

Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development

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SUMMARY

Reciprocal cell-cell interactions between the ureteric epithelium and the metanephric mesenchyme are needed to drive growth and differentiation of the embryonic kidney to completion. Branching morphogenesis of the Wolffian duct derived ureteric bud is integral in the generation of ureteric tips and the elaboration of the collecting duct system. Wnt11, a member of the Wnt superfamily of secreted glycoproteins, which have important regulatory functions during vertebrate embryonic development, is specifically expressed in the tips of the branching ureteric epithelium. In this work, we explore the role of Wnt11 in ureteric branching and use a targeted mutation of the Wnt11 locus as an entrance point into investigating the genetic control of collecting duct morphogenesis. Mutation of the Wnt11 gene results in ureteric branching morphogenesis defects and consequent kidney hypoplasia in newborn mice. Wnt11 functions, in part, by maintaining normal expression levels of the gene encoding glial cell-derived neurotrophic factor (Gdnf). Gdnf encodes a mesenchymally produced ligand for the Ret tyrosine kinase receptor that is crucial for normal ureteric branching. Conversely, Wnt11 expression is reduced in the absence of Ret/Gdnf signaling. Consistent with the idea that reciprocal interaction between Wnt11 and Ret/Gdnf regulates the branching process, Wnt11 and Ret mutations synergistically interact in ureteric branching morphogenesis. Based on these observations, we conclude that Wnt11 and Ret/Gdnf cooperate in a positive autoregulatory feedback loop to coordinate ureteric branching by maintaining an appropriate balance of Wnt11-expressing ureteric epithelium and Gdnf-expressing mesenchyme to ensure continued metanephric development.

Key words: Wnt11, Metanephric kidney, Ureteric branching morphogenesis, Ret, Gdnf, Epithelial mesenchymal interaction, Mouse

INTRODUCTION

The adult metanephric kidney of mammals is primarily derived from two embryonic tissue sources: the ureteric epithelium and the metanephric mesenchyme. Metanephric development is launched with an outgrowth of the Wolffian duct, termed the ureteric bud, into the neighboring uninduced metanephric mesenchyme (Saxen, 1987). The classical co-culture experiments of Grobstein have demonstrated that the arborization of ureteric epithelium into the mature collecting duct system, and the terminal differentiation of mesenchyme into functional nephrons, is dependent upon continued cell-cell interactions between the component ureteric epithelium and mesenchyme (Grobstein, 1953). Genetically or chemically induced perturbation of either component tissue or of signaling between these tissues obstructs metanephric growth and differentiation (Davies and Bard, 1998; Davies and Davey, 1999; Lechner and Dressler, 1997).

Epithelial branching morphogenesis is common to the development of the kidney, lung, pancreas and other ductal organs, and involves the regulated growth and branching of an epithelial primordium within a mesenchymal environment. The Ret/Gdnf signaling pathway is a major regulator of ureteric branching in the metanephric kidney (Airaksinen and Saarma, 2002; Davies and Bard, 1998; Lechner and Dressler, 1997; Manie et al., 2001). Glial cell-derived neurotrophic factor (Gdnf), a member of the TGFβ superfamily, functions as a ligand secreted by the metanephric mesenchyme that binds to the Ret tyrosine kinase receptor and GFRα1 co-receptor, both of which are expressed within the ureteric epithelium (Durbec et al., 1996; Pachnis et al., 1993; Sariola and Saarma, 1999; Vega et al., 1996). Targeted mutagenesis of Gdnf, Ret or Gfra1 results in failed ureteric bud morphogenesis and consequently kidney agenesis (Schuchardt et al., 1994; Sanchez et al., 1996; Schuchardt et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). Conversely, ectopic activation of the Ret/Gdnf pathway induces the appearance of supernumary ureteric tips. Implantation of Gdnf-coated beads into kidney explant cultures stimulates ectopic ureteric tip formation from the Wolffian duct (Brophy et al., 2001; Pepicelli et al., 1997; Sainio et al., 1997).
Similarly, in the Foxc1 mutant, an expanded mesenchymal Gdnf expression domain is the target of ectopic ureteric bud invasion from the Wolffian duct resulting in multi-lobular kidneys (Kume et al., 2000). Based on these and cell migration studies using MDCK cells, Gdnf has been proposed as a mesenchymally localized chemoattractant that promotes Wolffian duct derived ureteric bud outgrowth (Tang et al., 1998).

Several members of the Wnt gene family are expressed in the developing kidney. Wnt genes encode secreted glycoproteins with important roles in regulating cell proliferation, tissue patterning, and morphogenesis during vertebrate embryogenesis (Wodarz and Nusse, 1998). The Wnt ligands are thought to elicit their cellular responses by binding to transmembrane Frizzled receptors (Bhanot et al., 1996). Among the Wnt members, Wnt11, Wnt7b, Wnt6, Wnt2b, and Wn-4 have been reported to be in unique domains within the embryonic mouse kidney (Kispert et al., 1996; Lin et al., 2001; Stark et al., 1994). Wnt11 is unique in that it shows a striking expression pattern in the branching ureteric tips suggesting a possible function in regulating ureteric branching morphogenesis (Kispert et al., 1996). In addition to its kidney expression, Wnt11 is expressed in multiple embryonic tissues, including the node, heart primordium, somites, branchial arches, and limb buds (Kispert et al., 1996). Analysis of zebrafish silberblick (slb), a mutation in zebrafish wnt11, and experiments in Xenopus suggest that Wnt11 signals through the planar cell polarity (PCP), and not the canonical β-catenin pathway, to regulate convergence and extension movements during gastrulation that elongate the axis (Heisenberg et al., 2000; Tada and Smith, 2000). Recently, Wnt11 has been implicated in the regulation of cardiogenesis in Xenopus (Pandur et al., 2002).

In the kidney, Wnt11 is expressed in the tips of the branching ureter at all stages of ureteric development (Kispert et al., 1996). In addition, the implantation of Gdnf coated beads causes induction of ectopic ureteric tips and upregulation of Wnt11 at these sites (Pepicelli et al., 1997; Sainio et al., 1997). Furthermore, genetic and chemical perturbation of sulfated proteoglycan synthesis blocks ureteric branching and simultaneously results in loss of Wnt11 expression (Bullock et al., 1998; Kispert et al., 1996). These experiments indicate a correlation between the formation of ureteric tips, the appearance of Wnt11 expression and the initiation of ureteric branching.

In order to determine the function of Wnt11 during metanephric kidney development, we generated a targeted knockout mutation of the Wnt11 locus. We report here the phenotypic analysis of the Wnt11 mutant mice and show that Wnt11 is required for embryonic viability and also for normal ureteric branching morphogenesis. In the absence of Wnt11 function, branching morphogenesis is abnormal resulting in kidney hypoplasia. We show that Wnt11 regulates ureteric branching, at least in part, by regulating mesenchymal Gdnf expression. Ureteric Wnt11 expression is reciprocally dependent upon Ret/Gdnf signaling. Wnt11 and Ret mutants genetically interact in the branching morphogenesis process. We propose that the Wnt11 and Ret/Gdnf signals may participate in a positive, autoregulatory feedback loop to coordinate branching of the ureteric epithelium and hence normal morphogenesis of the normal kidney.

**MATERIALS AND METHODS**

**Construction of the targeting vector, gene targeting and generation of Wnt11 knockout mice**

The targeting construct, containing 4.8 kb of 5′ and a 4.0 kb of 3′ homology regions (Fig. 1A), was transfected into 5×10^5 R1 ES cells derived from 129Sv strain (Nagy et al., 1993) using a BioRad gene pulser. ES cell clones were selected positively with G418 for presence of the PGK-neo cassette (Swiatek et al., 1994) and negatively with FIAU for absence of the MC1TK cassette (Mansour et al., 1988; Stark et al., 1994). Surviving colonies were isolated, trypsinized and seeded onto mouse embryonic feeder cells or onto non-coated 24-well plates for DNA isolation. Southern blot analysis was used to detect a restriction fragment length polymorphism (RFLP) on DNA purified from 240 colonies by using a 2 kb EcoRV genomic fragment as a 3′ probe (Fig. 1A). Spel digestion leads to generation of a 20 kb fragment in the wild-type allele, whereas replacement of exons IV and V of the Wnt11 gene with PGK-neo will introduce additional Spel sites and leads to the generation of a 10 kb mutant Spel fragment (Fig. 1A). One out of 240 screened ES cell clone showed homologous recombination in the Wnt11 locus. The clone was subjected to Southern blot analysis using the 5′ probe and a neo probe to confirm the targeting event (Fig. 1A). The correctly targeted ES cell line was used to generate chimeras by routine blastocyst injection. Germine transmission of the targeted allele was monitored by RFLP analysis using the 5′ probe and Spel digestion on a Southern blot. All subsequent genotyping was carried out by Southern blotting or PCR. Southern blotting was performed using Amersham Pharmacia Biotech Hybond N+ membranes according to the manufacturer’s guidelines. Radioactive probes were labeled using Random Primers DNA Labeling System (Invitrogen). For PCR, genotyping, the wild-type allele was identified using primers 5′CTGGCACTGTCCAAGAGCTCC3′ and 5′AGCTCAGTGAGGAGCAGT3′, which amplify a 220 bp fragment. The mutant allele was identified using primers 5′GGATCCGACGATGTGCAC3′ and 5′TACCGGTGGATGTGGATGTGGC3′ which amplify a 250 bp fragment. The PCR conditions are 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute.

The Ret mutant allele has been described (Schuchardt et al., 1996). The Ret mutant embryos were genotyped by PCR as described in Schuchardt et al. (Schuchardt et al., 1996). In double mutant crosses to examine genetic interactions, Southern blot genotyping with BamHI digestion and probing with the neo gene identified a 1.1 kb band unique to the Ret mutation and a 3.8 kb band unique to the Wnt11 mutation.

**Mouse crosses**

All Wnt11 phenotypic analysis was performed with mice maintained in the 129/Sv background. The Ret mutant allele was obtained from F. Costantini and maintained in the 129/Sv background. To visualize ureteric branching morphogenesis, male mice expressing the Cre recombinase under control of the HoxB7 enhancer were crossed to Rosa26 YFP females (Srinivas et al., 2001; Yu et al., 2002). Kidneys from HoxB7 Cre; Rosa26 YFP embryos were dissected and examined under fluorescence using a GFP filter set on a Nikon SMZ1500 stereoscope.

**RNA isolation and RT-PCR**

Total RNA was isolated from PI kidneys using TRI reagent (Sigma) and treated with RNase free DNase (Gibco BRL). RT-PCR was performed using the SuperScript Plasmid System (Invitrogen) with the Wnt11 forward primer 5′GAAATTCGGAGAGCTCGAGA3′ and the Wnt11 reverse primer 5′TCTAGAGACCCACCTGAGGAAAG3′. PCR products were digested with EcoRI and XbaI and cloned into pCR2.1 (Invitrogen). PCR products were sequenced using ABI BigDye cycle sequencing. Wild-type and mutant Wnt11 cDNA sequences were compared to genomic sequence obtained from the Celera database.
Regulation of ureteric branching

Histology and quantitating kidney size

Kidneys were fixed in 4% paraformaldehyde and taken through a graded alcohol series in preparation for paraffin wax sectioning. Sections were cut at 6 μm and stained with Hematoxylin/Eosin. Kidney size was quantified throughout the whole kidney by counting absolute numbers of glomeruli in Hematoxylin/Eosin stained sections. Glomeruli were identified by the presence of a Bowmann’s capsule and capillary tuft.

Immunohistochemistry

For whole-mount immunocytochemistry, same stage E12.5 kidneys were fixed in methanol prior to antibody staining. Kidneys were rehydrated, blocked in PBS/0.1% Triton X-100/1% dry milk/2% BSA and stained with a 1:20 dilution of α pan-cytokeratin mAb (Sigma) at 4°C overnight. After washes in PBS/0.1% Triton X-100, staining was visualized with a 1:2000 dilution of Alexa 568 goat-anti-mouse secondary antibody (Molecular Probes). Confocal images were taken on a Zeiss LSM510 Axioplan confocal microscope.

In situ hybridization

Whole-mount in situ hybridization was performed based on the method described by Wilkinson (Wilkinson and Nieto, 1993). Digoxigenin-UTP labeled antisense riboprobes were prepared from the following templates Wnt11 (XhoI/T3), Ret (RamHI/T7), Pax2 (XbaI/T3), Emx2
Wnt11

Germline integrants were identified and bred to homozygosity. V in mouse 129 ES cells (Fig. 1A). Homologous recombinant Wnt11 locus was mutated by targeted deletion of exons IV and X in mouse 129 ES cells (Fig. 1A). Homologous recombinant Wnt11 locus was mutated by targeted deletion of exons IV and V in the targeted allele. Southern blot and genomic PCR analyses (Fig. 1B). Southern blot analysis was also used to confirm the absence of exons IV and V in the targeted allele.

Analysis of the Wnt11 allele

RT-PCR analysis on RNA from Wnt11 –/– P1 kidneys identified a cDNA of ~1.4 kb in agreement with the predicted size of a transcript resulting from deletion of exons IV and V (Fig. 1C). In Wnt11 mutants, a stable transcript is made containing exons I-III upstream and exons VI and VII downstream of the targeted deletion. Sequence analysis of the Wnt11 mutant cDNA demonstrated the fusion of exon III to exon VI, and conceptual translation of the open reading frame predicts that only the N-terminal 28 amino acids, including the signal peptide sequence, matches the wild-type sequence while the reading frame downstream of the deletion is out of frame (Fig. 1D). Thus, the targeted allele is expected to eliminate wild-type Wnt11 function.

Wnt11 –/– mutants show lethality in utero

All Wnt11 –/– mutant pups died by 2 days post-partum (pp). In addition, of 152 genotyped pups, only 13% were Wnt11 –/– indicating an earlier lethality (Table 1). Analysis of E12.5 embryos revealed a statistically significant (\( \chi^2 \) test, \( P<0.001 \)) deviation from expected Mendelian ratios. The cause of the early lethality was not investigated but could correlate with potential roles for Wnt11 in node and cardiac signaling that has been associated with axis elongation and cardiac morphogenesis in zebrafish (Heisenberg et al., 2000) and Xenopus (Pandur et al., 2002) embryogenesis, respectively.

Smaller kidneys in Wnt11 mutants

Examination of Wnt11 –/– genitourinary systems from newborn pups revealed that Wnt11 –/– mutant kidneys have 64% the normal number of glomeruli compared with their wild-type littermates (\( P=0.0001 \)) whereas Wnt11 +/+ kidneys were normal (Fig. 2 and Table 2). The smaller kidney phenotype was completely penetrant. Despite the size difference, Wnt11 –/– kidneys were histologically normal with normal nephron organization along the corticomedullary axis (Fig. 2C,D). The smaller, but otherwise normal, kidneys suggested that Wnt11 signaling at

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### RESULTS

**Wnt11 targeting**

The Wnt11 gene is spread over seven exons (Fig. 1A). The Wnt11 locus was mutated by targeted deletion of exons IV and V in mouse 129 ES cells (Fig. 1A). Homologous recombinant 129 ES cells were isolated and used to generate chimeric mice. Germline integrants were identified and bred to homozygosity. Wnt11 homozygous mutant mice (Wnt11 –/–) were identified by Southern blot and genomic PCR analyses (Fig. 1B). Southern blot analysis was also used to confirm the absence of exons IV and V in the targeted allele.

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Table 2. Wnt11<sup>–/–</sup> kidneys have reduced numbers of nephrons

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of glomeruli (±s.d.)</th>
<th>n</th>
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<tr>
<td>Wnt11&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>1934±148</td>
<td>6</td>
</tr>
<tr>
<td>Wnt11&lt;sup&gt;+/–&lt;/sup&gt;</td>
<td>1804±52</td>
<td>4</td>
</tr>
<tr>
<td>Wnt11&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>1237±135</td>
<td>6</td>
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</table>

In order to quantify kidney size, the numbers of nephron glomeruli in Wnt11<sup>+/+</sup>, Wnt11<sup>+/–</sup> and Wnt11<sup>–/–</sup> P1 kidneys were counted from 6 μm Hematoxylin and Eosin stained sections. Wnt11<sup>+/+</sup> kidneys contain ~64% (P=0.0001) the number of glomeruli compared with Wnt11<sup>+/+</sup>, Wnt11<sup>–/–</sup> kidneys are not statistically significantly different in size from genotypically wild-type kidneys.

The tips of ureteric branches may be required for normal branching.

Wild-type branching pattern

The early stages of ureteric branching morphogenesis in vivo have not been previously described in detail (for reviews, see Sariola and Sainio, 1997; al-Awqati and Goldberg, 1998; Davies and Davey, 1999). In order to better understand ureteric branching morphogenesis and the role of Wnt11 in this process, we visualized the time course of ureteric branching morphogenesis using whole-mount in situ hybridization with a Ret antisense probe (Fig. 3A-F), a marker strongly upregulated in the ureteric tips (Pachnis et al., 1993), and a YFP reporter protein that was specifically expressed within the ureteric epithelium in response to a ureteric epithelial specific HoxB7 Cre transgene (Fig. 3G-I) (Srinivas et al., 1999; Yu et al., 2002).

In the mouse, the Wolffian duct-derived ureteric bud invades the metanephric mesenchyme on E10.5. By E11.5, the ureteric bud has undergone a single branching event giving rise to two ampullae or the ‘T stage’. By E12.0, each ampulla has a triangular shape with Ret and Wnt11 expression increased at the tip vertices (arrowheads in Fig. 3A,D,G). The regions of increased Ret expression pre-figure the appearance of new ureteric tips. By E12.25, each ampulla is undergoing a trifurcation to give rise to a total of six new ureteric buds (Fig. 3B,E,H). The trifurcation is a stereotyped branching event. These trifurcations appear to give rise to three ureteric tips simultaneously and do not appear to result from rapid sequential bifurcations. In 42% of E12.5 kidneys, a seventh ampulla emerges from the bifurcation point of the T (arrowheads in insets in Fig. 3; Table 3). Ret and Wnt11 are expressed at high levels in the six newly forming ureteric tips and are downregulated in the stems. By E12.5, each of these six morphologically distinct tips start to undergo a round of dichotomous branching (Fig. 3C,F,I). Ret and Wnt11 continue to be highly expressed in the six ureteric tips. We have concentrated our analysis of kidney branching morphogenesis between the E11.5 and E12.5 stages because at this time the individual ureteric tips are easily identified and the kidney branching pattern is readily discernable.

Defects in the Wnt11<sup>–/–</sup> ureteric branching pattern

The α-cytokeratin antibody stains renal epithelia and, prior to the formation of mature nephrons, visualizes the early morphology of the metanephric kidney. The mesenchyme of the developing kidney is populated by mesenchymal cells, which undergo a rotation during these stages such that the mediolateral axis present at E11.5 has translated to a dorsoventral axis by E12.5. Both left and right kidneys show similar patterns of branching. Double-headed arrows in A and B indicate orientation [anterior (A), posterior (P), dorsal (D) and ventral (V)]. Scale bars: 100 μm.
architecture of the branching ureteric duct network. Importantly, α-cytokeratin antibody stained kidneys from E12.5 Wnt11−/− embryos show loss of ureteric tips and some ampullae with abnormal morphology where ampullae do not appear to be well separated and continue to share a common lumen (data not shown). Quantification of ureteric tips at this stage in Wnt11−/− kidneys shows a statistically significant difference from wild type (χ² test, P<0.001; Table 3).

Table 3. Quantification of ureteric tips loss in Wnt11−/− kidneys

<table>
<thead>
<tr>
<th>Number of ureteric tips</th>
<th>Wild type (%) (n=57)</th>
<th>Wnt11−/− (%) (n=11)</th>
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<tr>
<td>8</td>
<td>5</td>
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</tr>
<tr>
<td>7</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>36</td>
</tr>
</tbody>
</table>

Ureteric tips from E12.5 wild-type and Wnt11−/− kidneys hybridized with Ret antisense probe were counted. The percentage of kidneys (n=57 for wild type, 11 for mutant) with eight, seven, six, five or four tips is shown. Wnt11−/− kidneys show a statistically significant (P<0.001) difference from wild-type frequencies for the distribution of ureteric tips.

To address the branching phenotype more thoroughly, we examined expression of Ret and Wnt11 at several stages. In all experiments, only kidneys from equivalent stage wild-type and mutant embryos were compared. Embryos were staged according to lung branching pattern whereby only embryos with the identical pattern of lung branching were used (see Materials and Methods). In E11.5 Wnt11−/− kidneys, the ureteric bud has undergone one round of branching giving rise to two ampullae by the T-stage of E11.5 suggesting that the timing of ureteric bud invasion into the mesenchyme and first branching event occur on schedule (Fig. 4A-D). At E12.0, the two ampullae of the T-stage appear smaller than those of wild-type kidneys possibly reflecting retarded growth (compare Fig. 4G with 4E). Each trifurcation is retarded in tip formation by E12.25 (Fig. 4I-L). Wnt11 expression levels are markedly reduced in the tips of mutant kidneys, despite the fact that the Wnt11 antisense probe is identical to sequences common to wild-type and Wnt11 mutant transcripts 3’ of the targeted deletion. The early defects result in the loss of ureteric branches when these have clearly resolved from the trifurcation at E12.5, though Ret and Wnt11 are strongly expressed at the branch points (Fig. 4M-P). Thus, the timecourse analysis of the Wnt11−/− kidney phenotype shows a retarded morphogenesis that results in a defect in branching trifurcation resulting in loss of ureteric tips. The loss of ureteric tips at these early stages is a likely explanation for the small kidney phenotype observed in Wnt11−/− newborns. Nevertheless, some ureteric tips do form in Wnt11−/− kidneys and continue to grow and branch during later kidney development, suggesting other signals may be operating to support continued ureteric branching (see Discussion).

Wnt11 and Ret/Gdnf signaling in ureteric branching morphogenesis

As Gdnf is an important regulator of ureteric branching, we sought to determine whether Gdnf expression was normal in Wnt11−/− kidneys. In wild-type E12.5 kidneys, Gdnf is intensely expressed in mesenchymal cells surrounding the branching ureteric epithelium (Fig. 5A). Importantly, Gdnf expression is downregulated in Wnt11−/− kidneys at this time, suggesting that Wnt11 expression in the ureteric epithelium is required for normal Gdnf expression in the
adjacent mesenchyme (Fig. 5B). Gdnf is expressed normally in earlier E12.0 Wnt11/+/− kidneys (data not shown). By contrast, mesenchymal expression of Pax2, an important regulator of mesenchymal differentiation (Dressler et al., 1990; Torres et al., 1995), is relatively unaffected in E12.5 Wnt11/−/− kidneys (compare Fig. 5C with 5D), suggesting that the reduction in Gdnf expression is specific.

We next determined whether Wnt11 expression might be dependent upon Ret/Gdnf signaling in the developing collecting duct. The Ret/Gdnf pathway has been shown to play a crucial role in ureteric epithelial branching. The Ret targeted mutation, therefore, eliminates signaling in the kidney by all Gdnf family ligands. Ureteric bud invasion into the metanephric mesenchyme fails in most Ret and Gdnf mutants, resulting in kidney agenesis (Schuchardt et al., 1994; Schuchardt et al., 1996). However, in some Ret−/− mutants, the ureteric bud enters the mesenchymal mesenchyme and undergoes a single bifurcation event to generate the T-stage kidney by E12.0 (Schuchardt et al., 1996). Interestingly, Wnt11 expression is dramatically reduced in these mutants (compare Fig. 5E with 5F), suggesting that ureteric Wnt11 expression is dependent upon Ret/Gdnf signaling within the ureteric epithelium. By contrast, Emx2 is expressed at comparable levels throughout the entire branching ureteric epithelium in wild-type and Ret−/− kidneys (compare Fig. 5G with 5H) (Miyamoto et al., 1997; Pellegrini et al., 1997). Thus, Wnt11 is specifically downregulated in Ret−/− kidneys.

Fig. 5. Wnt11 and Ret/Gdnf signals are mutually dependent. Gdnf is downregulated in Wnt11−/− kidneys. Gdnf expression in E12.5 wild-type (A) kidneys is found in the mesenchyme surrounding the non-staining ureteric epithelium. Mesenchymal Gdnf expression is reduced in E12.5 Wnt11−/− kidneys (B). By contrast, Pax2 continues to be expressed in Wnt11−/− kidney mesenchyme at E12.5 (compare D with C). Wnt11 expression is reduced in Ret−/− kidneys. Wnt11 expression in wild-type (E) E12.0 kidneys marks the forming ureteric tips during the trifurcation stage. Wnt11 expression is dramatically reduced in E12.0 Ret−/− kidneys (arrows in F). However, Emx2 continues to be expressed in Ret−/− ureteric epithelium comparable with wild type (compare H with G). Kidneys are oriented anterior towards the top and posterior towards the bottom.

Genetic interactions between Wnt11 and Ret

The observation that Wnt11 and Gdnf expression levels are mutually interdependent in E12.5 kidneys, prompted us to ask whether Wnt11 genetically interacts with members of the Ret/Gdnf pathway. We crossed the Ret mutation into the Wnt11 genetic background to generate Ret+/−; Wnt11+/− compound heterozygotes and Ret−/−; Wnt11−/− mutant mice. As shown in Fig. 6 and Table 4, Wnt11+/−; Ret−/− E18.5 kidneys are 52% (P=0.007) the size of same stage wild-type kidneys, indicating a genetic interaction between the Wnt11 and Ret mutations in the compound heterozygote state (Fig. 6E,F). Removal of another copy of Wnt11 demonstrates dose-dependent interactions between Wnt11 and Ret. Ret−/−; Wnt11−/− kidneys are 67% (P=0.0001) the size of Ret+/−; Wnt11+/− and 44% (P=0.0008) the size of Wnt11+/− kidneys, again suggesting a genetic interaction between the Ret/Gdnf and Wnt11 pathways (compare Fig. 6H with 6E-G). Ret−/−; Ret−/− kidneys are not significantly different in size from genotypically wild-type kidneys (P=0.65).

We also investigated the pattern of ureteric branching at E12.5 in these genetic combinations using the Ret probe. The observation that ureteric branching in Ret+/−; Wnt11−/− kidneys appears normal at E12.5, yet the E18.5 kidneys are smaller than wild-type controls suggests that both Ret and Wnt11 are also required for branching morphogenesis throughout later embryonic kidney development after E12.5. Analysis of E12.5 ureteric branching morphogenesis using in situ hybridization with a Ret antisense probe reveals that ureteric branching is severely affected in Ret+/−; Wnt11+/− kidneys (Fig. 6L). The E12.5 Ret+/−; Wnt11+/− kidneys show branching defects that are more severe than those observed in Wnt11+/− kidneys (Fig. 6K). E12.5 kidneys from Ret−/−; Wnt11+/− embryos have two to four ureteric tips compared with the seven found in wild type. Thus, reducing Ret activity appears to enhance the effects of a loss of Wnt11 signaling on the branching process.

DISCUSSION

Wnt11 is required for normal kidney development

Our analysis reveals a genetic requirement for Wnt11 in kidney ureteric branching morphogenesis. Previous studies on Wnt11 during kidney development raised correlative evidence for Wnt11 function in ureteric branching. First, Wnt11 is expressed

Table 4. Wnt11 and Ret mutations synergistically interact to yield kidney hypoplasia

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of glomeruli (±s.d.)</th>
<th>n</th>
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<tbody>
<tr>
<td>Ret+/−; Wnt11+/−</td>
<td>1358±258</td>
<td>4</td>
</tr>
<tr>
<td>Ret−/−; Wnt11+/−</td>
<td>1447±278</td>
<td>4</td>
</tr>
<tr>
<td>Ret−/−; Wnt11−/−</td>
<td>1055±161</td>
<td>4</td>
</tr>
<tr>
<td>Ret+/−; Wnt11−/−</td>
<td>702±40</td>
<td>5</td>
</tr>
<tr>
<td>Ret−/−; Wnt11−/−</td>
<td>469±131</td>
<td>10</td>
</tr>
</tbody>
</table>

Average total numbers of glomeruli in E18.5 kidneys from Ret;Wnt11 double mutant combinations are shown. Ret+/−; Wnt11+/− kidneys contain fewer glomeruli than wild-type controls. Ret−/−; Wnt11+/− kidneys are much smaller than either Ret+/− or Wnt11+/− kidneys by themselves. Ret−/−; Wnt11−/− kidneys are not statistically significantly different in size from genotypically wild-type controls. Glomeruli were counted from 6 μm coronal Hematoxylin/Eosin stained kidney sections.
at the tips of the ureteric epithelium where branching morphogenesis is occurring, suggesting that Wnt11 may participate in regulating branching events (Kispert et al., 1996). Second, Wnt11 expression directly correlates with genetically and chemically induced gain and loss of ureteric tips (Kispert et al., 1996; Pepicelli et al., 1997; Sainio et al., 1997). Our observations demonstrate that Wnt11 is required for collecting duct development and suggest that Wnt11 signaling is most probably one component of reciprocal signaling mechanisms that act between the ureteric epithelium and metanephric mesenchyme to regulate the Ret/Gdnf signaling pathway to control normal ureteric branching.

Initial ingrowth of the ureteric bud into the mesenchyme appears to be independent of Wnt11 function as all Wnt11 mutant kidneys examined had progressed to the T-stage on schedule. The progression of ureteric branching to the T-stage in Wnt11+/– mutants is associated with normal Gdnf expression (data not shown). However, an abnormal branching pattern comprised of retarded morphogenesis and loss or ureteric tips was observed in Wnt11+/– kidneys from the T stage. These branching defects are associated with a reduction in mesenchymal Gdnf expression. Given that Gdnf can function as a chemoattractant, low Gdnf levels may result in lower outgrowth promoting activity and decreased numbers of ureteric tips as is observed in Wnt11+/– mutants. The loss of ureteric tips early in metanephric development results in significantly smaller kidney size by birth.

In wild type, Wnt11 is expressed robustly in ureteric tips during all stages of metanephric development, suggesting a potential role for Wnt11 in branching morphogenesis throughout kidney development. The defects in Wnt11+/– kidneys correlate with a trifurcation and trifurcations are observed at later stages of kidney development (A.M., unpublished), again pointing to a larger role for Wnt11 in branching. Our analysis of Wnt11 in a genetically sensitized Ret+/– background supports this hypothesis, as Wnt11+/+; Ret+/– E18.5 kidneys are significantly reduced in size compared with controls, even though the branching pattern at E12.5 is indistinguishable from wild type. Furthermore, we observe that Wnt11+/+; Ret+/– kidneys are smaller than Wnt11+/– kidneys, even though the branching at E12.5 in the compound heterozygotes is unaffected. This may suggest that ureteric branching is differentially sensitive to the level of Wnt11 and Ret/Gdnf signals at different times during kidney development. Thus, the genetic interaction studies in the sensitized Ret+/– background reveal a wider requirement for Wnt11 in ureteric branching beyond the stages analyzed here.

Although it is clear that Wnt11 is required for normal ureteric branching, considerable branching morphogenesis still occurs in Wnt11+/– mutant kidneys. As the targeted allele encodes only the first 28 amino acids of the total 354 amino acid wild-type Wnt11 ligand, our Wnt11 allele most probably encodes a nonfunctional peptide. Thus, no residual Wnt11 signaling should remain in Wnt11+/– kidneys.

A second possibility that might explain the branching in Wnt11+/– kidneys is the functional redundancy of another Wnt in the ureteric epithelium. Though Gdnf expression is reduced in E12.5 Wnt11+/– kidneys, Gdnf expression appears normal by E13.5 (data not shown) suggesting that the kidney has invoked a compensatory mechanism to support continued branching in the absence of Wnt11 activity. Although other Wnt genes, including Wnt7b. Wnt6 and Wnt15 are expressed in the branching ureter proximal to the Wnt11 domain, none extend into the ureteric tips themselves nor do any of these Wnt expression domains alter in the ureteric epithelium in Wnt11+/– kidneys. Of these, Wnt6 is weakly expressed throughout the ureteric epithelium at the stages studied here. However, renal tubulogenesis induction assays suggest that Wnt11 and Wnt6 have different activities (Kispert et al., 1998; Itaranta et al., 2002).
An alternative explanation for branching in Wnt11−/− kidneys is the influence of other functionally redundant signaling pathways regulating branching. Multiple fibroblast growth factor (Fgf) ligands and their receptors are expressed during metanephric development and can modulate ureteric branching (Qiao et al., 2001). Among the Fgfs, mesenchymally expressed Fgf7 has been proposed as a modulator of ureteric growth and branching (Qiao et al., 1999). Like Wnt11, Fgf7 does not appear to be required for ureteric bud invasion into the mesenchyme, but is required for subsequent elaboration of the collecting duct system, as Fgf7−/− mutants have normally patterned but smaller kidneys. Whether Gdnf expression is dependent upon Fgf7 is not known. Kidney culture experiments have shown that members of the TGFβ and bone morphogenetic protein families can also modulate ureteric branching (Grisaru et al., 2001; Piscione et al., 1997). Finally, the stroma is known to provide signals promoting ureteric Ret expression and ureteric outgrowth (Batourina et al., 2001; Mendelsohn et al., 1999). Wnt2b is expressed at sites of epithelial/ mesenchymal interaction in multiple organs (Lin et al., 2001).

In the kidney, Wnt2b is expressed in the presumptive stromal cell population. In kidney explant culture experiments, incubation of ureteric buds with NIH3T3 cells expressing Wnt2b results in increased ureteric branching. This result has been interpreted as evidence that Wnt2b present in the stroma promotes, either directly or indirectly, branching of the ureteric epithelium. Wnt2b mutants have not been reported. Therefore, it is likely that multiple signaling pathways acting from different cellular populations are integrated by the ureteric epithelium and metanephric mesenchyme to maintain appropriate Ret/Gdnf signal levels to support collecting duct morphogenesis, and one such signal appears to be Wnt11.

The silberblick (slb) mutation demonstrates a requirement for Wnt11 in regulating convergence/extension movements during zebrafish gastrulation (Heisenberg et al., 2000) and Wnt11 appears to have a similar role in Xenopus (Djiane et al., 2000; Tada and Smith, 2000). Wnt11 is thought to signal through a planar cell polarity (PCP) pathway to regulate cytoskeletal rearrangements, thus coordinating polarized cell movement during vertebrate gastrulation. Recently, a role for Wnt11 PCP signaling has been demonstrated in Xenopus cardiogenesis (Pandur et al., 2002). We found no similar absolute requirement for Wnt11 in either mouse gastrulation or cardiac development. This may reflect a difference in the genetic regulation of gastrulation between mouse and zebrafish or it may reflect a functional redundancy in mouse. Although Wnt11 is required for viability during the embryonic and post-partum stages, these lethali ties do not arise from the kidney defects we describe here.

Although our analysis advances Wnt11 as a modulator of Ret/Gdnf signaling, Wnt11 may have other roles in the branching process. Wnt11 PCP signaling employs Rho kinase 2 (Rok2), Rho GTPase and Jun N-terminal kinase (JNK) to effect changes in actin cytoskeleton organization (Marlow et al., 2002; Mlodzik, 2002). In the kidney, Wnt11 may regulate branching morphogenesis by causing cytoskeletal reorganization within the plane of the ureteric epithelium. These additional roles for Wnt11 in ureteric branching await further investigation.

**Wnt11 and Ret/Gdnf signals cooperate in a regulatory circuit to control ureteric branching morphogenesis**

Three observations suggest that Wnt11 and Ret/Gdnf signals cooperate to regulate ureteric branching morphogenesis. First, mesenchymal Gdnf expression is dependent upon ureteric Wnt11 signal. Second, Wnt11 expression is reciprocally dependent upon Ret/Gdnf signaling within the ureteric epithelium. Third, Wnt11 and Ret mutants synergistically interact during ureteric branching morphogenesis, suggesting both pathways are functioning cooperatively and inter-dependently in a common branching process.

What is not clear is whether Wnt11 acts as a paracrine factor to regulate Gdnf expression directly in the metanephric mesenchyme or if Wnt11 itself is a direct transcriptional target of the Ret/Gdnf signaling pathway. Mesenchymal Gdnf expression is known to be dependent upon at least two transcription factors, Pax2 and Sox11 (Brophy et al., 2001; Miyamoto et al., 1997; Nishinakamura et al., 2001). Indeed, cell culture experiments and analysis of cis-regulatory regions in the Gdnf gene indicate that Pax2 may be a direct regulator of Gdnf expression (Brophy et al., 2001). Wnt-mediated regulation of Pax gene expression in the kidney has been reported in Wnt4 mutants where Pax8 and Pax2 expressions are absent in the pre-tubular aggregates (Stark et al., 1994). We failed to observe any obvious alteration in Pax2 levels in Wnt11−/− kidneys.

In addition, our results suggest that Wnt11 expression is dependent upon Ret/Gdnf signaling within the ureteric epithelium and the Wnt11 locus may therefore be a downstream target of Ret/Gdnf signaling, consistent with our earlier observations where implantation of Gdnf-coated beads into kidney explant cultures significantly upregulated Wnt11 expression (Pepicelli et al., 1997; Sainio et al., 1997). Upon ligand binding, Ret activates multiple downstream signaling pathways (reviewed by Airaksinen and Saarma, 2002; Manie et al., 2001). Inhibition of PI-3 kinase activity with the small molecule LY294002 prevented Gdnf-induced ectopic ureteric outgrowth in kidney explant culture, implicating PI-3 kinase signaling in ureteric morphogenesis (Tang et al., 2002). Whether Wnt11 expression is altered in these experiments has not been addressed.

The genetic interactions observed in Ret+/−; Wnt11+/− kidneys suggest that the Wnt11 and Ret/Gdnf signaling pathways function serially and not in parallel. The Wnt11 and Ret/Gdnf signals may participate in a positive, autoregulatory feedback loop to coordinate ureteric branching by maintaining a balance between appropriate amounts of Gdnf-expressing mesenchyme with Wnt11-expressing ureteric tips. Wnt11 levels may inform the mesenchyme as to the number of ureteric buds present. Therefore, this regulatory network may function as a counting mechanism for the developing kidney to determine the extent of branching, convey this information to the mesenchyme and respond with a matching level of outgrowth-promoting Gdnf.

**Wnt genes and branching morphogenesis**

Other Wnt genes have also been proposed to play roles in branching morphogenesis. In addition to Wnt2b (discussed earlier), the Wnt4−/− knockout mouse has been used to demonstrate a requirement for Wnt4 function in progesterone
induced mammary epithelium branching morphogenesis during pregnancy (Brisken et al., 2000). However, substantial branching still occurs in grafted Wnt4−/− ductal tissue at later stages of pregnancy, implying that Wnt4 may act in concert with other Wnt genes in this tissue. Although past studies of Wnt genes have focused on their roles in growth and patterning, future investigations may uncover other examples of these genes in morphogenetic processes during vertebrate development.

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