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

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Accepted 31 March 2004

Development 131, 3345-3356
Published by The Company of Biologists 2004
doi:10.1242/dev.01200

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 Spry genes contribute to reciprocal epithelial-mesenchymal and stromal signalling controlling ureteric branching, which involves the coordination of *Ffg/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.,

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).

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/Gndf/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) *Fgf7* and retinoic acid appear to fulfil some of the criteria (Barasch et al., 1997; Batourina et al., 2001; Mason et al., 1994; Qiao et al., 1999).

Sprouty was identified in an essential gene in the branching process of the *Drosophila* tracheal system that apparently antagonizes both the Fgf and epidermal growth factor (Egf) signalling pathways (Casci et al., 1999; Hacohen et al., 1998; Wong et al., 2002). Four mouse *Sprouty* homologues have been identified to date and shown to be expressed during embryogenesis (Mailleux et al., 2001; Zhang et al., 2001). In the kidney, the Wilms tumor suppressor protein 1 gene that encodes a transcription factor crucial for nephrogenesis (Kreidberg et al., 1993) regulates *Spry1* as one target gene (Gross et al., 2003). This finding suggests a morphogenetic role for the Spry proteins in the developing kidney in vivo.

We have previously determined the expression pattern of the mouse *Spry1*, *Spry2* and *Spry4* genes in the developing kidney (Zhang et al., 2001). As Spry gene expression is prominent in the ureteric bud, this raised the possibility that the above factors might contribute to kidney organogenesis by controlling the inductive signalling pathways. This was addressed by expressing human *SPRY2* in the bud in vivo, which led to severe kidney defects and included either complete unilateral agenesis of the kidney, a reduction in its size or a division into separate lobes with an ectopic ureteric bud. *Spry2* signalling contributes to kidney development by coordinating *Wnt11/Gdnf/Fgf7* signalling, as expression of these genes was reduced in mutant cases and ectopic *Gdnf/Fgf7* signalling rescued ureteric bud branching in vitro. We propose that *Spry2* controls ureteric bud branching during kidney assembly by regulating the reciprocal cooperation between *Wnt11/Gdnf*-mediated epithelial-mesenchymal and stromal *Fgf7* signalling.

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 *Bam*HI/*Not*I 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 *Bam*HI/*Eco*RI 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′-GAATTCATGGAGGCCAGAGCTCAGAGTGG and 3′-CGCGGATCCCTATGTTGGTTTTTCAAAGTTCC primers and cloned to the expression vector, as indicated in Fig. 1A. The ready-made construct was excised from the vector backbone with *Sal*I (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′GAGTCCAAACCGGGCCCCTCTGC3′ (P1); 5′CGAG GAG CAGGCTTGAGCCCAGG3′ (P2) 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 *Bam*HI and run on a 1% agarose gel, blotted and probed with a [^{32}P]ATP-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,

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 (Fisher 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) (Mailleux 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 immunoassay 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; Kispert 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* (Bellusci et al., 1997), *Bmp4* (Bellusci 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 *Egf* 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 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 6 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′-diaminiobenzidine (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 DMLB fluorescence 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,J,K). As the third alternative, the presence of human *SPRY2* in the ureteric bud led to the

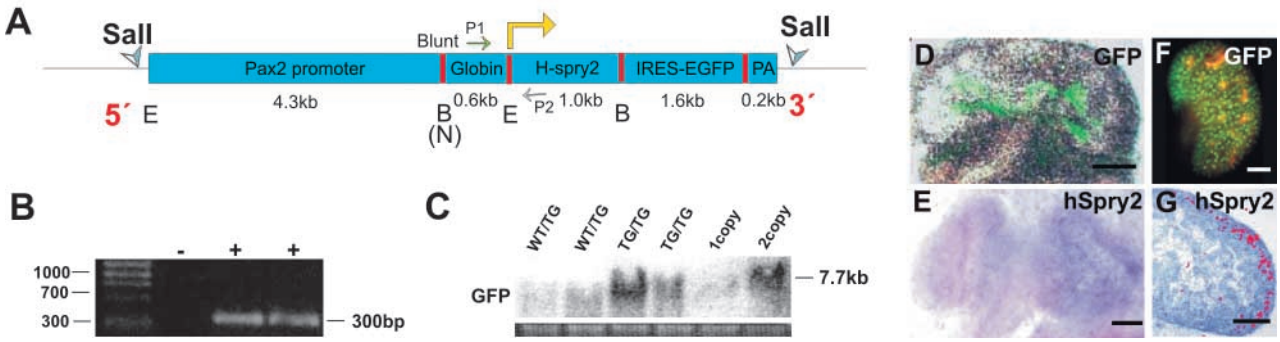

Fig. 1. Targeted expression of human *SPRY2* and *EGFP* in the ureteric bud in vivo. (A) Schematic structure of the expression construct generated by inserting a PCR fragment of human *SPRY2* cDNA into the *Eco*RI and *Bam*HI sites of a plasmid containing *IRES-EGFP*. The fragment was excised with *Eco*RI and *Ssp*I, and inserted into the *Eco*RI and *Sma*I sites of a modified bluescript vector. A *β-globin* splice acceptor was then cut with *Bam*HI and *Eco*RV, blunted and inserted into a blunted *Not*I site (N) downstream of the *Pax2* promoter. The fragment containing the *Pax2* promotor and *β-globin* was removed from the vector with *Eco*RI and ligated upstream of the human *SPRY2* cDNA, *IRES EGFP-PA*. The yellow arrow indicates the start site of transcription. B, *Bam*HI; *Eco*RI site, (N) defective *Not*I site; E, *Eco*RI. (B) An expected 300 bp fragment was amplified with P1 and P2 primers in PCR, indicating the presence of the transgene in the genome of the carriers. (C) The copy number of the transgene was estimated by Southern blotting using *GFP* as a probe. Control of loading is indicated below. The *Pax2* promoter drives expression of *EGFP* in the ureteric bud of a kidney at E11.5 (D) and E17.5 (F). The human *SPRY2* gene is also expressed in the kidney at E12.5 (E) and E17.5 (G), as judged by in situ hybridization. WT, wild type; TG, transgene carrier. Scale bars: 100 μm.

Table 1. Humans *SPRY2* transgene-induced lethality

Stage	Normal	TG	Lethality (%)
E15.5	40	22	45
E17.5	40	12	70
Newborn	47	10	47
2 weeks	39	4	90
1 month	84	8	91

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.

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

	WT/TG kidneys				TG/TG kidneys			
Stage	Number analysed	Reduced (%)	Cystic (%)	Ectopic (%)	Number analysed	Reduced (%)	Cystic (%)	Ectopic (%)
E15.5	42	40 (95.24)	0	2 (4.76)	23	17 (73.91)	5 (21.74)	1 (4.35)
E17.5	9	33 (84.62)	2 (5.13)	4 (10.25)	12	10 (83.33)	2 (16.67)	0
Newborn	150	122 (81.33)	10 (6.67)	18 (12.00)	26	20 (76.92)	4 (15.39)	2 (7.69)
Total	231	195 (84.42)	12 (5.19)	24 (10.39)	61	47 (77.05)	11 (18.03)	3 (4.92)

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.

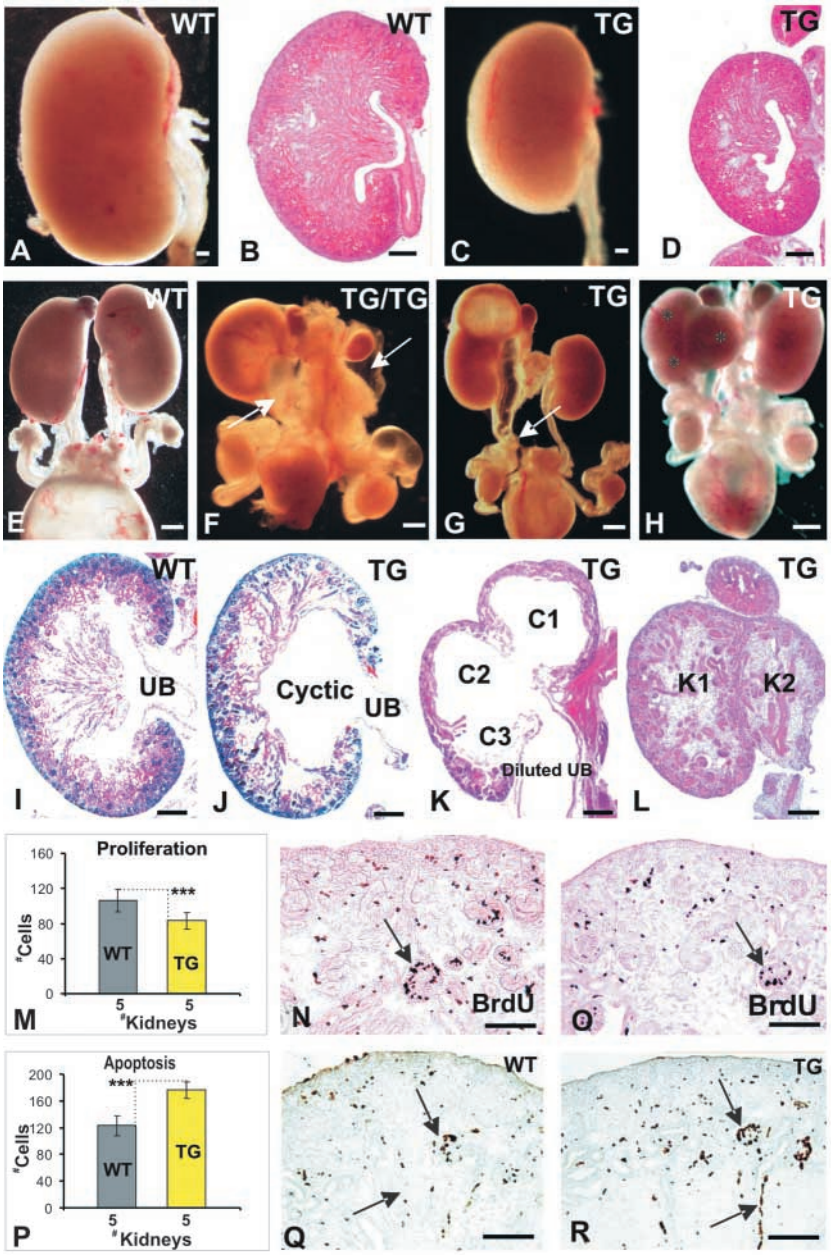

Fig. 2. Human *SPRY2* expression leads to severe defects in kidney development. When compared with the kidney of a wild-type embryo (A,B), human *SPRY2* (TG) expression leads to reduced size of the organ (C,D; E17.5), unilateral agenesis (F, arrow on the right), cystogenesis with a blind-ended hydroureter (F,G,K; F,G, arrows), unilateral lobularization of the kidney (H, stars; E17.5) with cysts (C1-C3 in K; E17.5) or an ectopic second ureteric bud (K1 and K2 in L) when compared with wild-type (WT) controls (A,B,E,I; E17.5). Reduction in the size of the kidney is associated with reduced cell proliferation (M; ***P<0.005) between the wild-type and transgenic organ in derivatives of the ureteric bud and glomeruli (N,O arrows; E15.5). Human *SPRY2* expression also leads to stimulation of apoptosis (P; ***P<0.005) in derivatives of the ureteric bud and glomeruli (Q,R arrows). Scale bars: 100 μm. K1, host kidney; K2, ectopic kidney; C1-C3, cystic lobules).

regulate 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*

Stage	Genotype	Number of glomeruli	Number of kidneys
E17.5	WT	682±148	5
	TG	513±178	6
NB	WT	1630±197	5
	TG	958±113	6

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.

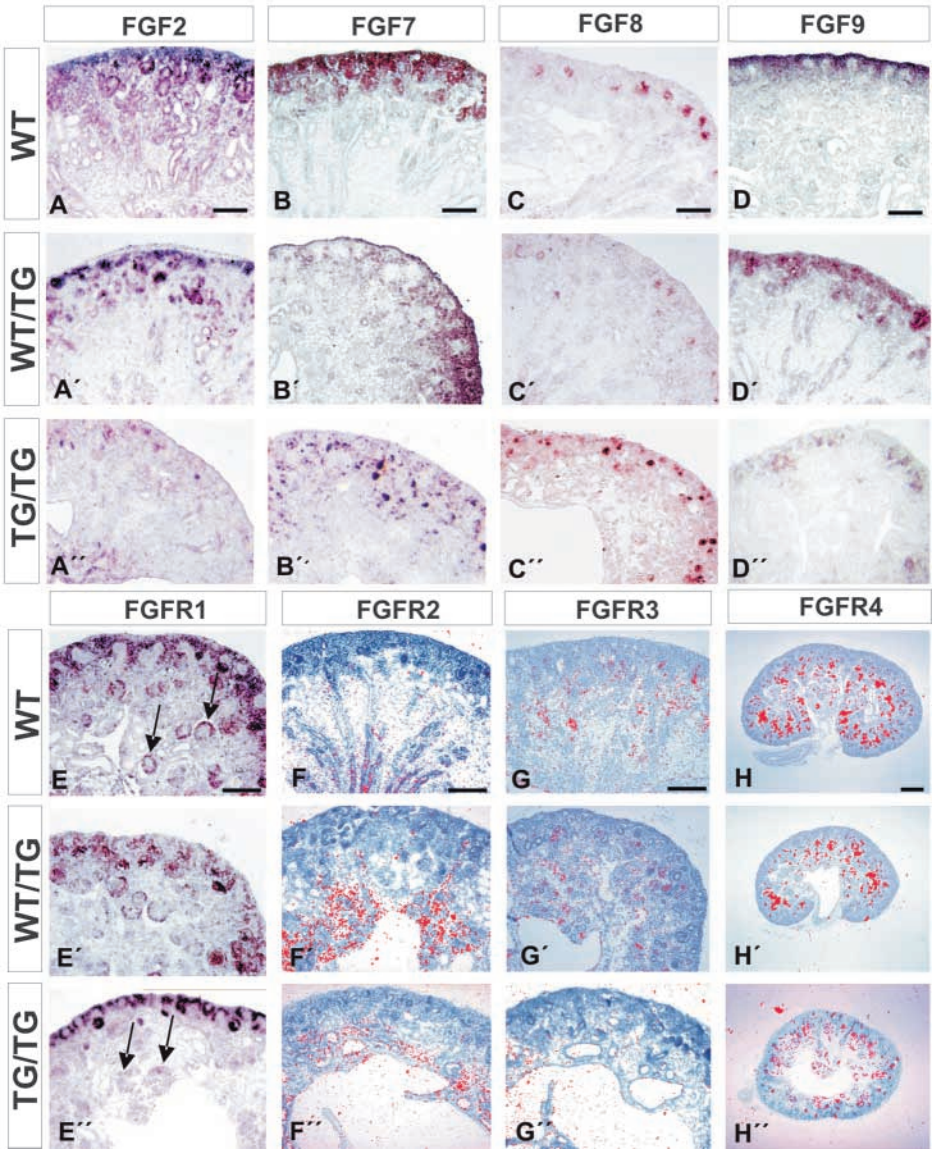

Fig. 3. Expression of candidate targets for human *SPRY2*-mediated antagonism of Fgf genes and Fgfr genes in the embryonic kidney. *Fgf2* is expressed in the ureteric bud, mesenchymal cells and assembling nephrons, and human *SPRY2* expression leads to reduced *Fgf2* gene expression in the ureteric bud (compare A with A′,A″). Expression of *Fgf7*, a stromal marker (Finch et al., 1995), is also reduced because of human *SPRY2* expression when compared with the normal kidney (compare B with B′,B″), while *Fgf8* remains unchanged (C,C′,C″). *Fgf9* is again reduced, especially when the transgene is inherited from both the female and male (compare D,D′ with D″). *Fgfr1*, which is expressed in mesenchymal cells and in developing nephrons (E arrows), is not detected in more mature nephrons, owing to human *SPRY2* expression (compare E,E′ with E″). Expression of *Fgfr2*, *Fgfr3* and *Fgfr4* appeared to be unchanged in the presence of human *SPRY2* gene expression (F-H″). (A-H″) E15.5. Scale bars: 100 μm. Fgfr, Fgf receptor; WT, wild type; TG, explants containing human *SPRY2*.

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′-C′; 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 (Fig. 4A and Fig. 5A), and human *SPRY2* expression led to a notable reduction at these stages (Fig. 4A′) relative to controls at E12.5 and E15.5 (Fig. 5, compare A to A′,A″). Irrespective of the reduction in *Gdnf* expression, expression of *Ret*, which encodes one of the identified receptors for *Gdnf*, was unchanged at the corresponding developmental stages (Fig. 4, compare B with B′; Fig. 5, compare B with B′,B″). Consistent with the diminished *Gdnf* expression, *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′; Fig. 5, compare C with C′,C″).

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

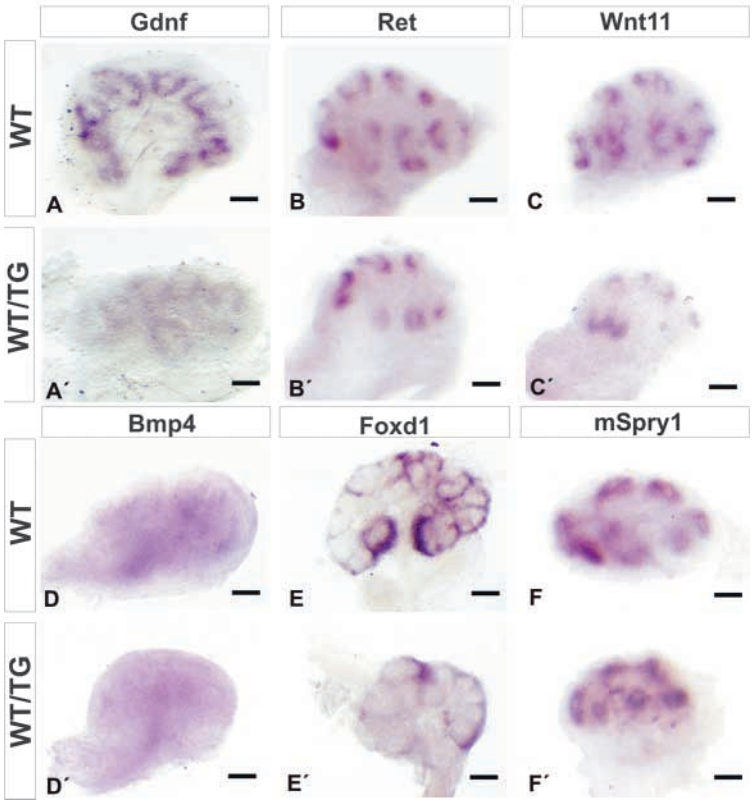

Fig. 4. Ectopic human *SPRY2* in the ureteric bud leads to deregulated expression of *Wnt11/Gdnf* and *Foxd1* and is associated with reduced branching. (A) *Gdnf* is expressed in the kidney mesenchyme, its gene encoding the *Ret* receptor and another gene *Wnt11* in the ureteric bud (B,C). At the same developmental stage, *Gdnf* (A′) and *Wnt11* (C′) expression is reduced in response to human *SPRY2* expression, while *Ret* remains unchanged (B′). Note that there are less ureteric tips in the kidneys expressing human *SPRY2*, as indicated by the tip markers (compare B,C with B′,C′). While *Bmp4* expression shows no notable changes (D,D′), the stromal marker *Foxd1* is clearly reduced due to human *SPRY2* expression (compare E with E′). Mouse *Spry1* expression remains constant irrespective of expression of the human *SPRY2* transgene (F,F′). (A-F′) E12.5. Scale bars: 100 μm. WT, wild type; TG, explants containing human *SPRY2*.

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 Fgfr and Egfr signalling upstream of the ERK-MAP kinase pathway (Hanafusa et al., 2002). As an initial attempt to characterize the mechanism of signal transduction controlled by human *SPRY2* in these

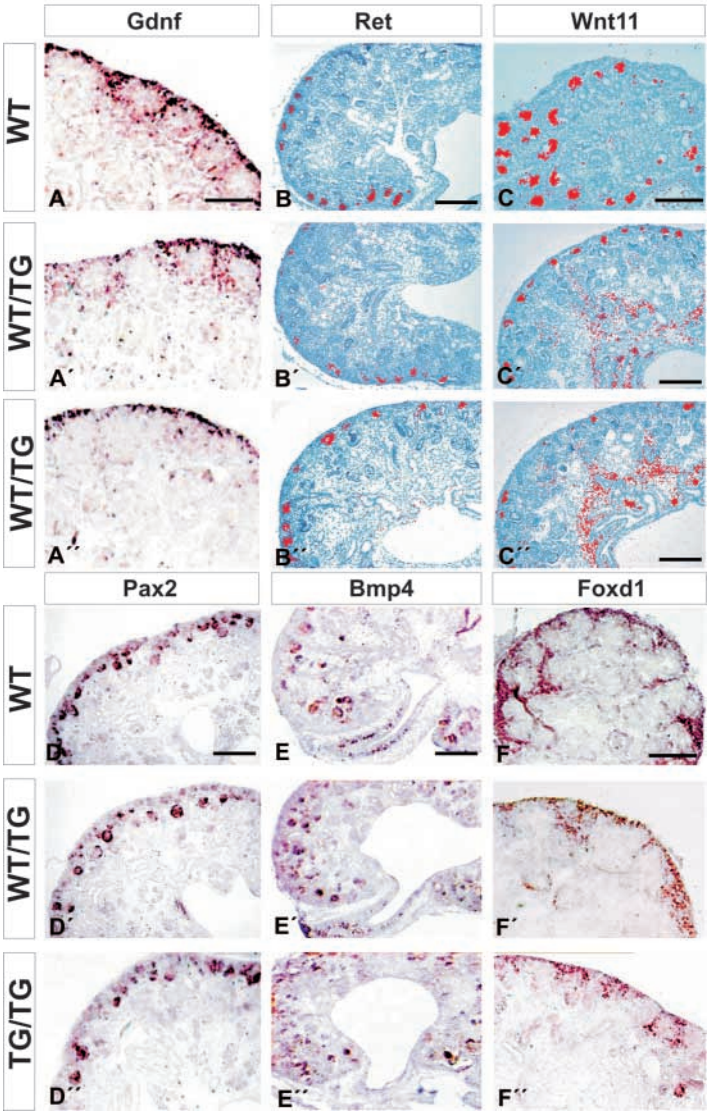

Fig. 5. Expression of mesenchymal, epithelial and stromal markers in the embryonic kidney expressing human *SPRY2*. *Gdnf*, which is expressed in the nephrogenic mesenchyme, is downregulated by human *SPRY2* (compare A with A′ and A″), while its *Ret* receptor encoding gene remains normally expressed 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.

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 Fgf/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.

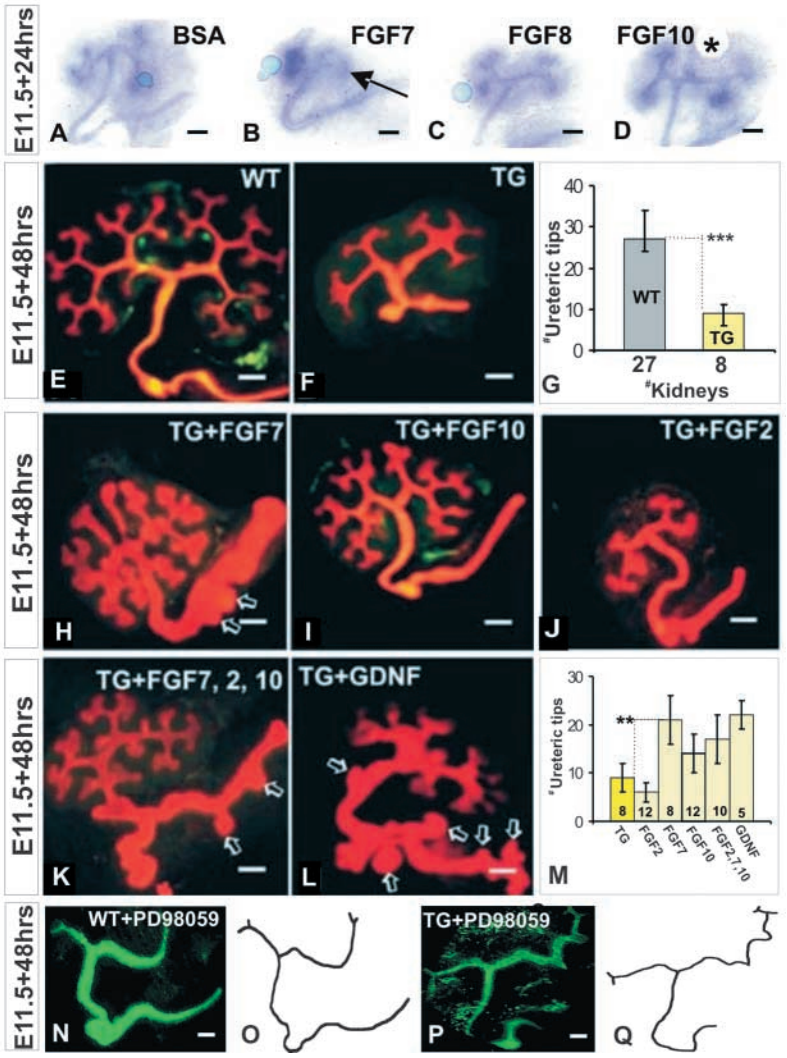

Fig. 6. Human *SPRY2* mediates its effect via Fgf genes and resembles the phenotype induced by inhibition of the ERK/MAPK pathway. Although local application of an agarose bead soaked with BSA, Fgf8 or Fgf10 (A,C,D) does not alter *Spry2* gene expression, a Fgf7-soaked bead (B) induces its expression in culture when compared with the contralateral side that serves as a control (arrow). 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.

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 expression of *Gdnf* and *Wnt11* genes. *Wnt11* has recently been shown to be a functional signal around the same time as the phenotype of the human *SPRY2* transgenic embryos becomes noticeable (Majumdar et al., 2003). In the case of *Wnt11* deficiency, *Gdnf* expression is also reduced, suggesting that *Wnt11* may regulate *Gdnf* expression in kidney mesenchyme, either directly or via another signal (Fig. 7). The *Wnt11* gene, in turn, is also regulated by Ret signalling, as *Wnt11* expression is reduced in the kidneys of *Ret*-deficient embryos (Majumdar et al., 2003) (Fig. 7). The downregulated expression of *Wnt11* and *Gdnf* genes in kidneys expressing human *SPRY2* and the finding that the ureteric bud defect in the transgenic kidneys was substantially reversed via stimulated *Gdnf* signalling in vitro, support the hypothesis that human *SPRY2* contributes to cooperation between the *Wnt11/Gdnf/Ret* pathways during early kidney development perhaps by directly antagonising *Ret* signalling (Fig. 7).

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 (Fisher 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

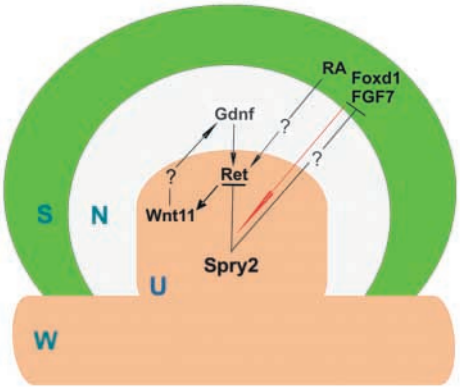

Fig. 7. Schematic model of some mediators of inductive tissue interactions in the embryonic kidney. Ret, a tyrosine kinase receptor, and its ligand Gdnf are essential for kidney development (Airaksinen and Saarma, 2002). *Wnt11* is necessary for *Gdnf* expression, while *Ret* signalling regulates *Wnt11* expression (Majumdar et al., 2003). Stromal components in the retinoic acid (RA) signalling system also contribute to *Ret* expression (Batourina 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 hypo/dysplastic 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.

We thank Hannele Härkman, Sanna Kalmukoski, Johanna Kekolahti-Liias and Mervi Matero for their excellent technical assistance; Dr Gregory Dressler for the *Pax2* promotor; and Dr Juha Tuukkanen (Biocenter Oulu and Department of Anatomy and Cell Biology, University of Oulu, Oulu, Finland) for the morphometry. This work was supported financially by the Academy of Finland (41001, 49986), the Sigrid Jusélius Foundation and the European Union (QLRT-2000-01275).

References

Airaksinen, M. S. and Saarma, M. (2002). The GDNF family: signalling, biological functions and therapeutic value. *Nat. Rev. Neurosci.* **3**, 383-394.

Barasch, J., Qiao, J., McWilliams, G., Chen, D., Oliver, J. A. and Herzlinger, D. (1997). Ureteric bud cells secrete multiple factors, including bFGF, which rescue renal progenitors from apoptosis. *Am. J. Physiol.* **273**, F757-F767.

Batourina, E., Gim, S., Bello, N., Shy, M., Clagett-Dame, M., Srinivas, S., Costantini, F. and Mendelsohn, C. (2001). Vitamin A controls epithelial/mesenchymal interactions through Ret expression. *Nat. Genet.* **27**, 74-78.

Bellusci, S., Henderson, R., Winnier, G., Oikawa, T. and Hogan, B. L. (1996). Evidence from normal expression and targeted misexpression that bone morphogenetic protein (BMP-4) plays a role in mouse embryonic lung morphogenesis. *Development* **122**, 1693-1702.

Bellusci, S., Grindley, J., Emoto, H., Itoh, N. and Hogan, B. L. (1997).

Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* **124**, 4867-4878.

Bertram, J. F., Soosaipillai, M. C., Ricardo, S. D. and Ryan, G. B. (1992). Total numbers of glomeruli and individual glomerular cell types in the normal rat kidney. *Cell Tissue Res.* **270**, 37-45.

Brenner, B. M. and Milford, E. L. (1993). Nephron underdosing: a programmed cause of chronic renal allograft failure. *Am. J. Kidney Dis.* **21**, 66-72.

Brophy, P. D., Ostrom, L., Lang, K. M. and Dressler, G. R. (2001). Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene. *Development* **128**, 4747-4756.

Casci, T., Vinos, J. and Freeman, M. (1999). Sprouty, an intracellular inhibitor of Ras signaling. *Cell* **96**, 655-665.

Colvin, J. S., Bohne, B. A., Harding, G. W., McEwen, D. G. and Ornitz, D. M. (1996). Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat. Genet.* **12**, 390-397.

Colvin, J. S., Feldman, B., Nadeau, J. H., Goldfarb, M. and Ornitz, D. M. (1999). Genomic organization and embryonic expression of the mouse fibroblast growth factor 9 gene. *Dev. Dyn.* **216**, 72-88.

Crossley, P. H. and Martin, G. R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.

Davies, J. A. and Bard, J. B. (1998). The development of the kidney. *Curr. Top. Dev. Biol.* **39**, 245-301.

DePamphilis, M. L., Herman, S. A., Martinez-Salas, E., Chalifour, L. E., Wirak, D. O., Cupo, D. Y. and Miranda, M. (1988). Microinjecting DNA into mouse ova to study DNA replication and gene expression and to produce transgenic animals. *Biotechniques* **6**, 662-680.

Dolbeare, F. (1995). Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part II: Oncology, chemotherapy and carcinogenesis. *Histochem. J.* **27**, 923-964.

Dono, R., Texido, G., Dussel, R., Ehmke, H. and Zeller, R. (1998). Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. *EMBO J.* **17**, 4213-4225.

Dressler, G. R., Deutsch, U., Chowdhury, K., Nornes, H. O. and Gruss, P. (1990). Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development* **109**, 787-795.

Drummond, I. A., Mukhopadhyay, D. and Sukhatme, V. P. (1998). Expression of fetal kidney growth factors in a kidney tumor line: role of FGF2 in kidney development. *Exp. Nephrol.* **6**, 522-533.

Dudley, A. T. and Robertson, E. J. (1997). Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. *Dev. Dyn.* **208**, 349-362.

Dudley, A. T., Godin, R. E. and Robertson, E. J. (1999). Interaction between FGF and BMP signaling pathways regulates development of metanephric mesenchyme. *Genes Dev.* **13**, 1601-1613.

Dunn, N. R., Winnier, G. E., Hargett, L. K., Schrick, J. J., Fogo, A. B. and Hogan, B. L. (1997). Haploinsufficient phenotypes in BMP4 heterozygous null mice and modification by mutations in Gli3 and Alx4. *Dev. Biol.* **188**, 235-247.

Durbec, P., Marcos-Gutierrez, C. V., Kilkenny, C., Grigoriou, M., Wartiowaara, K., Suvanto, P., Smith, D., Ponder, B., Costantini, F., Saarma, M. et al. (1996). GDNF signalling through the Ret receptor tyrosine kinase. *Nature* **381**, 789-793.

Ekblom, P. (1981). Formation of basement membranes in the embryonic kidney: an immunohistological study. *J. Cell Biol.* **91**, 1-10.

Finch, P. W., Cunha, G. R., Rubin, J. S., Wong, J. and Ron, D. (1995). Pattern of keratinocyte growth factor and keratinocyte growth factor receptor expression during mouse fetal development suggests a role in mediating morphogenetic mesenchymal-epithelial interactions. *Dev. Dyn.* **203**, 223-240.

Fisher, C. E., Michael, L., Barnett, M. W. and Davies, J. A. (2001). Erk MAP kinase regulates branching morphogenesis in the developing mouse kidney. *Development* **128**, 4329-4338.

Ford, M. D., Cauchi, J., Greferath, U. and Bertram, J. F. (1997). Expression of fibroblast growth factors and their receptors in rat glomeruli. *Kidney Int.* **51**, 1729-1738.

Furthauer, M., Reifers, F., Brand, M., Thisse, B. and Thisse, C. (2001). sprouty4 acts in vivo as a feedback-induced antagonist of FGF signaling in zebrafish. *Development* **128**, 2175-2186.

Gross, I., Morrison, D. J., Hyink, D. P., Georgas, K., English, M. A., Mericskay, M., Hosono, S., Sassoon, D., Wilson, P. D., Little, M. et al. (2003). The receptor tyrosine kinase regulator Sprouty1 is a target of the tumor suppressor WT1 and important for kidney development. *J. Biol. Chem.* **278**, 41420-41430.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y. and Krasnow, M. A. (1998). sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* **92**, 253-263.

Hall, A. B., Jura, N., DaSilva, J., Jang, Y. J., Gong, D. and Bar-Sagi, D. (2003). hSpry2 is targeted to the ubiquitin-dependent proteasome pathway by c-Cbl. *Curr. Biol.* **13**, 308-314.

Hanafusa, H., Torii, S., Yasunaga, T. and Nishida, E. (2002). Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nat. Cell Biol.* **4**, 850-858.

Hatini, V., Huh, S. O., Herzlinger, D., Soares, V. C. and Lai, E. (1996). Essential role of stromal mesenchyme in kidney morphogenesis revealed by targeted disruption of Winged Helix transcription factor BF-2. *Genes Dev.* **10**, 1467-1478.

Itaranta, P., Lin, Y., Perasaari, J., Roel, G., Destree, O. and Vainio, S. (2002). Wnt-6 is expressed in the ureter bud and induces kidney tubule development in vitro. *Genesis* **32**, 259-268.

Kispert, A., Vainio, S. and McMahon, A. P. (1998). Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. *Development* **125**, 4225-4234.

Kispert, A., Vainio, S., Shen, L., Rowitch, D. H. and McMahon, A. P. (1996). Proteoglycans are required for maintenance of Wnt-11 expression in the ureter tips. *Development* **122**, 3627-3637.

Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D. and Jaenisch, R. (1993). WT-1 is required for early kidney development. *Cell* **74**, 679-691.

Kuschert, S., Rowitch, D. H., Haenig, B., McMahon, A. P. and Kispert, A. (2001). Characterization of Pax-2 regulatory sequences that direct transgene expression in the Wolffian duct and its derivatives. *Dev. Biol.* **229**, 128-140.

Kuure, S., Vuolteenaho, R. and Vainio, S. (2000). Kidney morphogenesis: cellular and molecular regulation. *Mech. Dev.* **92**, 31-45.

Lin, Y., Liu, A., Zhang, S., Ruusunen, T., Kreidberg, J. A., Peltoketo, H., Drummond, I. and Vainio, S. (2001a). Induction of ureter branching as a response to Wnt-2b signaling during early kidney organogenesis. *Dev. Dyn.* **222**, 26-39.

Lin, Y., Zhang, S., Rehn, M., Itaranta, P., Tuukkanen, J., Heljasvaara, R., Peltoketo, H., Pihlajaniemi, T. and Vainio, S. (2001b). Induced repatterning of type XVIII collagen expression in ureter bud from kidney to lung type: association with sonic hedgehog and ectopic surfactant protein C. *Development* **128**, 1573-1585.

Mailleux, A. A., Tefft, D., Ndiaye, D., Itoh, N., Thiery, J. P., Warburton, D. and Bellusci, S. (2001). Evidence that SPROUTY2 functions as an inhibitor of mouse embryonic lung growth and morphogenesis. *Mech. Dev.* **102**, 81-94.

Majumdar, A., Vainio, S., Kispert, A., McMahon, J. and McMahon, A. P. (2003). Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. *Development* **130**, 3175-3185.

Mason, I. J., Fuller-Pace, F., Smith, R. and Dickson, C. (1994). FGF-7 (keratinocyte growth factor) expression during mouse development suggests roles in myogenesis, forebrain regionalisation and epithelial-mesenchymal interactions. *Mech. Dev.* **45**, 15-30.

Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pedersen, R. A., Werb, Z. and Derynck, R. (1995). Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* **376**, 337-341.

Minowada, G., Jarvis, L. A., Chi, C. L., Neubuser, A., Sun, X., Hacohen, N., Krasnow, M. A. and Martin, G. R. (1999). Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* **126**, 4465-4475.

Miyazaki, Y., Oshima, K., Fogo, A., Hogan, B. L. and Ichikawa, I. (2000). Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter. *J. Clin. Invest.* **105**, 863-873.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K. and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-79.

Nagy, A., Gertsenstein, M., Vintersten, K. and Behringer, R. (2003). *Manipulating the Mouse Embryo: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory.

Parr, B. A., Shea, M. J., Vassileva, G. and McMahon, A. P. (1993). Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119**, 247-261.

Pepicelli, C. V., Kispert, A., Rowitch, D. H. and McMahon, A. P. (1997).

GDNF induces branching and increased cell proliferation in the ureter of the mouse. *Dev. Biol.* **192**, 193-198.

Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J. et al. (1996). GDNF is required for kidney development and enteric innervation. *Cold Spring Harb. Symp. Quant. Biol.* **61**, 445-457.

Qiao, J., Uzzo, R., Obara-Ishihara, T., Degenstein, L., Fuchs, E. and Herzlinger, D. (1999). FGF-7 modulates ureteric bud growth and nephron number in the developing kidney. *Development* **126**, 547-554.

Qiao, J., Bush, K. T., Steer, D. L., Stuart, R. O., Sakurai, H., Wachsman, W. and Nigam, S. K. (2001). Multiple fibroblast growth factors support growth of the ureteric bud but have different effects on branching morphogenesis. *Mech. Dev.* **109**, 123-135.

Raatikainen-Ahokas, A., Hytonen, M., Tenhunen, A., Sainio, K. and Sariola, H. (2000). BMP-4 affects the differentiation of metanephric mesenchyme and reveals an early anterior-posterior axis of the embryonic kidney. *Dev. Dyn.* **217**, 146-158.

Rosenquist, T. A. and Martin, G. R. (1996). Fibroblast growth factor signalling in the hair growth cycle: expression of the fibroblast growth factor receptor and ligand genes in the murine hair follicle. *Dev. Dyn.* **205**, 379-386.

Ryan, G., Steele-Perkins, V., Morris, J. F., Rauscher, F. J., 3rd and Dressler, G. R. (1995). Repression of Pax-2 by WT1 during normal kidney development. *Development* **121**, 867-875.

Sainio, K., Suvanto, P., Davies, J., Wartiovaara, J., Wartiovaara, K., Saarma, M., Arumae, U., Meng, X., Lindahl, M., Pachnis, V. et al. (1997). Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. *Development* **124**, 4077-4087.

Santos-Ocampo, S., Colvin, J. S., Chellaiah, A. and Ornitz, D. M. (1996). Expression and biological activity of mouse fibroblast growth factor-9. *J. Biol. Chem.* **271**, 1726-1731.

Sasaki, H. and Hogan, B. L. (1994). HNF-3 beta as a regulator of floor plate development. *Cell* **76**, 103-115.

Saxen, L. (1987). *Organogenesis of Kidney*. Cambridge: Cambridge University Press.

Stark, K., Vainio, S., Vassileva, G. and McMahon, A. P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* **372**, 679-683.

Tang, M. J., Cai, Y., Tsai, S. J., Wang, Y. K. and Dressler, G. R. (2002). Ureteric bud outgrowth in response to RET activation is mediated by phosphatidylinositol 3-kinase. *Dev. Biol.* **243**, 128-136.

Tefft, D., Lee, M., Smith, S., Crowe, D. L., Bellusci, S. and Warburton, D. (2002). mSprouty2 inhibits FGF10-activated MAP kinase by differentially binding to upstream target proteins. *Am. J. Physiol. Lung Cell Mol. Physiol.* **283**, L700-L706.

Torres, M., Gomez-Pardo, E., Dressler, G. R. and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. *Development* **121**, 4057-4065.

Vainio, S. and Lin, Y. (2002). Coordinating early kidney development: lessons from gene targeting. *Nat. Rev. Genet.* **3**, 533-543.

Vainio, S., Karavanova, I., Jowett, A. and Thesleff, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* **75**, 45-58.

Vainio, S. J., Itaranta, P. V., Perasaari, J. P. and Uusitalo, M. S. (1999). Wnts as kidney tubule inducing factors. *Int. J. Dev. Biol.* **43**, 419-423.

Vize, P. D., Woolf, A. S. and Bard, J. B. L. (2003). *The Kidney: From Normal Development to Congenital Disease*. USA: Elsevier.

Walshe, J. and Mason, I. (2000). Expression of FGFR1, FGFR2 and FGFR3 during early neural development in the chick embryo. *Mech. Dev.* **90**, 103-110.

Wilkinson, D. G., Bhatt, S. and McMahon, A. P. (1989). Expression pattern of the FGF-related proto-oncogene int-2 suggests multiple roles in fetal development. *Development* **105**, 131-136.

Wong, E. S., Fong, C. W., Lim, J., Yusoff, P., Low, B. C., Langdon, W. Y. and Guy, G. R. (2002). Sprouty2 attenuates epidermal growth factor receptor ubiquitylation and endocytosis, and consequently enhances Ras/ERK signalling. *EMBO J.* **21**, 4796-4808.

Yamaguchi, F., Saya, H., Bruner, J. M. and Morrison, R. S. (1994). Differential expression of two fibroblast growth factor-receptor genes is associated with malignant progression in human astrocytomas. *Proc. Natl. Acad. Sci. USA* **91**, 484-488.

Zhang, S., Lin, Y., Itaranta, P., Yagi, A. and Vainio, S. (2001). Expression of Sprouty genes 1, 2 and 4 during mouse organogenesis. *Mech. Dev.* **109**, 367-370.