5 ± 2.8). Values are means ± SE. P < 0.0001; n = 17 (control) and n = 12 (mutant).
Frs2αUB−/− (Fig. 6, B and B’) kidneys, including interdigitating fingers (arrowheads). Also, TUNEL staining was similar in control and Frs2αUB−/− kidneys with few apoptotic cells in the developing medulla (Fig. 6, C and D). These findings are in stark contrast to Fgfr2UB−/− mice that develop aberrantly thickened cortical stroma with no interdigitating fingers and that demonstrate excessive cortical stromal apoptosis (47). Thus Frs2αUB−/− mice appear to have much milder renal abnormalities than Fgfr2UB−/− mice.

**Mice with Point Mutations in the Frs2α Binding Site of Fgfr2 have Phenotypically Normal Kidneys**

To further clarify the relationship between Fgfr2 and Frs2α in the ureteric bud (i.e., whether Frs2α mediates signals from Fgfr2), we generated mice with point mutations in the Frs2α binding site of Fgfr2 (that effectively abrogate interactions between the 2 molecules) (9). Using E11.5 organ culture, we observed no differences in ureteric branch morphogenesis between Fgfr2LR/LR mice (named for the amino acids changed to alanine), heterozygous (Fgfr2LR/%), and wild-type littermates (not shown). Ureteric tip counts were not statistically different among any of the genotypes (wild-type 45.15 ± 8.09, Fgfr2LR/− 47.00 ± 8.07, Fgfr2LR/LR 45.88 ± 6.06). At P30, we observed no overt differences between mutants and controls. Measurements in male mice confirmed no differences in body weights (wild-type 19.77 ± 3.32 g, Fgfr2LR/LR 19.55 ± 5.67 g); kidney weights (wild-type 0.144 ± 0.033 g, Fgfr2LR/LR 0.152 ± 0.053 g); kidney long axes (wild-type 0.872 ± 0.080 cm, Fgfr2LR/LR 0.884 ± 0.120 cm); or kidney surface areas (wild-type 0.410 ± 0.070, Fgfr2LR/LR 0.422 ± 0.115 cm²). Similarly, there were no differences between male Fgfr2LR/LR and wild-type renal cortex-to-medulla ratios (data not shown). Thus the renal abnormalities in the Frs2αUB−/− mice are not recapitulated in Fgfr2LR/LR mice.

**Downstream Targets Etv4 and Etv5 are Unaltered in Frs2αUB−/− Kidneys**

To further investigate the reasons for the abnormalities in Frs2αUB−/− kidneys, we examined the expression of two known downstream targets of Frs2α in other systems, namely, Etv4 and Etv5 in E13.5 kidneys. Surprisingly, we observed no differences in expression of Etv4 and Etv5 in Frs2αUB−/− kidneys compared with controls (Fig. 7). A robust Etv4 and Etv5 signal was present in ureteric tips in both mutants and controls (arrowheads). Etv4 and, to a lesser extent, Etv5 were also present in mesenchymal-derived tissues in both mutants and controls (arrows). Thus the renal defects seen in Frs2αUB−/− mice are not secondary to obvious changes in Etv4 or Etv5 expression.
Expression of Sprouty1, Gdnf, Ret, and Wnt11 in Frs2α⁻/⁻ Kidneys

Given that Sprouty1 is a known antagonist of Frs2α (and Fgfr2) signaling, we assayed for whether its overexpression could explain the hypoplastic phenotype seen in Frs2α⁻/⁻ kidneys. As shown, however, expression of Sprouty1 appears unaltered in mutants at E11.5 or E12.5 by in situ hybridization compared with controls (Fig. 8). Furthermore, real-time PCR measurements in whole kidneys at E12.5 confirmed no significant differences between mutants and controls (89% Sprouty1 mutant expression vs. controls, P = 0.294). Thus renal hypoplasia in Frs2α⁻/⁻ mice cannot be explained by an overexpression of Sprouty1.

Given the critical importance of Gdnf, Ret, and Wnt11 in ureteric branching morphogenesis, we next examined their expression in Frs2α⁻/⁻ and control mice at E12.5. As shown (Fig. 9), Gdnf expression appeared similar between mutants and controls by in situ hybridization; however, the Ret and Wnt11 signal appeared less robust in Frs2α⁻/⁻ compared with controls. Real-time PCR measurements confirmed that Ret and Wnt11 expression was decreased in the mutants (Ret, 47% compared with controls, P = 0.032; Wnt11, 63% compared with controls, P = 0.035). Real-time PCR measurements of Gdnf revealed a trend toward decreased expression in the mutants, although it did not reach statistical significance (63% compared with controls, P = 0.072).

Fig. 7. Etv4 and Etv5 expression in E13.5 Frs2α⁻/⁻ kidneys. A and A': lightfield (A) and dark field (A') images of control tissues stained for Etv5 and counterstained with hematoxylin showing normal renal tissue organization (arrows). B and B': lightfield (B) and darkfield (B') images of Frs2α⁻/⁻ section stained for Etv5 showing normal renal tissue organization (arrows). C and C': lightfield (C) and darkfield (C') images of control tissues stained for Etv4 and counterstained with hematoxylin showing expression of Etv4 in the ureteric tips (arrowheads) and the surrounding mesenchyme (arrows). D and D': lightfield (D) and darkfield (D') images of Frs2α⁻/⁻ section stained for Etv4 showing normal renal tissue organization (arrowheads) and the surrounding mesenchyme (arrows). Scale bars = 100 μm.
DISCUSSION

In this report, we observed that Frs2α is expressed in ureteric epithelium starting at E11.5 and that it is deleted from the ureteric lineage in Frs2αUB−/− mice. The consequence of the deletion is renal hypoplasia secondary to ureteric branching defects. Interestingly, the renal defects do not appear as severe as in Fgfr2UB−/− kidneys. Furthermore, mice with point mutations in the Frs2α binding site on Fgfr2 have no apparent renal defects. Expression of Ev4 and Ev5, downstream targets of Frs2α signaling in other systems, was unaltered in Frs2αUB−/− kidneys, as was Sprouty1, a known Frs2α antagonist. Conversely, expression of Ret and Wnt11 was clearly diminished in mutant kidneys and Gdnf trended toward decreased expression. These findings are discussed below.

Frs2α was known to be expressed in mesenchymal-derived tissues in the kidney at later stages of embryonic development (13). We now show that the docking protein is also expressed as early as E11.5 in ureteric lineages, strongly in the ureteric trunk, and to a lesser extent in ureteric tips. As renal development proceeds, ureteric expression becomes much more pronounced in the tips. Thus Frs2α has overlapping expression with two receptor tyrosine kinases that it associates with, namely, Fgfr2, which is present throughout the ureteric tree, and Ret, which is confined to ureteric tips after E12.5 (17, 28, 47). Finally, in the Frs2αUB−/− mice, Frs2α mRNA and protein appears absent in the ureteric lineage.

Given that Frs2α is a major docking protein for both Fgfr2 and Ret, we strongly suspected that deletion in the ureteric tree would lead to renal abnormalities. Indeed, Frs2αUB−/− mice do develop renal hypoplasia, including an undergrowth of nephrons secondary to a reduction in ureteric branching morphogenesis. Interestingly, however, deletion of this common receptor adapter molecule is milder than conditional ureteric deletion of Fgfr2 or global deletion of Ret, which leads to severe ureteric branching and stromal mispatterning and renal agenesis, respectively (38, 47). One possibility is that the Frs2αUB−/− mice are actually hypomorphic, thereby leading to less severe abnormalities. This is not likely, however, given that ureteric expression of Frs2α (both mRNA and protein) appears absent in the mutants. Also, the Hoxb7creEgfpr line used to generate the Frs2αUB−/− mice has been shown to delete other floxed lines well, including the Fgfr2Lcpl/Lcpl mice (5, 47). Another possibility is that there may be redundant signaling through other receptor tyrosine kinases in the ureteric bud, such as Fgfr1, that may damp the ureteric defects in Frs2αUB−/− mice. This also appears unlikely given that conditional deletion of Fgfr1 from the ureteric bud resulted in no renal abnormalities and that combined Fgfr1/Fgfr2 conditional targeting in the ureteric bud was no more severe than deleting Fgfr2 alone. The most likely explanation is that signaling through other docking proteins, such as phospholipase C_γ (PLC_γ) or Crk 2 in Fgfr2 or SHC or DOK family proteins in Ret, is critical in regulating receptor functions in the ureteric bud (19, 35). To further clarify the role of Frs2α signaling downstream of Fgfr2, we complemented the Frs2α conditional knockout by generating a mouse with point mutations in the Frs2α binding site on Fgfr2. A previous publication has shown that when amino acids Leu-424 and Arg-426 in Fgfr2 (LR) were replaced by Ala residues, Frs2α was unable to dock with Fgfr2, whereas signaling through other adapters such as PLC_γ was unaffected (9). Interestingly, the inability of Fgfr2 to engage Frs2α in the Fgfr2LR/LR mice resulted in no renal abnormalities. In contrast, mice with a point mutation in the Frs2α binding site of Ret (Y1062), develop a remarkably similar phenotype to the Frs2αUB−/− mice, namely, mild renal hypoplasia (19); thus Frs2α may be acting through Ret in the ureteric bud. Frs2α is also a major docking adapter molecule for a number of other receptor tyrosine kinases such as neurotropin receptors and anaplastic lymphoma kinase. While several reports have documented expression of Trks in mam-
malian kidneys (1, 8, 44), only TrkB mutations have resulted in kidney abnormalities (10). Furthermore, TrkB/H11002/H11002 mice had mild abnormalities in the juxtaglomerular apparatus and no global renal hypoplasia. Finally, Alk knockout mice did not display an abnormal kidney phenotype (4).

To further interrogate the reasons for the renal abnormalities in the Frs2α/H9251UB/H11002/H11002 mice, we examined expression of two known downstream targets in other systems, namely, Etv4 and Etv5. Surprisingly, we detected no changes in ureteric (or mesenchymal) Etv4 or Etv5 signal in Frs2αUB/-/- mutants vs. controls. Excluding the possibility that the mutant mice represent hypomorphs (which is unlikely for reasons discussed earlier), it is possible that there are small changes in Etv4 and Etv5 expression below the sensitivity of in situ hybridization. It is more likely, however, that signaling through other docking proteins compensates for the absence of Frs2α/H9251.

We next examined whether there were perturbations in other developmental genes that could explain the renal hypoplasia in the Frs2αUB/-/- mice. Despite the fact that Sprouty1 is a known Frs2α antagonist, we detected no increase in expression either by in situ hybridization or real-time PCR. In contrast, we did detect a decrease in both Ret and Wnt11 expression by both in situ hybridization and real-time PCR. Moreover, perturbations in Ret (as noted earlier) or Wnt11 signaling can lead to renal hypoplasia (19, 23). Furthermore, there appear to be synergistic actions of the two molecules, in that compound heterozygous mice (Ret/H11001/H11002/Wnt11/H11001/H11002) mice develop renal hypoplasia (23). Wnt11/H11002 and Wnt11/Ret compound heterozygous mice also had a reduction in Gdnf expression (23); thus the reduced expression of Wnt11 and Ret in the Frs2αUB/-/- mice may explain the trend toward decreased Gdnf expression. Finally, a reduction in Ret and Wnt11 expression in Frs2αUB/-/- mice likely explains the ureteric bud branching abnormalities and subsequent hypoplasia.

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