Sprouty proteins regulate ureteric branching by coordinating reciprocal epithelial Wnt11, mesenchymal Gdnf and stromal Fgf7 signalling during kidney development

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Summary

The kidney is a classic model for studying mechanisms of inductive tissue interactions associated with the epithelial branching common to many embryonic organs, but the molecular mechanisms are still poorly known. Sprouty proteins antagonize tyrosine kinases in the Egf and Fgf receptors and are candidate components of inductive signalling in the kidney as well. We have addressed the function of sprouty proteins in vivo by targeted expression of human sprouty 2 (SPRY2) in the ureteric bud, which normally expresses inductive signals and mouse sprouty 2 (Spry2). Ectopic SPRY2 expression led to postnatal death resulting from kidney failure, manifested as unilateral agenesis, lobularization of the organ or reduction in organ size because of inhibition of ureteric branching. The experimentally induced dysmorphology associated with deregulated expression of Wnt11, Gdnf and Fgf7 genes in the early stages of organogenesis indicated a crucial role for sprouty function in coordination of epithelial-mesenchymal and stromal signalling, the sites of expression of these genes. Moreover, Fgf7 induced Spry2 gene expression in vitro and led with Gdnf to a partial rescue of the SPRY2-mediated defect in ureteric branching. Remarkably, it also led to supernumerary epithelial bud formation from the Wolffian duct. Together, these data suggest that Sprr genes contribute to reciprocal epithelial-mesenchymal and stromal signalling controlling ureteric branching, which involves the coordination of Fgf/Wnt11/Gdnf pathways.

Key words: Sprouty, Kidney morphogenesis, Fgf, Ureteric branching, Wnt11, Ret, Gdnf, Metanephric mesenchyme, Mouse

Introduction

The mammalian metanephric kidney has served as a useful model for studying the inductive signalling that takes place between heterotypic cell types, such as those of epithelial and mesenchymal tissues, and is associated with the generation of epithelial branches (Saxen, 1987; Vize et al., 2003). In the embryonic kidney, such signalling occurs between the ureteric bud and the metanephric mesenchyme, and is sequential and reciprocal in nature, but only specific signals involved have been identified to date, and the molecular mechanisms are still poorly understood (Vainio and Lin, 2002).

Organogenesis of the kidney is initiated when the ureteric bud forms as an outgrowth from the Wolffian duct. The bud invades the adjacent metanephric blastema, which has obtained a bias by this stage when induced initially by signals from the ureter to generate nephrons (Itaranta et al., 2002). As in many other developing organs, the epithelial signals lead first to morphological condensation of the mesenchymal cells in the kidney. A selected pool of such cells then go on to transform into the epithelium in order to assemble the nephrons and into its associated terminally differentiated cell types (Vainio and Lin, 2002; Vize et al., 2003). Alongside this ureteric branching, each of the ureteric tips acts inductively by secreting as yet unknown factors that lead to the initiation of nephrogenesis in the mesenchymal cells that flank the ureter bud (Saxen, 1987). The number of tubules assembled during organogenesis depends on the degree of ureteric branching. Hence nephrogenesis and ureteric branching both contribute to the secretory and excretory capacities of the mature kidney (Brenner and Milford, 1993).

A member of the transforming growth factor β (Tgfβ) superfamily of secreted signals, the glial cell line-derived neurotrophic factor (Gdnf), and its receptor, Ret, are crucial for the initiation of kidney organogenesis, in that they regulate ureteric bud development (Airaksinen and Saarma, 2002; Davies and Bard, 1998; Kuure et al., 2000). Gdnf is expressed in mesenchymal cells that are adjacent to the ureteric bud, which expresses Ret and a co-receptor for Gdnf, Gfra1 (Durbec et al., 1996; Pichel et al., 1996). Ectopic Gdnf signalling can induce bud formation from the Wolffian duct (Brophy et al.,
Besides Gdnf/Ret, other classes of secreted signals such as the Wnt genes and bone morphogenetic proteins (Bmps) regulate kidney development (Dudley et al., 1999; Dudley and Robertson, 1997; Dunn et al., 1997; Miyazaki et al., 2000; Moore et al., 1996). Genes from these families are expressed in epithelial and mesenchymal tissues and are also essential for their development. Wnt4, for example, is expressed in mesenchymal pretubular aggregates, and nephrogenesis fails in the event of its deficiency. Wnt6, Wnt7b and Wnt11 are expressed in the ureteric bud, and Wnt11, at least, is functional in ureteric bud branching in vivo (Itaranta et al., 2002; Kispert et al., 1996; Stark et al., 1994; Vainio et al., 1999). The pattern of early bud branching is defective in Wnt11-deficient kidneys, and this is associated with deregulated expression of the Gdnf genes.

Ret signalling is also necessary to maintain Wnt11 gene expression in the ureteric bud. These findings suggest that coordination of the Wnt11/Gdnf/Ret pathways is associated with epithelial branching during kidney development (Majumdar et al., 2003).

The Bmps that mediate secondary inductive epithelial-mesenchymal interaction during organogenesis (Vainio et al., 1993) are also important secreted signals for kidney development. Bmp4, for example, is expressed in the kidney mesenchyme and may in turn antagonize ureteric bud development (Dunn et al., 1997; Miyazaki et al., 2000). Like Wnt11, Bmp4 also contributes to organogenesis by controlling the expression of Gdnf (Raatikainen-Ahokas et al., 2000).

In addition to the epithelial and mesenchymal signals, it has become evident that the renal stroma is a source of inducers that contribute to organogenesis. This suggestion is based on the fact that disruption of a winged helix transcription factor, the Foxd1 (previously BF-2) gene, which is expressed by cells of the renal stroma, impairs kidney development via reduced ureteric branching and associated nephrogenesis (Hatini et al., 1996). The stromal signals remain elusive, however, even though fibroblast growth factor (Fgf) expression of these genes was with epithelial branching during kidney development. This was addressed by in situ hybridization.

Materials and methods

Generation of the expression construct and transgenic mouse lines

Expression of human SPRY2 in the embryonic kidney was achieved using a 4.3 kb BamHI/NcoI 5' fragment of the Pax2 gene containing the promoter (Kuschert et al., 2001; Ryan et al., 1995). A plasmid with the internal ribosome entry site (IRES), enhanced green fluorescent protein (EGFP) and a SV40 early mRNA polyadenylation signal (Clontech) were cloned 3' in a 0.64 kb BamHI/EcoRI fragment of the rabbit-globin gene (Sasaki and Hogan, 1994), which provided the splice acceptor and donor sites for the construct. The SPRY2 cDNA was sequenced from the dbEST (R552589), amplified by means of a polymerase chain reaction (PCR) with a 5'-GAATTCTAGGGCCAGCTCAGTGG and 3'-GGCGGATTCCCTATGGTTGTTTTTCATCGGTTCC primers and cloned to the expression vector, as indicated in Fig. 1A. The ready-made construct was excised from the vector backbone with SalI (Fig. 1A) and used in 5.0 ng/μl for microinjection into the fertilized eggs (C57Bl6:Dba) by standard methods (Nagy et al., 2003) to generate the transgenic mouse lines.

The presence of the transgene in the genome was analysed in the DNA samples isolated from ear clips by PCR with 5'GAATTCATGGCCAGCTCAGTGG and 3'-GGCGGATTCCCTATGGTTGTTTTTCATCGGTTCC primers (Fig. 1A). A 300 bp fragment was detected in all the transgenic mouse lines, as expected (Fig. 1B). Southern blotting with the cDNAs of the SPRY2 or GFP as probes was used to assay the copy number and the amount of the transgene in the litters (Fig. 1C). Tail genomic DNA was digested with BamHI and run on a 1% agarose gel, blotted and probed with a [32P]-labelled 771 bp fragment of GFP. The copy number of the transgene in the genome was estimated on the basis of the intensity of the GFP gene relative to controls (DePamphilis et al., 1988). The phenotypes were analysed by backcrossing the founders with the C57BL/6 strain. Expression of the transgene was monitored by analysing GFP fluorescence, and expression of SPRY2 by in situ hybridization.

Histological analysis of phenotypes

Kidneys were isolated from E11.5, E12.5, E15.5 and E17.5 embryos and newborn mice, and samples were fixed in Bouin’s solution, embedded in paraffin wax and cut by standard methods. Serially sectioned slices were stained with Haematoxylin and Eosin. The number of glomeruli was defined as described previously (Bertram et al., 1992).

Organ culture and the use of growth factors

Culture conditions for the isolated embryonic kidneys were as reported elsewhere (Lin et al., 2001a; Lin et al., 2001b; Vainio et al., 1993). Fgf2, Fgf7, Gdnf (Pepro Tech) and Fgf10 (R&D Systems) were used in the organ cultures. Fgf2, 50-250 ng/ml of growth factor in the media (Qiao et al., 2001); Fgf7, 100 ng/ml (Qiao et al., 1999); Fgf10, 2001; Pepicelli et al., 1997; Sainio et al., 1997) and knockout of the ligand or receptors for it will perturb kidney development by inhibition of ureteric branching, indicating a crucial organogenetic role (Tang et al., 2002; Vainio and Lin, 2002).
500 ng/ml (Qiao et al., 2001); and Gdnf, 100 ng/ml (Sainio et al., 1997). The same amounts of bovine serum albumin (BSA, Sigma) served as controls. The drug PD98059 was applied to the medium to a final concentration of 20 μM (Fishel et al., 2001), and the kidneys were subcultured as indicated in the Results section. The numbers of ureteric bud tips in the experimentally manipulated explants were evaluated using Student’s t-test.

Fgf-soaked beads were prepared and used as reported earlier (Lin et al., 2001a; Sainio et al., 1997; Vainio et al., 1993). The Affi-gel blue agarose beads (100-200 mesh, 75-150 nm in diameter, 100 beads/unit volume, BioRad Laboratories Hercules, CA) were incubated in pools separately with human recombinant Fgf2 (50 μg/ml) (Minowada et al., 1999), Fgf7 (200 μg/ml) (Lin et al., 2001b), Fgf8 (50-100 μg/ml) (Minowada et al., 1999) or Fgf10 (100 μg/ml) (Mäelle et al., 2001), washed and combined individually with isolated kidneys and co-cultured for 24 hours. BSA beads served as controls and were treated and used identically to those soaked with growth factor.

**Localization of diagnostic markers**

The Troma-I antibody against cytokeratin EndoA used to monitor ureteric branching was from the Developmental Studies Hybridoma Bank (USA), while the antibody against the brush-border antigen used for visualizing induced tubules was from Dr Aaro Miettinen (University of Helsinki, Finland) (Ekblom, 1981). FITC-conjugated donkey anti-rabbit IgG and TRITC-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. The immunostaining was performed on a whole mount basis as described previously (Lin et al., 2001a; Lin et al., 2001b; Sainio et al., 1997).

The whole-mount and non-radioactive section in situ hybridization were performed as described previously (Zhang et al., 2001), as was radioactive in situ hybridization (Parr et al., 1993, Kispet et al., 1998). Wild-type and transgenic kidneys were processed in the same test tube (whole mounts) or at the same time (histological sections) to allow the comparison of staining intensities. A minimum of five hybridizations/marker gene/stage were performed in order to evaluate changes in gene expression.

The entire SPRY2 cDNA was used to synthesize a riboprobe for detecting transgene expression in embryonic kidneys. The other probes have been described earlier and were obtained as gifts: Fgfr1 (Yamaguchi et al., 1994), Fgfr2, Fgfr3, Fgfr4 and Fgfr7 (Rosenquist and Martin, 1996), Fgf2 (Wilkinson et al., 1989), Fgf8 (Crossley and Martin, 1995), Fgf9 (Colvin et al., 1996; Colvin et al., 1999; Santos-Ocampo et al., 1996), Fgf10 (Bellucci et al., 1997), Bmp4 (Bellucci et al., 1996), Ret and Gdnf (Sainio et al., 1997), Wnt11 (Kispert et al., 1996), Pax2 (Dressler et al., 1990), Foxd1 (Hatini et al., 1996), and Egfr (Miettinen et al., 1995).

**Analysis of changes in cell proliferation and apoptosis**

BrdU, an analogue of thymidine that is incorporated into DNA during the S-phase of the cell cycle (Dolbeare, 1995), served as an indicator of cell entry into mitosis. The cell proliferation kit (RPN20, Amersham Biosciences, UK) was used as suggested by the manufacturer. Briefly, pregnant mice were injected intraperitoneally with a solution containing with bromodeoxyuridine (BrdU, Sigma B-5002) at a dose of 50 mg/kg/body weight. The mice were sacrificed at two hours post-injection and the kidneys were dissected in ice-cold PBS and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C. dehydrated and embedded in paraffin wax. Sections (5 μm) were cut and the BrdU incorporated was detected with the specific antibody provided in the kit.

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) method was used to monitor apoptosis. The paraffin wax was removed from the 5 μm sections with xylene and the tissues were rehydrated and incubated with 20 μg/ml of Proteinase K for 15 minutes at 37°C. Unspecific binding was reduced with a hydrogen peroxide/methanol solution. Fragmented DNA was labelled using a reaction mixture, according to the manufacturer’s instructions (Roche). Bound probes were detected using 3,3-diaminobenzidine (DAB) as a substrate (Vectastain ABC kit, Vector Laboratories).

**Photography and image analysis**

Processed samples were photographed on Ektachrome Tungsten 64 Collagenor slide film (Kodak, USA), or with a digital camera (DC100 Leica) connected to an Olympus SZH10 stereo microscope. The composites were assembled with the Adobe Photoshop v5.0 and Corel Draw programs. All the cultured kidneys, with or without growth factors, were analysed and photographed for expression of SPRY2 and GFP at selected time points with a Leica MZFLIII stereo microscope and an Olympus DP50 digital camera connected to a Leica MZFLIII stereo microscope.

**Results**

The Pax2 promoter directs transgene expression to the ureteric bud

We and others have reported previously that Spry2 is expressed in the ureteric bud and the metanephric mesenchyme (Gross et al., 2003; Zhang et al., 2001). This suggested a putative role during kidney development in vivo. To address this possibility, a 4.3 kb fragment 5′ of the Pax2 gene containing the promoter that drives reporter gene expression in the ureteric bud (Kuschert et al., 2001; Ryan et al., 1995) was used (Fig. 1A). All of the three independent transgenic mouse lines generated (Fig. 1B,C; data not shown) revealed targeted transgene expression in the ureteric bud, as was expected (Fig. 1D-G). GFP expression was clearly detected in the ureteric bud at E11.5, when it had generated its first branch (Fig. 1D), and coincided with human SPRY2 expression (Fig. 1E). Transgene expression persisted in the bud at E15.5 and E17.5 (Fig. 1F,G). Hence the promoter targeted expression of human SPRY2 and the reporter gene to the ureteric bud.

Expression of the human SPRY2 gene leads to postnatal mortality

Crossing of transgenic mice with another carrier or with wild-type background mice was performed in order to assay the consequences of human SPRY2 expression for survival. Genotyping of the litters from such crosses indicated a gradual loss of those individuals that expressed the human SPRY2. Approximately 10% of the transgenic mice were recovered two weeks post partum (Table 1). We concluded that normal Spry protein signalling is crucial for postnatal survival. Subsequent studies were performed on the survivors and their litters. As human SPRY2 was targeted to the ureteric bud of the embryonic kidney, the likely reason for the transgene-induced death was deregulated kidney development.

Ectopic human SPRY2 expression leads to severe defects in kidney development

Closer studies of the kidneys of embryos that expressed human SPRY2 (Fig. 1C) revealed three main phenotypes (Table 2). The kidney was either reduced in size (Fig. 2A-I) or completely degenerated unilaterally (Fig. 2F, arrow on the right). Human SPRY2 expression led to the formation of cystic kidneys with a blind-ended hydroureter, which typically occurred also unilaterally (Fig. 2F,G,I,K). As the third alternative, the presence of human SPRY2 in the ureteric bud led to the
formation of two or even three separate lobes, which were frequently cystic with an ectopic second ureteric bud (Fig. 2H,K,L).

The reduction in the size of the kidneys that expressed SPRY2 correlated with a diminished number of glomeruli (Table 3). The kidneys that expressed the SPRY2 gene at E17.5 had 76% of the number of glomeruli found in the wild type, while the corresponding number in newborn mice was even less, amounting to 58%. Hence the phenotype became gradually more severe. This suggests that reduced ureteric bud branching is the likely reason for the smaller organ size, as ureteric bud signalling controls the degree of nephrogenesis via branching (Saxen, 1987).

The reason for the reduction in size in the transgenic kidney at the cellular level was likely to be either deregulated cell proliferation and/or induced apoptosis. Although mitotic cells at the S phase were localized to the nephrons in the normal manner, apoptotic cells were detected both there and in the epithelial collecting duct, which is a derivative of a ureteric bud expressing human SPRY2 (Fig. 2N,O,Q,R). More detailed determination of the mitotic index revealed a 29% reduction in the number of cells in the S phase of the cell cycle, whereas the value for cells in apoptosis demonstrated a 41% increase at E15.5 in the transgenic kidney compared with the wild-type samples (Fig. 2M,P). Together, these results suggest that the deregulated development of kidneys expressing human SPRY2 is probably due to inhibition of cell proliferation and enhanced apoptosis.

Expression of the genes encoding Fgf and Egf proteins, their receptors and mouse Spry1 in response to human SPRY2 expression

Spry proteins have been implicated in the control of Fgf and Egf signalling and organogenesis (Mailleux et al., 2001; Minowada et al., 1999). As Fgf protein activity may similarly

Table 1. Humans SPRY2 transgene-induced lethality

<table>
<thead>
<tr>
<th>Stage</th>
<th>Normal</th>
<th>TG</th>
<th>Lethality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15.5</td>
<td>40</td>
<td>22</td>
<td>45</td>
</tr>
<tr>
<td>E17.5</td>
<td>40</td>
<td>12</td>
<td>70</td>
</tr>
<tr>
<td>2 weeks</td>
<td>47</td>
<td>10</td>
<td>47</td>
</tr>
<tr>
<td>Newborn</td>
<td>39</td>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>1 month</td>
<td>84</td>
<td>8</td>
<td>91</td>
</tr>
</tbody>
</table>

Data are collected from crosses between wild types and mice that expressed the human SPRY2 transgene. Such crosses were expected to generate 50% transgenic embryos (TG) or mice. Deviation from this value was judged to be the result of the transgene-induced lethality.

Table 2. Phenotypes associated with kidney malformations by human SPRY2 expression

<table>
<thead>
<tr>
<th>Stage</th>
<th>WT/TG kidneys</th>
<th>TG/TG kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number analysed</td>
<td>Reduced (%)</td>
</tr>
<tr>
<td>E15.5</td>
<td>42</td>
<td>40 (95.24)</td>
</tr>
<tr>
<td>E17.5</td>
<td>9</td>
<td>33 (84.62)</td>
</tr>
<tr>
<td>Newborn</td>
<td>150</td>
<td>122 (81.33)</td>
</tr>
<tr>
<td>Total</td>
<td>231</td>
<td>195 (84.42)</td>
</tr>
</tbody>
</table>

The left panel indicates those embryos and newborn mice that were scored carriers of the hSprouty2 transgene in their genome while those on the right column represent samples that are obtained from crosses of two transgenic mouse. Inheritance of the transgene from the female and male increases the % of cytogenesis and induce also unilateral agenesis of the kidney.
Sprouty proteins and kidney development (Qiao et al., 1999), the involvement of human SPRY2 in Fgf-mediated signalling was also analysed. Of the Fgf proteins, Fgf2 is normally expressed in the ureteric bud and nephrogenic mesenchyme during their morphogenesis (Fig. 3A) (Drummond et al., 1998), while its expression is substantially reduced in the presence of human SPRY2 expression (Fig. 3, compare A with A’ and A’’). Fgf7, which is expressed in the peripheral mesenchymal stromal cells that surround the ureteric bud and collecting duct in the wild type (Fig. 3B) (Mason et al., 1994), showed reduced expression in the presence of ectopic human SPRY2, as also did another Fgf, mesenchymal Fgf9 (Fig. 3, compare B with B’,B’’ and D with D’,D’’). Tubular Fgf8 expression remain unchanged between the normal and transgenic samples (Fig. 3C,C’,C’’), as was the case with Egf (data not shown).

It was of interest to consider whether human SPRY2 would similarly influence the expression of Fgf/Egf receptors, the prime candidate targets of Spry protein-mediated antagonism. Fgfr1 was present in kidney mesenchymal cells regardless of their genetic status, as reported earlier (Dudley et al., 1999; Ford et al., 1997; Walshe and Mason, 2000), but its expression was lost in the more mature nephrons in the presence of human SPRY2, unlike the situation in the controls (Fig. 3, compare E with E’,E’’), suggesting that human SPRY2 expression has an influence on the maturation of the nephron, apparently owing to deregulated ureteric bud inductive signalling. The expression of Fgf receptors 2-4 appeared to be normal in all the samples analysed (Fig. 3F-H’’), as was the case for Egfr (data not shown), even though the latter is implicated in the epithelial branching process in the embryonic lung, for example (Miettinen et al., 1995).

Another Spry gene, Spry1 is expressed in the ureteric bud and may be associated with generation of the ectopic human SPRY2 phenotypes. Spry1 expression was normal regardless of the presence of human SPRY2 (Fig. 4F,F’). Hence, besides controlling Fgfr/Egfr, human SPRY2 signalling may also regulate other pathways in the kidney.

Table 3. Reduction in the number of glomeruli induced by ectopic human SPRY2

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype</th>
<th>Number of glomeruli</th>
<th>Number of kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td>E17.5</td>
<td>WT</td>
<td>682±148</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>513±178</td>
<td>6</td>
</tr>
<tr>
<td>NB</td>
<td>WT</td>
<td>1630±197</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>958±113</td>
<td>6</td>
</tr>
</tbody>
</table>

In the kidney, ectopic SPRY2 reduces the number of glomeruli from the wild-type embryo (P<0.005) at E17.5 by 24% and by 48% in the newborn mouse, respectively. The number of glomeruli were counted from sections as indicated in the Materials and methods.
Human SPRY2 expression leads to changes in Gdnf and Wnt11 expression during the early stages of organogenesis

Organogenesis of the kidney starts at E10.5 by ingrowth of the ureteric bud into the metanephric mesenchyme, followed by subsequent branching of the epithelial bud. As the human SPRY2 gene was expressed at the early stages of kidney development, these were the prime targets for further functional studies. Analysis of transgenic kidneys expressing human SPRY2 indicated early inhibition of organogenesis. The ureteric bud of the kidney of a mouse embryo at E12.5 branched to a lesser extent than that of a wild-type mouse at the corresponding developmental stage (Fig. 4, compare A-C with A’,A’’; other data not shown).

As Gdnf/Ret signalling is critical for the regulation of ureteric bud growth in the early stages of kidney organogenesis (Sainio et al., 1997), and has recently shown to be coordinated by Wnt11 (Majumdar et al., 2003), these genes were candidates for being influenced by human SPRY2. Gdnf is expressed in kidney mesenchymal cells at E12.5 and E17.5

Wnt11, a target gene for Gdnf signalling (Majumdar et al., 2003; Pepicelli et al., 1997), was also expressed in lesser amounts in the ureteric bud at E12.5 and E17.5 because of human SPRY2 expression (Fig. 4, compare C with C’,C’’). Consistent with the diminished Gdnf expression, the genes encoding the secreted signalling factor Bmp4 and the transcription factor Pax2, which are both implicated in kidney development (Miyazaki et al., 2000; Raatikainen-Ahokas et al., 2000; Torres et al., 1995), appeared to be normal at E12.5 and E15.5 regardless of human SPRY2 expression (Fig. 4D,D’,Fig. 5D-E’’). This supports the more specific notion that Spry protein signalling influences Wnt11/Gdnf cooperation (Majumdar et al., 2003), perhaps via regulation of Ret in the ureteric bud.

Human SPRY2 expression in the ureteric bud leads to changes in the stromal component of the embryonic kidney

The winged helix transcription factor Foxd1 is required for ureteric bud development and nephrogenesis via signals that
are secreted by the stromal cells (Hatini et al., 1996). Human SPRY2 signalling evidently influenced the stromal component, as Foxd1, a marker of these cells, was severely reduced at E12.5 and E15.5 in the transgenic kidneys relative to controls (Fig. 4, compare E with E'; Fig. 5, compare F with F',F'').

**Induction of Spry2 gene expression by Fgf7**

The Spry genes are induced in response to Fgf proteins, and are thought to antagonize Fgf signalling in order to control the cellular response to these factors (Mailleux et al., 2001). As the expression of three Fgf proteins in the kidney was altered because of human SPRY2 expression, we tested whether these Fgf proteins also regulated Spry2 gene expression. Agarose beads soaked with Fgf proteins were combined with kidneys isolated from E11.5 embryos derived from transgenic matings. The tissue was subcultured for 24 hours and changes in Spry2 expression were monitored. Although control beads soaked with BSA, Fgf8 or Fgf10 had no notable effect, beads releasing Fgf7 induced Spry2 expression in the nearby cells (Fig. 6A-D). Hence, Fgf7 expression is not only dependent on Spry2 function, but reciprocally, Fgf7 is also sufficient to coordinate Spry2 expression.

**Partial rescue of human SPRY2-mediated inhibition of kidney development by Fgf and Gdnf signalling**

The finding that ectopic human SPRY2 deregulated Fgf7 and Gdnf expression potentiated the hypothesis that human SPRY2 signalling is involved in the coordination of the reciprocal epithelial, mesenchymal and stromal signalling that controls kidney development via these factors. To address this point further, the development of kidneys expressing human SPRY2 was first monitored in organ culture by comparison with controls. Changes in ureteric bud branching and induced nephrogenesis were used as criteria for the progress of organogenesis and were identified with brush-border and cytokeratin antibody markers, which visualize the ureteric bud and the induced tubules (Fig. 6E-G; data not shown). The number of ureteric tips expressing human SPRY2 was reduced by 64.7% at 48 hours (Fig. 6G) and 80% at 144 hours (data not shown) relative to the wild-type kidneys in subculture, as estimated from kidneys immunoassayed with markers. It is significant that the reduced ureteric bud branching was associated with inhibition of mesenchymal tubulogenesis, as evaluated from the number of mesenchymal aggregates that expressed the Brush-border antigen (Fig. 6E,F). This is consistent with the reciprocal nature of inductive signalling between epithelium and mesenchyme ureteric branching and nephrogenesis. We conclude that human SPRY2 has an effect on kidney development under in vitro conditions, which provides an experimental model for more detailed studies.

We set out next to test whether the reduction in expression of Fgf and Gdnf resulting from human SPRY2 expression was causally associated with the phenotypes. For this purpose, the potential of these factors to rescue the defect caused by human SPRY2 was tested by exposing kidneys to them in organ culture. Fgf7, Fgf10 and GDNF all stimulated ureteric branching in the human SPRY2 kidneys, whereas Fgf2 had the opposite effect on this process relative to untreated kidneys also expressing the human SPRY2 gene (Fig. 6H-L). The quantification of the response to growth factors indicated in Fig. 6M is based on counting the number of epithelial tips. Surprisingly, the Wolffian duct of the human SPRY2 embryonic kidney appeared to be sensitized to Fgf7 and GDNF signalling, as these factors induced supernumerary bud formation from the rest of the Wolffian duct. Such an effect was not obtained in normal control kidneys when similarly exposed to these factors in the media (Fig. 6H,K,L, arrows and data not shown). The ectopic buds developed as a response to the growth factors during overnight culture of E11.5 kidneys expressing human SPRY2, and were documented with Fgf7 in 7/9 cases (77.8%) and with GDNF in 9/10 cases (90%).

Sprouty2 is thought to antagonize Fgf and Egfr signalling upstream of the ERK-MAP kinase pathway (Hanaoka et al., 2002). As an initial attempt to characterize the mechanism of signal transduction controlled by human SPRY2 in these
primary kidney rudiments, the inhibitor of the ERK1/2 pathway, PD98059, was used to specifically inhibit normal ureteric development (Fisher et al., 2001), thus demonstrating a phenotype that is related to that induced by human SPRY2 expression. However the drug had an additive effect on the human SPRY2-mediated defect in ureteric branching in all of the cases analysed. The bud was even further reduced in development and it generated a longer ureteric branch than in the untreated transgenic or wild-type control mice, as demonstrated also in skeletonized diagrams of the ureteric buds (Fig. 6N-Q). We conclude that besides Spry proteins other signal transduction pathways are also likely to be mediated via the ERK-MAP kinase pathway to contribute to ureteric bud development in the kidney.

Discussion
Ectopic human SPRY2 leads to severe defects in kidney organogenesis

Inductive signalling from the ureteric bud is known to regulate kidney development from the early stages onwards throughout organogenesis, but the mechanisms involved are still not well understood. To address these problems, human SPRY2, one of the Spry family of antagonists of receptor tyrosine kinases, which include the Fgf- and Egf-activated pathways, was targeted to the ureteric bud in vivo, and its expression was found to lead to postnatal death, probably owing to kidney failure. Hence, the in vivo genetic evidence points to a crucial role for Spry protein signalling in controlling the inductive signalling governing kidney development.

Human SPRY2-induced defects were manifested in complete agenesis, reduced size, lobularization or cystogenesis of the ureteric bud and an ectopic organ with a second complete ureteric bud was also seen to be formed because of human SPRY2-mediated signalling. Human SPRY2 expression also induced prenatal death, but the reason for this remains unknown at present. Degree of the severity of the kidney phenotypes are likely to be induced by the level of human SPRY2, as the crossing of two human SPRY2 carriers lead to an additive effect to the penetration of the kidney associated phenotypes. A higher level of human SPRY2 is expected to antagonize the associated inductive signal transduction pathway more efficiently, pointing to dose-dependent functioning of the pathway in the process. This point can be analysed once the tools become available. We conclude that the phenotypes caused by human SPRY2 expression suggest that Spry protein signalling is important in coordination of ureteric bud development during kidney organogenesis and that the mouse line serves as a useful model for studying the mechanisms of ureteric bud signalling and pattern formation during organogenesis.

Spry proteins have been implicated in the control of RTKs such as Fgfl/Egf receptors in other developmental systems, and as a target of Fgf signalling (Hacohen et al., 1998; Mailleux et al., 2001). Ectopic human SPRY2 was also found here to be associated with reduced Fgf2 expression in the ureteric bud and reduced Fgfr1 expression in the mesenchyme. In addition to these findings, stromally expressed Fgf7 also induced Spry2 gene expression. However, even though changed as a response to human SPRY2 expression, these factors are apparently not the primary targets of the human SPRY2 antagonism that is expected to take places in the ureteric bud. Deficiency in Fgf2 does not lead to any overt kidney phenotypes either (Dono et al., 1998). The fact that Fgf signalling induced Spry2 gene expression is consistent with the findings in the embryonic lung (Mailleux et al., 2001; Tefft et al., 2002) and in the zebrafish (Furthauer et al., 2001), for example, pointing that Fgf protein signalling controls Spry gene expression in these systems. However, in the kidney, the target of human SPRY2-mediated antagonism may include also other then the Fgf pathway.

Fig. 5. Expression of mesenchymal, epithelial and stromal markers in the embryonic kidney expressing human SPRY2. Gdnf, which is expressed in the nephrogenic mesenchyme, is downregulated by human SPRY2 (compare A with A’ and A”), while its Ret receptor encoding gene remains normally expressed irrespective of the presence of human SPRY2 (B-B”). Wnt11 expression is reduced in response to human SPRY2 expression (compare C with C’C”). Wnt11 also appears in the stromal cells of the transgenic kidney (C’C”). Pax2 and Bmp4 gene expression is unchanged (D-E”), while the stromal marker Foxd1 is reduced (F-F”) because of ectopic human SPRY2 (A-F”; E.15.5). Scale bar: 100 μm.
Coordination of Gdnf/Wnt11 signalling by human SPRY2 during early organogenesis

A deviation of kidney development from normal in response to human SPRY2 expression was already noted at E12.5, being manifested as a reduction in the number of ureteric tips relative to controls at the same developmental stage. This phenotype was associated with a reduction in the number of ureteric tips relative to controls at the same developmental stage. The asterisk in D indicates the site of the bead. (E) The kidney of a normal E11.5 embryo cultured for 48 hours shows an extensively branched ureteric bud (indicated in orange) and induced nephrons (indicated in green). (F) The kidney from an embryo expressing human SPRY2 has a ureteric bud with less branching and no nephrons at this stage of culture. (G) Quantification of the reduction in the degree of ureteric branching between wild-type and human SPRY2 kidneys (***P<0.005). A 48 hour culture. (H,K) Fgf7 and Gdnf, when administered to the culture media, alone (H) or in combination (K; Fgf2,7,10), lead to substantial recovery in the branching defect affecting the ureteric buds of the kidneys expressing human SPRY2 (compare H,I,K,L with F), whereas Fgf2 does not (J). Fgf7 signalling (H), a combination of Fgf7, Fgf2 and Fgf10 signalling (K), and GDNF signalling (L) induce the formation of supernumerary epithelial buds from the Wolffian duct (arrows), indicating that human SPRY2 sensitizes the kidney to these signals. (M) Quantification of the responses of human SPRY2 transgenic kidneys. Significant differences (**P<0.05), numbers of samples and growth factors analysed are indicated. Administration of PD98059, an inhibitor of ERK/MAP kinase, reduces branching of the ureteric bud in a normal kidney (compare N,O with E) and has an additive influence on the reduction in ureteric elongation when human SPRY2 is expressed (compare P,Q with F). Scale bar: 100 μm.

The signal transduction pathway regulated by Spry proteins is not known at present in the kidney. In other systems, Spry proteins interact with Cbl to control the activity of the receptor tyrosine kinase receptors (RTKs), while the Fgf proteins regulate the Spry-mediated antagonism by inducing degradation of Spry proteins, which then restores the sensitivity of the extracellular signal-regulated kinase (ERK) to Fgf signalling (Hall et al., 2003) and may serve as a mechanism in the embryonic kidney as well. Indeed the ERK/MAPK inhibitor PD98059 (2'-amino-3'-methoxyflavone) reduces ureteric branching in a wild-type embryonic kidney in vitro (Fish et al., 2001) and generates a phenotype that is related to that induced by human SPRY2, but the administration of PD98059 reduced ureteric branching even further in untreated kidneys expressing human SPRY2. We interpret these data as suggesting that, in addition to Spry proteins, other RTK signalling pathways, such as those activated by Fgf and Egf are transduced via the ERK/MAPK pathway in the embryonic kidney. As the Gdnf expression that mediates its signal transmission via an RTK receptor, Ret (Durbec et al., 1996), was indeed downregulated, Ret may be one primary candidate target for human SPRY2 antagonism in the ureteric bud (Fig. 7). As Ret is a important regulator of ureteric bud branching...
We noted that the expression of the stromal genes cells in kidney development and mesenchymal could be expected to reduce epithelial (Sainio et al., 1997), inhibition of its activity by Spry proteins Wnt11 and Saarma, 2002). and its ligand Gdnf are essential for kidney development (Airaksinen et al., 2001), perhaps indirectly. Sprouty signalling may contribute to the coordination of Wnt11/Gdnf/Ret pathways (Majumdar et al., 2003). As a RTK receptor effector, Sprouty may either directly antagonize Ret function or have an indirect effect via changes in reciprocal stromal signalling, such as Fgf7 or Foxd1-regulated signals (Hatini et al., 1996). Fgf7 signalling regulates Spry2 expression and is also influenced by human SPRY2 expression in the ureteric bud (red arrow), suggesting a link in inductive signalling between epithelial ureteric bud (U) and stromal cells (S). W, Wolffian duct; N, nephrogenic mesenchyme.

(Sainio et al., 1997), inhibition of its activity by Spry proteins could be expected to reduce epithelial Wnt11 and mesenchymal Gdnf gene expression (Majumdar et al., 2003) (Fig. 7), and consequently also ureteric branching, a possibility that warrants further investigation.

**Human SPRY2 expression and the role of stromal cells in kidney development**

We noted that the expression of the stromal genes Foxd1 and Fgf7 was altered because of ectopic human SPRY2 expression in the ureteric bud. Fgf7, which is normally expressed in the stromal cells, regulates kidney development via its receptors, which are thought to be located in the ureteric bud site of the targeted human SPRY2 expression. Moreover, Fgf7 knockout leads similarly to a reduction in the overall number of nephrons and the size of the kidney (Qiao et al., 1999), as attributed here to human SPRY2 expression. In further support of the speculation that ureteric bud signalling may also influence the stromal cells, human SPRY2 expression leads to a notable reduction in stromally located Foxd1 and Fgf7 expression (Fig. 7), and that the ectopic Fgf7 signalling that is normally localized to the stromal cells in the kidney (Qiao et al., 1999) also has an rescue effect to the deficiency in ureteric bud branching in kidneys expressing human SPRY2 in vitro. Batourina et al. (Batourina et al., 2001) proposed that a signalling loop from the stromal cells and ureteric bud regulates kidney development. Our data are consistent with a model that implies that, in addition to the stromal-ureteric bud interactions, the signalling is reciprocal, so that the ureteric bud also coordinates the stromal cells and involves activities of Spry2 (Fig. 7).

**Induction of supernumerary bud formation and ectopic organogenesis due to human SPRY2 expression**

In addition to the similarity between the phenotypes of human SPRY2 expression and Fgf7 deficiency, the consequences of the lack of function of other genes, the Bmp4 and Foxc genes, relate to the phenotypes observed in the presence of human SPRY2 expression. Like embryos expressing human SPRY2, the kidneys of Bmp4-deficient embryos are hypodysplastic and have hydroureter and double collecting ducts. Such phenotypes are thought to be related to human congenital anomalies of the kidney and urinary tract (CAKUT) (Miyazaki et al., 2000), and to be associated with reduction in the expression of Gdnf and Wnt11, which are also properties of ectopic human SPRY2. Like Bmp4 deficiency, human SPRY2 expression induces a complete second collecting duct system in vivo and in vitro. Moreover, in our case, exposure of the Wolffian duct to Fgf7 and Gdnf generated supernumerary epithelial buds not found in the non-transgenic kidneys and is the likely reason for the formation of the complete ectopic ureteric bud induced by human SPRY2 signalling. Hence, kidneys expressing human SPRY2 appear to be sensitised to generate ectopic buds from the Wolffian duct in response to Fgf7 and Gdnf signalling. Even though Bmp4 has been implicated in the budding process, we did not record any significant changes in its expression during early kidney development resulting from human SPRY2 expression. Hence, Spry protein signalling appears to be involved in the patterning mechanism that defines those cells that form the ureteric bud from the Wolffian duct. Our data suggest that this process may be distinct from the one caused by Bmp4. We conclude that Spry protein-regulated signalling is involved in coordination of the reciprocal epithelial, mesenchymal and stromal signalling via Wnt11, Gdnf and Fgf7 that controls the ureteric bud initiation and subsequent branching process during kidney development.

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**References**


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