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# Branching morphogenesis of the ureteric epithelium during kidney development is coordinated by the opposing functions of GDNF and Sprouty1

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**Abstract**

Branching of ureteric bud-derived epithelial tubes is a key morphogenetic process that shapes development of the kidney. Glial cell line-derived neurotrophic factor (GDNF) initiates ureteric bud formation and promotes subsequent branching morphogenesis. Exactly how GDNF coordinates branching morphogenesis is unclear. Here we show that the absence of the receptor tyrosine kinase antagonist Sprouty1 (*Spry1*) results in irregular branching morphogenesis characterized by both increased number and size of ureteric bud tips. Deletion of *Spry1* specifically in the epithelium is associated with increased epithelial *Wnt11* expression as well as increased mesenchymal *Gdnf* expression. We propose that *Spry1* regulates a Gdnf/Ret/Wnt11-positive feedback loop that coordinates mesenchymal–epithelial dialogue during branching morphogenesis. Genetic experiments indicate that the positive (GDNF) and inhibitory (Sprouty1) signals have to be finely balanced throughout renal development to prevent hypoplasia or cystic hyperplasia. Epithelial cysts develop in *Spry1*-deficient kidneys that share several molecular characteristics with those observed in human disease, suggesting that *Spry1* null mice may be useful animal models for cystic hyperplasia.


*Keywords:* Kidney development; Ureteric epithelium; Branching morphogenesis; GDNF; *Sprouty1*; Cystic kidney disease; Renal hypoplasia

## Introduction

Branching morphogenesis of ureteric bud-derived epithelial tubes is essential for development of the mammalian kidney (Saxen, 1987). The importance of branching morphogenesis for normal renal organogenesis is underscored by the demonstration that mutations affecting this process often result in kidney malformations such as multicystic renal dysplasia and hypoplasia (Matsell et al., 1996; Piscione and Rosenblum, 2002).

The first step in kidney development is the outgrowth of the ureteric bud (UB) from the caudal Wolffian duct (WD) (Saxen, 1987). UB outgrowth is induced by GDNF (Moore et al., 1996; Pichel et al., 1996; Sainio et al., 1997; Sanchez et al., 1996; Towers et al., 1998; Vega et al., 1996) in a process regulated by Sprouty1 (*Spry1*) (Basson et al., 2005). Reciprocal signaling between the UB epithelium and metanephric mesenchyme (MM) regulates subsequent growth and branching morphogenesis of the UB-derived epithelial tubes to form a tree-like

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structure, the ureteric tree that develops into the collecting system of the kidney. Multiple growth factors expressed in the metanephric mesenchyme have been proposed to play roles in morphogenesis of the ureteric tree (Schedl and Hastie, 2000). In particular, both *in vitro* and *in vivo* data provide compelling evidence for a central role for glial cell line-derived neurotrophic factor (GDNF) in orchestrating branching morphogenesis (Michael and Davies, 2004; Moore et al., 1996; Pepicelli et al., 1997; Pichel et al., 1996; Sanchez et al., 1996; Towers et al., 1998; Vega et al., 1996). The GDNF receptor RET and co-receptor GFRα1 are highly expressed in the tips of the branching ureteric tree and mice lacking these genes have severe defects in both ureteric bud formation and branching (Cacalano et al., 1998; Schuchardt et al., 1994). Furthermore, a proportion of *Gdnf* heterozygous mice exhibit unilateral renal agenesis or hypoplasia, indicating that kidney development is highly sensitive to modest changes in GDNF signaling (Cullen-McEwen et al., 2001; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Finally, blockade of GDNF/RET signaling during the culture of kidney rudiments *in vitro* inhibits branching morphogenesis (Davies et al., 1999; Ehrenfels et al., 1999; Fisher et al., 2001; Vega et al., 1996). Thus, a likely explanation for the reduction in kidney size in $Gdnf^{+/-}$ mice is a paucity of branching of the ureteric epithelium during development. *In vitro* evidence also suggests a role for members of the fibroblast growth factor (Fgf) family in branching morphogenesis (Qiao et al., 2001). *In vivo* support for this notion is provided by the demonstration that mice lacking *Fgf7* or *Fgf10* have smaller kidneys due to reduced branching morphogenesis of the ureteric tree (Ohuchi et al., 2000; Qiao et al., 1999). Similar defects are found in mice in which *Fgfr2* is deleted in the ureteric epithelium (Zhao et al., 2004). Finally, overexpression of a human *Spy2* transgene in mouse kidneys interferes with GDNF- and FGF-induced branching morphogenesis (Chi et al., 2004).

Ectopic ureteric buds develop in the majority of embryos lacking the receptor tyrosine kinase antagonist, *Spry1* (Basson et al., 2005), indicating an important role for this gene in the initiation of renal development. We now show that *Spry1* mutant kidneys have defects in every generation of branching morphogenesis of the ureteric tree irrespective of whether the kidneys are derived from a single or multiple ureteric buds. We conclude that *Spry1* functions throughout kidney development to regulate GDNF/RET-mediated epithelial morphogenesis.

## Materials and methods

### Mice

*Spry1* null ($Spry1^{-}$) and conditional null ($Spry1^{flox}$) mice (Basson et al., 2005) were maintained on a mixed genetic background. Tissue-specific knockouts were generated by breeding $Spry1^{flox/flox}$ females to *Hoxb7-cre;* $Spry1^{flox/+}$ males (Yu et al., 2002). Mice carrying a *Gdnf* null allele (Sanchez et al., 1996), were crossed to the $Spry1^{+/-}$ animals. $Spry1^{+/-}$;$Gdnf^{+/-}$ males were bred to $Spry1^{+/-}$ females to produce embryos and newborns that were analyzed for defects in kidney development. All experiments involving animals were approved by the Mount Sinai School of Medicine and Columbia University Institutional Animal Care and Use Committees (USA) and the Home Office (UK).

### Immunohistochemistry

Newborn kidneys were fixed with 4% paraformaldehyde in PBS for 2–4 h at 4°C, embedded in wax and 7 μm sections cut. After dewaxing and rehydration, sections were stained for 1.5 h at room temperature with primary antibodies specific for PAX2 (Covance, Cambridge Bioscience, Cambridge, UK), diphosphorylated ERK (Cell signaling, NEB, UK), phospho-histone H3 (Upstate Biotechnology, Lake Placid, NY), E-cadherin (RDI, Flanders, NJ), β-catenin (BD Transduction Labs, BD Biosciences, San Jose, CA) and polycystin-1 (Wilson et al., 1999). Antibody stains were developed using the Vector ABC Elite system followed by a DAB (3,3′-diaminobenzidine) or AEC color reaction according to the manufacturer's instructions (Vector Labs, Burlingame, CA). DAB-stained sections were counterstained with hematoxylin. For fluorescent detection, sections were stained with Alexa 488- or Alexa 568-conjugated species-specific secondary antibodies (Molecular Probes, Invitrogen, Carlsbad, CA) and examined on a Zeiss Axioskop2 mot plus confocal microscope equipped with LSM5 Pascal lasers.

### Transmission electron microscopy

Portions of wild-type and *Spry1* mutant kidneys were fixed in 3% glutaraldehyde in a 0.2 M sodium cacodylate buffer and embedded in Embed 812 plastic resin (Electron Microscopy Sciences, Hatfield, PA). One-micrometer plastic sections were cut, stained with methylene blue and azure II and observed by light microscopy. Representative areas were chosen for ultrathin sectioning. The ultrathin sections were stained with uranyl acetate and lead citrate. The sections were observed on a H7000 Hitachi transmission electron microscope.

### Metanephric kidney organ cultures

Metanephric kidneys were dissected from embryonic day (E)11.5 embryos in ice-cold $CO_2$-independent medium (Gibco, Invitrogen, Paisley, UK) containing 1% fetal bovine serum (Gibco, Invitrogen, Paisley, UK). Explants were cultured in DMEM (Gibco) supplemented with 290 μg/ml glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum in a 5% $CO_2$ at 37°C in a humidified atmosphere at the medium-air interface on Costar Transwell filters (0.4 μm). After culture, explants were fixed either in methanol for immunostaining or 4% paraformaldehyde in PBS for in situ hybridization. For immunostaining, explants were stained with anti-cytokeratin (Sigma, Surrey, UK) as described previously (Basson et al., 2005). Images were captured on a Zeiss Axioskop microscope attached to a Colorview12 digital camera. GFP expressing embryonic mouse kidneys were fixed for 1 h in 4% paraformaldehyde at room temperature. The kidneys were cleared with FocusClear (PacGen Biopharmaceuticals, Inc., Vancouver, BC, Canada) for 20 min at room temperature, then mounted in MountClear (PacGen) between 25 mm coverslips separated by a silicone spacer (Grace Bio-Labs, Bend, OR). Kidneys were examined with a Bio-Rad laser scanning confocal microscope equipped with a 10× water immersion lens, NA 0.3. Image stacks were collected by optically sectioning the kidneys at 4.4-μm intervals. Image stacks were volume-rendered using Volocity Software (Improvision, Lexington, MA).

### GFP time-lapse videography

E11.5 kidney rudiments from *Hoxb7-eGFP;*$Spry1^{+/-}$ by $Spry1^{+/-}$ crosses were cultured and time-lapse recordings were taken as described previously (Srinivas et al., 1999a; Watanabe and Costantini, 2004). Images were collected every 30 min for a 24-h period. To estimate the kinetics of branching morphogenesis and growth, the relative length of the ureteric bud generations was measured as described (Watanabe and Costantini, 2004). In addition, individual branched tips were counted at 5-h intervals.

### Whole-mount in situ hybridization

Tissue was fixed overnight in 4% paraformaldehyde in PBS at 4°C. In situ hybridization was carried out as described (Grieshammer et al., 2004). Probes for *Gdnf* were as described (Srinivas et al., 1999b), with the addition of a shorter *Gdnf* probe (Grieshammer et al., 2004). The *Wnt11* probe (Majumdar et al.,

2003) was kindly provided by A. McMahon (Harvard University, USA), the *Spry1* and *Spry2* probes (Minowada et al., 1999) by G. Martin (University of California, San Francisco, USA) and the *erm* probe (Hippenmeyer et al., 2002) was from Silvia Arber (University of Basel, Switzerland).

### *Biochemical analysis of GDNF signaling pathways*

The mouse inner medullary collecting duct cell line (mIMCD3) was transfected with a pSIREN-RetroQ vector (Clontech, Palo Alto, CA) containing 19 base long inserts corresponding to nucleotides 226–244 (relative to Spry1 translation start site) of mouse *Spry1* or inserts of random sequence as a control (Hammond et al., 2001). 48 h after transfection, short-term selection was started with 2 mg/ml puromycin (Sigma). 72 h later cells were serum starved for 24 h and stimulated with 100 ng/ml GDNF (R&D systems) for the indicated time intervals. Cells were lysed in ice-cold lysis buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 10 mM NaF, 2 mM sodium orthovanadate and one tablet of complete protease inhibitors for 50 ml (Roche)). The resulting proteins (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore). The membranes were probed with antibodies against phospho-44/42 MAP kinase (Santa Cruz), GAPDH (Chemicon) and Sprouty1 as described (Gross et al., 2001).

### *Estimation of newborn kidney sizes*

Freshly dissected newborn kidneys were measured in two dimensions: length along the long axis, and width (from point of ureter entry to the outside edge). The volumes of newborn kidneys were estimated using the following equation: estimated volume ($mm^3$) = length (mm) × $width^2$ $(mm)^2/2$ (Gilbert et al., 2003).

## Results

### *Abnormal branching morphogenesis of Spry1 null ureteric epithelium*

We previously described defects in ureteric bud formation in *Spry1*-deficient embryos at the initiation of metanephric kidney development (Basson et al., 2005). Whereas most adult *Spry1*$^{-/-}$ animals exhibited bi- or unilateral multiplex kidneys due to the production of supernumerary ureteric buds in the embryo, a significant percentage (28%, $n=25$) of *Spry1*$^{-/-}$ kidneys were attached to a single ureter. RTK signaling pathways are utilized throughout branching morphogenesis and the overexpression of *Spry2* inhibits this process (Chi et al., 2004). Therefore, *Spry1*$^{-/-}$ kidneys that developed from single ureteric buds, provided us with the opportunity to examine *Spry1* function during branching morphogenesis independently of defects that might be secondary to the formation of ectopic ureteric buds.

We produced control (*Spry1*$^{+/-}$) and mutant (*Spry1*$^{-/-}$) embryos that contained a *Hoxb7-eGFP* transgene (Watanabe and Costantini, 2004). The three-dimensional structures of E14.0 kidney rudiments from these animals, that express GFP throughout the ureteric epithelium, were examined by confocal microscopy. The mutant kidneys displayed more numerous ureteric bud tips, irrespective of whether kidney primordia were derived from single (Fig. 1B, b) or multiple ureteric buds (Fig. 1C, c) (see below for quantitative analyses). In addition, we observed a number of ureteric tree branches that failed to swell and bifurcate as they reached the perimeter of the kidney (asterisk in Fig. 1B, b). Thus, branching morphogenesis was quantitatively and qualitatively abnormal in the absence of *Spry1*, even when the ureteric tree was derived from a single UB.

The analyses of *ex vivo* kidney rudiments only provide information at a single developmental end-point. *In vitro* culture of early rudiments can faithfully recapitulate *in vivo* development and allows the study of branching morphogenesis in real time. Therefore, we isolated embryonic kidney rudiments at E11.5 and cultured them *in vitro* for 48 h. The ureteric tree in cultured *Spry1*$^{-/-}$ metanephric kidneys that developed from

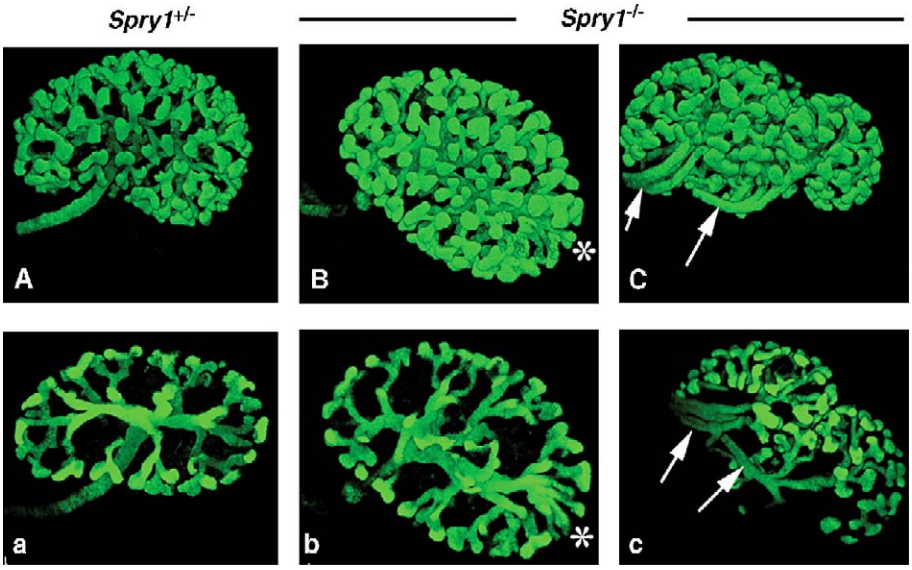


Fig. 1. Three-dimensional reconstruction of the structure of the ureteric tree of *Hoxb7-eGFP;Spry1*$^{+/-}$ (A, a) and *Hoxb7-eGFP;Spry1*$^{-/-}$ (B, b; C, c) kidneys at E14 imaged by confocal microscopy. Three-dimensional renderings from 110 confocal optical sections are shown in panels A–C, with the anterior poles of the kidneys orientated to the right. Panels a–c show 25 volume-rendered confocal sections through the center of the corresponding kidneys shown in panels A–C. Asterisks in panels B, b indicate abnormal ureteric tree tips in the anterior poles of the mutant kidneys and arrows in panels C, c indicate supernumerary ureters.

single ureteric buds also exhibited an abnormal branching pattern (Figs. 2A, B). In addition, the ureteric bud tips in *Spry1*$^{-/-}$ kidneys were dilated (Figs. 2A′, B′) and the branching pattern was highly irregular compared to controls. While it also appeared as if some tips might be fused at the perimeter of the developing kidneys (Figs. 2B, B′), confocal analysis indicated that the enlarged branch tips were overlapping yielding the abnormal appearance of these specimens (not shown).

*Spry1* is expressed in the ureteric tree and at much lower levels in the condensing mesenchyme of the developing metanephric kidney (Gross et al., 2003; Zhang et al., 2001) (Fig. 2E). To determine the primary site of *Spry1* function during branching morphogenesis, we examined the development of metanephric kidneys in which the *Spry1* gene was deleted only in the ureteric tree through the action of a *Hoxb7-cre* transgene (Yu et al., 2002). Our prior work indicated that deletion of *Spry1* in the Wolffian duct resulted in the formation of multiple ureters in nearly 3/4 mice (Basson et al., 2005). To avoid the effects of these multiple ureters, we focused on the phenotype of E11.5 *Hoxb7-cre;Spry1*$^{flox/flox}$ kidney explants derived from single ureteric buds. After *in vitro* culture for 40 h these conditional null kidney explants demonstrated a perturbed branching pattern characterized by wide ureteric trunks and tips, similar to that of *Spry1*$^{-/-}$ explants (Figs. 2C, D). This result indicated that *Spry1* functions continuously in the ureteric epithelium after initial ureteric bud formation to regulate branching morphogenesis. Furthermore, this result demontrates that these ureteric tree anomalies were not secondary to the loss of *Spry1* expression in the metanephric mesenchyme. In situ hybridization of these metanephric kidney explants using a *Spry1* probe to a portion of the open reading frame (Minowada et al., 1999) confirmed that the gene had been deleted from the ureteric bud epithelium, whereas expression in the metanephric mesenchyme remained intact (Figs. 2E, F).

### *Time-lapse analysis of branching morphogenesis reveals increased growth of Spry1 mutant kidneys*

Time-lapse photography was employed to explore the dynamics of the branching process in real time during *in vitro* culture (Watanabe and Costantini, 2004). Two *Spry1*$^{-/-}$ E11.5 kidney rudiments are shown in Fig. 3: (a) an explant with supernumerary buds (Fig. 3A, arrow) and (b) an explant with a single major ureteric bud. Note that in both cases the mutant ureteric bud tips were significantly wider than controls (Fig. 3Aa, b and (Basson et al., 2005)). During a 24-h culture period, control ureteric buds branched in a reiterative fashion to produce ureteric trees composed of regularly spaced tips of similar sizes (Fig. 3A and Supplementary Movie 1A). The mutant kidneys clearly did not follow this stereotypical branching morphogenesis program regardless of the presence or absence of extra ureteric buds (Fig. 3A and Supplement Figs. 1B, C). In the *Spry1*$^{-/-}$ rudiments, ureteric bud tips were significantly larger in length and caliper than in the controls and instead of undergoing mainly dichotomous branching, these wide ureteric bud stalks and tips formed multiple outbuddings resulting in highly irregular structures at the end of the culture (Fig. 3A and Supplementary Movie 1B, C).

Measuring the lengths of ureteric bud segments at each branch generation and summing these branch lengths showed a slow, steady growth rate in wild-type kidneys and a 2- to 3-fold greater rate of elongation in mutant kidneys (Fig. 3B). Some of the kidneys even showed five branch generations while the wild-type kidneys only displayed four ramifications in an equivalent period of *in vitro* culture (Fig 3B). In the *Spry1*$^{-/-}$ kidneys the 4th generation of branching, beginning at 15 h of culture was accentuated and growth appeared to accelerate after this point. The number of ureteric bud tips also rapidly increased in *Spry1*$^{-/-}$ ureteric buds between 15 and 24 h of

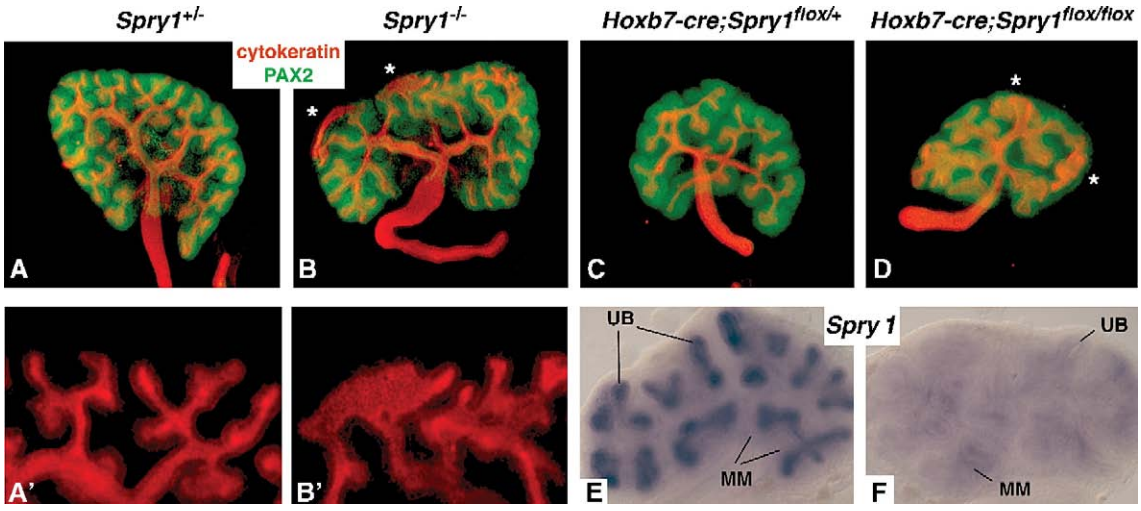


Fig. 2. Branching morphogenesis defects in *Spry1*$^{-/-}$ and *Hoxb7-cre;Spry1*$^{flox/flox}$ metanephric kidneys. (A, B) A comparison between *Spry1*$^{+/-}$ (A, A′) and *Spry1*$^{-/-}$ (B, B′) branched ureteric trees after 48-h *in vitro* culture of E11.5 kidney explants. Note the regular, reiterative branching pattern in control (A) vs. irregular pattern in mutant (B) organs. Wide, abnormally shaped ureteric bud tips (higher magnification in panel B′) are indicated by asterisks in the *Spry1*$^{-/-}$ kidney (B). (C, D) Comparison between *Hoxb7-cre, Spry1*$^{flox/+}$ (C) and *Hoxb7-cre;Spry1*$^{flox/flox}$ (D) E11.5 kidneys after 40-h culture. Note the wide, irregularly shaped tips, indicated by asterisks in panel D. (E, F) *Spry1* in situ hybridization indicating normal *Spry1* mRNA expression in the ureteric bud tips (UB) and the metanephric mesenchyme (MM) (E), and specific loss of expression in the UB in *Hoxb7-cre;Spry1*$^{flox/flox}$ kidney explants at ~E13.5 (F).

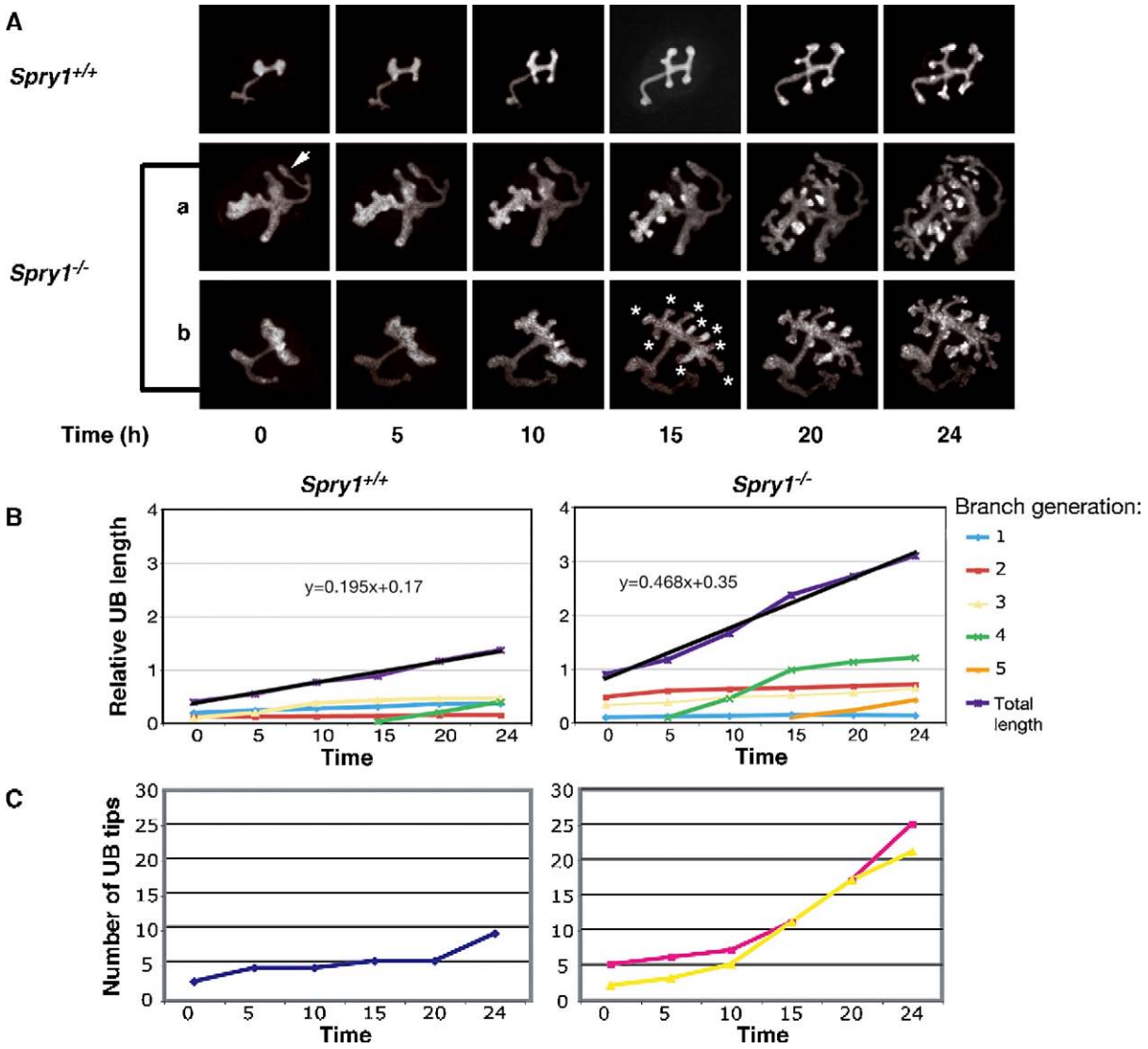


Fig. 3. Analyses of ureteric bud branching morphogenesis in real-time using a *Hoxb7-eGFP* transgene. (A) E11.5 (0 h) *Hoxb7-eGFP;Spry1*$^{+/+}$ ureteric buds are T-shaped at the beginning of culture. Iterative branching morphogenesis results in a tree-like structure with regularly spaced tips after 24 h. Two E11.5 *Hoxb7-eGFP;Spry1*$^{-/-}$ explants are shown. The top panel (a) shows an example of a wide, abnormally shaped ureteric bud with a clear ectopic ureteric bud (arrow) in an explant. The wide ureteric bud forms multiple bud tips by 15 h that grow and branch further to result in a highly complex, abnormal structure after 24 h. The ectopic ureteric bud initiates some branching morphogenesis after 24 h. An example of a *Hoxb7-eGFP;Spry1*$^{-/-}$ explant that has a relatively normal T-shaped ureteric bud at E11.5 is shown in the bottom panel (b). By 15 h multiple bud tips are observed (asterisks) that results in an abnormal ureteric structure after 24 h of culture. (B) Relative UB segment length was determined for each branch generation and a representative plot of the data is presented. Note the rapid increase in growth during the fourth generation branching of the mutant kidneys. Black linear regression lines with indicated slopes demonstrate the overall growth rate of the entire ureteric tree. (C) The number of ureteric bud tips was counted at each 5 h of culture to reveal increased rate of bud tip production in *Spry1*$^{-/-}$ explants between 15 and 24 h of culture compared to controls. See Supplementary data for real-time movies.

culture (Fig. 3C). These observations clearly suggest a continuing role for *Spry1* in regulating the extent and rate of ureteric bud branching morphogenesis.

### *Abnormalities in gene expression during ureteric branching morphogenesis*

The formation of dilated, almost cystic ureteric bud tips with multiple outbuddings is remarkably similar to anomalies in ureteric bud branching produced upon implantation of GDNF beads into normal kidney explants (Pepicelli et al., 1997) (http://cpmcnet.columbia.edu/dept/genetics/kidney/movies.html).

We therefore investigated whether or not the *Spry1*$^{-/-}$ mutant kidney explants displayed aberrant expression of genes of the GDNF/RET signaling pathway. *Gdnf* expression is normally restricted to the metanephric mesenchyme surrounding ureteric bud tips (Hellmich et al., 1996; Suvanto et al., 1996). Surprisingly, the expression of *Gdnf* appeared more abundant and in a more extensive portion of the *Spry1*$^{-/-}$ mesenchyme (compare Figs. 4A, A′ with B, B′). *Wnt11* expression is normally induced in ureteric bud tips upon activation of RET by GDNF (Pepicelli et al., 1997) and genetic experiments have confirmed that *Wnt11* is almost absent in *Ret*$^{-/-}$ kidneys (Majumdar et al., 2003). In the absence of *Spry1*,

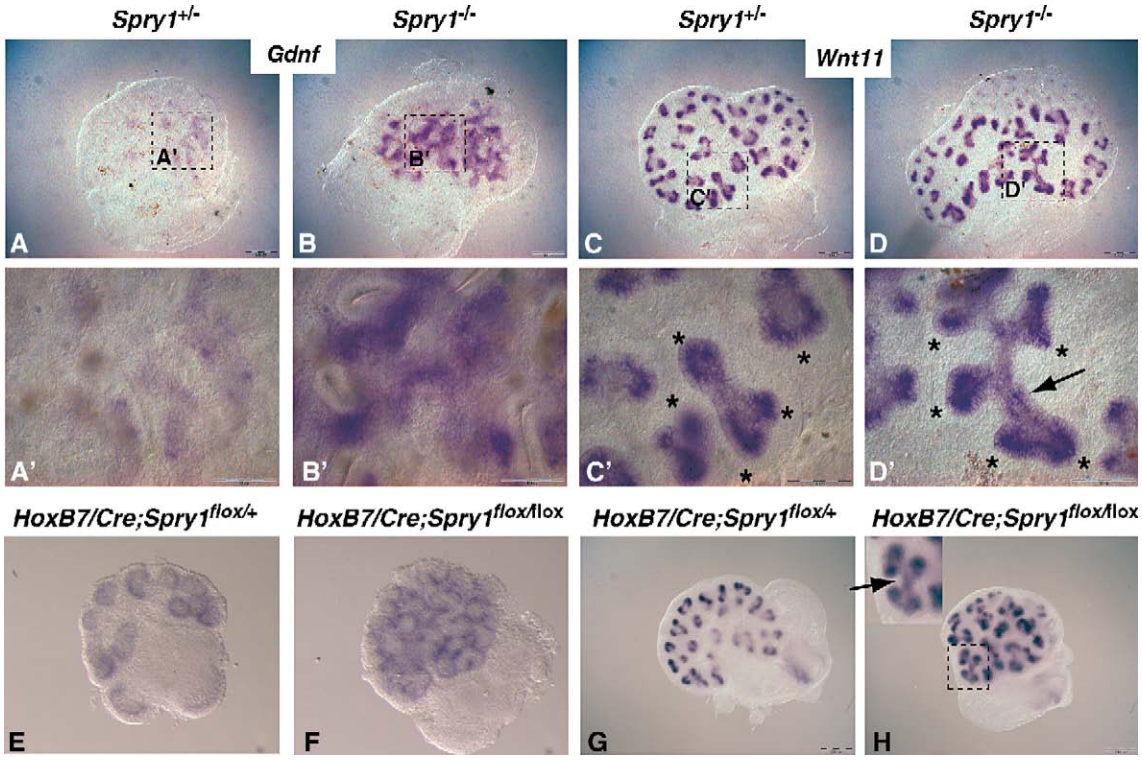


Fig. 4. In situ hybridization for *Gdnf* and *Wnt11* mRNA indicates hyperactivation of GDNF/RET/WNT11 signaling loop. (A, B) Comparison of *Gdnf* expression in $Spry1^{+/-}$ with $Spry1^{-/-}$ E11.5 explants after 48 h of culture. (A′, B′) Higher power views of panels A and B, respectively. (C, D) *Wnt11* expression in $Spry1^{+/-}$ and $Spry1^{-/-}$ kidney explants. (C′, D′) Higher power views of panels C and D, respectively indicating the expansion of *Wnt11* expression to ureteric stalks connecting individual ureteric bud tips (asterisks) in the mutant (arrow in panel D′). (E, F) Comparison of *Gdnf* and *Wnt11* (G, H) expression between *Hoxb7-cre;*$Spry^{flox/+}$ and $Spry1^{flox/flox}$ explants. Inset in panel H shows a higher power view of the indicated area to show expansion of *Wnt11* expression to ureteric stalks (arrow). Scale bars=200 μm, apart from panels C′ and D′ where they are 100 μm.

*Wnt11* mRNA was often detected in the connecting segments between tips further apart than 200 μm. In $Spry1^{+/-}$ explants, *Wnt11* expression does not persist in stalk areas between tips once the ureteric tree has grown to the point where tips are more than 200 μm apart (compare Figs. 4C, C′ with Figs. 4D, D'). This result suggested that these cells experienced increased GDNF signaling. A positive autoregulatory feedback loop that regulates *Gdnf*, *Ret* and *Wnt11* expression was described

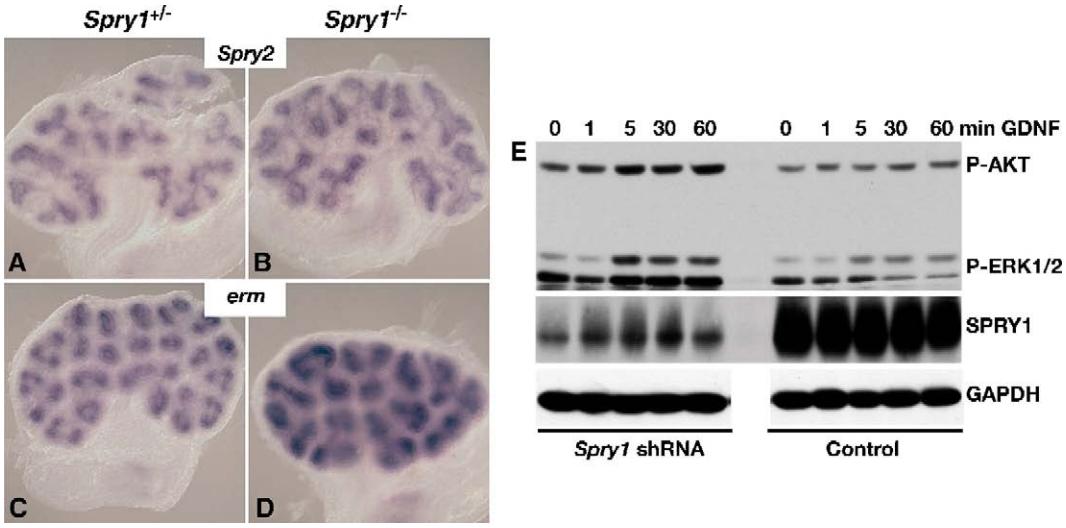


Fig. 5. The loss of *Spry1* results in hyperactive RTK signaling. The expression of genes downstream of RTK signaling, *Spry2* (A, B) and *erm* (C, D) were compared between control $Spry1^{+/-}$ and mutant $Spry1^{-/-}$ kidney explants. (E) Immunoblots of whole cell lysates from mIMCD3 cells transfected with *Spry1*-specific or control shRNA, stimulated with GDNF and blotted with antibodies to activated phospho-AKT (P-AKT), activated phospho-ERK1/2 (P-ERK1/2), SPRY1 and GAPDH are shown. Note that both AKT and ERK1/2 were hyperactivated in cells with reduced amounts of SPRY1.

previously (see Fig. 8). Strong genetic support for this model comes from the observation that *Gdnf* expression is drastically reduced in *Wnt11*$^{-/-}$ kidneys (Majumdar et al., 2003). Thus, the expanded mesenchymal *Gdnf* expression in *Spry1* mutant kidneys may be secondary to the hypersensitivity of ureteric epithelium to GDNF signals. In this scenario, increased and ectopic *Wnt11* expression in the ureteric tree induces *Gdnf* upregulation in the mesenchyme, thus closing the positive feedback loop resulting in perpetuation of elevated GDNF/RET signaling (see Fig. 8).

In order to obtain evidence for such a mechanism, we also examined *Gdnf* and *Wnt11* expression in *Hoxb7-cre; Spry1*$^{flox/flox}$ kidney explants. In these kidneys where *Spry1* had been deleted only in the ureteric bud, we also observed a significant expansion of *Gdnf* mRNA expression throughout the mesenchyme (Figs. 4E, F), indicating that upregulation of *Gdnf* message in the mesenchyme occurred in response to deregulated GDNF/RET signaling in the ureteric bud and was not due to the loss of *Spry1* in the mesenchyme. *Wnt11* was also abnormally expressed in the ureteric epithelium in between buds, suggesting that the absence of *Spry1* resulted in hypersensitivity to RET-mediated signals (Figs. 4G, H).

### *Increased RTK signaling in the absence of Spry1*

To find more evidence that the ureteric epithelium is subjected to increased RTK signaling in the absence of *Spry1*, we analyzed the expression of genes normally regulated by these signals. The expression of *Spry2* was slightly increased in *Spry1*-deficient kidneys (Figs. 5A, B), whereas *erm* (Hippenmeyer et al., 2002) was significantly upregulated (Figs. 5C, D). To directly demonstrate the ability of Spry1 to affect GDNF/RET signaling we used a shRNA vector to reduce Spry1 expression in the IMCD3 kidney inner medullary renal collecting duct cell line. Upon stimulation of serum-starved cells with GDNF, both phospho-ERK as well as phospho-AKT were elevated in cells depleted for Spry1 relative to cells transfected with a control shRNA vector (Fig. 5E). These data demonstrate that in accordance with the gene expression data, Spry1 inhibits the signaling pathways activated by GDNF. The results further indicate that Spry1 can interfere with multiple signaling pathways downstream of the receptor tyrosine kinase, RET.

### *The Spry1 and Gdnf genes interact during branching morphogenesis to regulate kidney size*

The data presented thus far suggested that the branching morphogenesis defects in *Spry1*-deficient kidneys were due to hyperresponsiveness to GDNF/RET signaling. To find genetic evidence to confirm this hypothesis, we compared the phenotypes of *Spry1*$^{-/-}$ and *Spry1*$^{-/-}$;*Gdnf*$^{+/-}$ embryonic kidneys. The highly irregular branching pattern of *Spry1*$^{-/-}$ kidneys, characterized by dilated and ectopic segments of the ureteric tree was significantly normalized when the *Gdnf* gene dosage was reduced (Figs. 6A′–C′). This observation provided

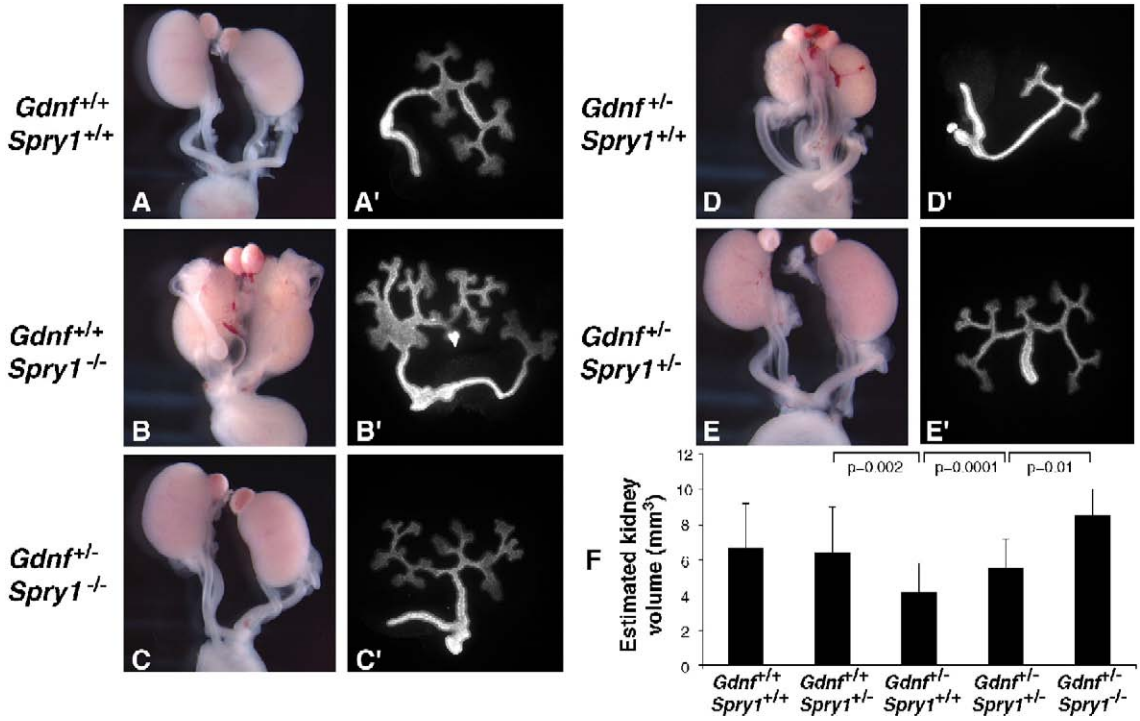


Fig. 6. Branching morphogenesis of the ureteric tree is regulated by the interplay between *Gdnf* and *Spry1*. (A–E) Newborn kidneys and urogenital tracts with the indicated genotypes are shown. (A′–E′) Representative ureteric trees in E11.5 metanephric kidney explants cultured for 48 h and stained for cytokeratin expression, are shown for each of the genotypes. Note that relatively normal branching patterns correlated with the formation of normal newborn kidneys and the correlation between efficiency of growth and branching morphogenesis and newborn kidney sizes. (F) Newborn kidney volumes were estimated and show statistically significant differences (two-tailed *T* test) in the size of *Spry1*$^{+/+}$, *Gdnf*$^{+/-}$, compound heterozygote *Gdnf*$^{+/-}$;*Spry1*$^{+/-}$ and *Gdnf*$^{+/-}$;*Spry1*$^{-/-}$ kidneys.

direct genetic evidence for the hypothesis that deregulation of GDNF/RET signaling was responsible for abnormal branching morphogenesis of *Spry1*$^{-/-}$ ureteric tree at time points later than the initial branching from the Wolffian duct.

*Gdnf*$^{+/-}$ mice frequently display unilateral kidney agenesis and the kidneys that do form in such mice are small (Cullen-McEwen et al., 2001). These observations suggested that ureteric bud development might be sensitive to relatively subtle changes in the dose of GDNF. In order to address this possibility, we examined a number of *Gdnf*$^{+/-}$ kidneys and explants. A significant proportion ($n$=15/39) of *Gdnf*$^{+/-}$ kidneys were severely hypoplastic (defined as 50% smaller than the average wild-type or *Spry1*$^{+/-}$ kidney) (Fig. 6D), compared to 0/45 of *Gdnf*$^{+/+}$ kidneys (Fig. 6A). Moreover, *Gdnf*$^{+/-}$ kidney explants fail to branch more than twice during a 48-h culture period (Fig. 6D′), confirming that ureteric bud branching morphogenesis was sensitive to reduced levels of GDNF. By contrast only 4/51 of *Gdnf*$^{+/-}$;*Spry1*$^{+/-}$ kidneys showed such extreme hypoplasia (Fig. 6E), indicating a significant ($p$=0.0018 vs. *Gdnf*$^{+/-}$) rescue of the *Gdnf*$^{+/-}$ phenotype. As expected, *Gdnf*$^{+/-}$;*Spry1*$^{+/-}$ explants (Fig. 6E′) showed a branching pattern comparable to normal kidneys (Fig. 6A′).

We also compared estimated mean kidney sizes of the different genotypes. On average *Gdnf*$^{+/-}$ newborn kidneys were 38% smaller than control *Spry1*$^{+/+}$ or *Spry1*$^{+/-}$ kidneys ($p$=0.002). Upon deletion of one allele of *Spry1*, the average size of the *Gdnf*$^{+/-}$;*Spry1*$^{+/-}$ kidneys increased by 35% but remained smaller than wild-type kidneys. Deleting the second allele of Spry1 yielded another 52% increase in estimated kidney volume yielding kidneys comparable in size to wild type (Fig. 6F). This indicates that *Spry1* affects *Gdnf* function in kidney development in a dose-dependent manner and show that *Spry1* and *Gdnf* operate closely together to orchestrate branching morphogenesis of the ureteric tree during kidney development.

### *Sprouty1 null mice as an experimental model for cystic renal hyperplasia*

The majority of *Spry1*$^{-/-}$ mice are born with multiple ureters, hydroureter and multiplex, disorganized kidneys that often contain large epithelial cysts (Figs. 7A, B) (Basson et al., 2005). Different types of cystic kidney disease can be classified by the segment of the nephron or collecting system that is affected (Wilson, 2004). Dolichus biflorus agglutinin (DBA)

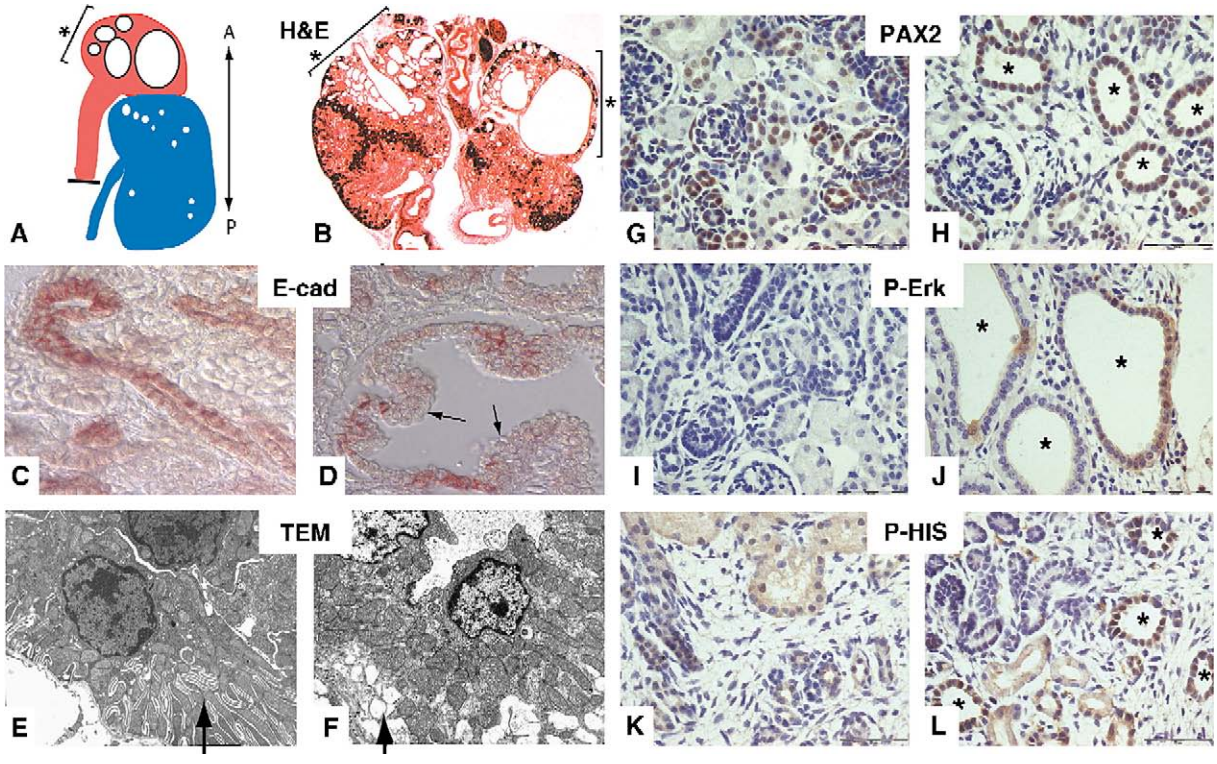


Fig. 7. Renal epithelia in newborn *Spry1*$^{-/-}$ kidneys exhibit structural and molecular changes characteristic of cystic kidney disease. (A) Diagram showing how anterior (A) portions of mutant kidneys (asterisks) that are attached to obstructed ureters develop large cysts (pink), whereas the more posterior (P) portions attached to normal, draining ureters only contain a few small cysts (blue) (Ichikawa et al., 2002). (B) H&E stained sections through both kidneys from a representative *Spry1*$^{-/-}$ newborn animal showing multiplex kidneys and renal cysts. Note the prevalence of large cysts in the anterior renal lobes (asterisks). Molecular and structural alterations characteristic of human cystic kidney diseases are compared between control *Spry1*$^{+/-}$ (C, E, G, I, K) and mutant *Spry1*$^{-/-}$ (D, F, H, J, L) kidneys. E-cadherin (E-cad) is highly expressed in all control tubular epithelium cells (C) whereas patches of cells have lost expression in the mutants (arrows in panel D). Transmission electron micrographs (TEM) show regular adhesions of epithelial cells to the basement membrane (arrow in panel E), disrupted in knockouts (arrow in panel F). High PAX2 expression (brown) in nephrogenic zones of control kidneys (G) and in epithelia lining renal cysts (asterisks in panel H). Many cystic epithelial cells contain high levels of diphosphorylated ERK (P-Erk) (J), which is undetectable in control tissue (I). Few actively cycling cells are detected in control tissue (K) by an antibody to phospho-histone H3 (P-HIS), whereas most cystic epithelial cells (L) are actively cycling.

staining revealed that most of the epithelial cysts in *Spry1*$^{-/-}$ animals were derived from the collecting system of the kidney (not shown). The collecting system develops from the ureteric bud, suggesting that defects in the embryonic ureteric epithelium may underlie this phenotype. However, macroscopic cysts were not distributed evenly throughout many of the mutant kidneys, but were present mainly in the anterior poles of the mutant kidneys that were attached to abnormal ureters (Figs. 7A, B). These observations support the conclusion made from human and experimental models that ureteric obstruction is associated with the formation of renal cysts (Ichikawa et al., 2002; Woolf et al., 2004). However, smaller cysts were evident throughout the mutant kidneys (Figs. 7A, B) suggesting that defects in morphogenesis of the ureteric tree may also predispose the collecting system to cyst formation.

We further characterized the nature of the epithelial cysts in *Spry1*$^{-/-}$ kidneys to determine to what extent the *Spry1*$^{-/-}$ cysts resemble those observed in human cystic kidney diseases. Alterations in adhesive properties of tubular epithelial cells may be associated with the development of cystic phenotypes in polycystic and possibly other cystic kidney diseases (Wilson and Burrow, 1999). Hence we determined whether the expression of E-cadherin, a key component of adherens junctions, was affected in *Spry1*$^{-/-}$ cystic epithelia. Large patches of cells lining the cysts in these kidneys were devoid of E-cadherin, suggesting that they had undergone alterations in their normal adhesive properties (Figs. 7C, D). Furthermore, whereas the normal branching ureteric epithelium consists of a single layer of epithelial cells (Fig. 7C), the cysts in *Spry1* mutant kidneys often contained disorganized clusters of E-cadherin negative cells, which appeared loosely attached to one another (Fig. 7D). A recent study showed that polycystin-1/E-cadherin-containing complexes are disrupted in human autosomal dominant polycystic kidney disease, resulting in depletion of E-cadherin from the cell membrane (Roitbak et al., 2004). One implication of this finding is that the loss of E-cadherin from the cell surface may be associated with the development of cysts. Interestingly, we could observe alterations in the expression and subcellular localization of polycystin-1 (PKD1) in cystic *Spry1*-deficient epithelia. Staining of normal kidney sections with an antibody to PKD1 revealed a punctate cytoplasmic localization at the basal poles of the polarized epithelia (Supplementary Fig. 1A). By contrast, many *Spry1*-deficient cystic epithelial cells seem to be completely devoid of PKD1 (asterisks in Supplementary Movie 1B) and some cells lining macrocysts show strong apical localization of PKD1 (arrows in Supplementary Fig. 1). We could not find any evidence for changes in the subcellular localization of polycystin-2 within these cells (not shown). Finally, we examined the integrity of epithelial basement membrane adhesions by transmission electron microscopy (TEM), as these were found to be abnormal in other models of cystic kidney disease (Liu et al., 2002). We found that some of the *Spry1*$^{-/-}$ epithelial cells have indeed lost their normal, regular adhesion to the basement membrane (Figs. 7E, F), a defect that may underlie the apparent loss of apical–basal polarization.

The PAX2 transcription factor is normally expressed in immature tubular epithelial cells in the nephrogenic zone of newborn kidneys (Fig. 7G) and down-regulated as the tubules mature. Human cystic epithelial cells aberrantly maintain high levels of PAX2 (Winyard et al., 1996). In *Spry1*$^{-/-}$ kidneys, PAX2 was also present at high levels in the cystic epithelia (Fig. 7H). Cystic epithelial cells in human renal dysplasia express abnormally high levels of activated, diphosphorylated Erk (P-Erk) (Omori et al., 2002). As Sprouty proteins are negative regulators of the ras/MAPK pathway (Cabrita and Christofori, 2003; Kim and Bar-Sagi, 2004), we expected cells in the *Spry1* mutant to express elevated levels of activated P-Erk. Indeed, high levels of P-Erk were readily detected in many of the epithelial cells lining such microcysts in these kidneys (Fig. 7J), whereas P-Erk was almost undetectable in normal kidneys (Fig. 7I). These data are in agreement with our findings on the action of Spry1 on P-Erk activity *in vitro* (Fig. 5E). Elevated expression of PAX2 and activated Erk in cystic epithelia are associated with a hyperproliferative state (Omori et al., 2002; Winyard et al., 1996). In agreement with this notion, immunohistochemical analyses revealed that many *Spry1*$^{-/-}$ cystic epithelial cells expressed high levels of phospho-histone H3, an indicator of cells in metaphase (Figs. 7K, L). These studies indicated that epithelial cysts that formed in *Spry1*$^{-/-}$ kidneys share several characteristics with cysts found in human disease.

## Discussion

Through a combination of genetic and biochemical experiments we have described a key function for Sprouty1 in preventing the uncontrolled activation of a Gdnf/Ret/Wnt11 signaling loop that coordinates epithelial–mesenchymal cross-talk during branching morphogenesis of the ureteric tree. In the early phases of kidney development *Gdnf* expression in the metanephric mesenchyme is unchanged in the absence of *Spry1* (Basson et al., 2005). We now show that *Gdnf* expression is augmented during later phases of kidney development, branching morphogenesis of the *Spry1*-deficient ureteric tree. Defects in branching morphogenesis were detected in *Spry1*$^{-/-}$ embryonic kidneys even if derived from a single ureteric bud.

The etiology of renal cystic diseases is highly complex and may involve multiple signaling pathways that regulate a myriad of cellular behaviors such as cell proliferation, apoptosis, adhesion and polarity (Piscione and Rosenblum, 2002; Shah et al., 2004; Wilson, 1997, 2004). Deregulation of growth factor receptor-regulated signaling pathways may underlie the development of a subset of these disorders. Indeed, a previous study reported that misexpression of a ligand-independent oncogenic isoform of the RET receptor (RET9) in the Wolffian duct led to cystic dilation, hyperplasia and the development of renal cysts in developing kidneys (de Graaff et al., 2001). Misexpression of *Fgf8* in the Wolffian duct also resulted in the formation of epithelial cysts (Kuschert et al., 2001). Other growth factor signaling systems such as EGF/TGFα/EGFR (Du and Wilson, 1995; Pugh et al., 1995) and HGF/c-Met (Takayama et al., 1997) were also implicated in the development of cystic kidney

diseases. The signaling pathways responsible for cystogenesis are largely unknown but several studies pointed to the possible deregulation of the ras/MAPK pathway (Omori et al., 2002; Shen and Cotton, 2003; Sorenson and Sheibani, 2002; Yamaguchi et al., 2000).

Some studies suggested that obstruction of urinary outflow may be an important factor in cystogenesis (Woolf et al., 2004; Yang et al., 2001). In support of the obstruction model, we observed larger cysts in kidney portions that were attached to obstructed ureters in the *Spry1* mutants. We showed that the renal cysts shared several characteristics with human disease. Notably, PAX2 and P-Erk were expressed in the cystic epithelia. Interestingly, in studies where ureters were surgically obstructed during fetal development, cyst formation was associated with similar molecular alterations as observed in patients, suggesting that many of these changes may be secondary to obstruction and not causative (Yang et al., 2001). However we also observed smaller cysts in regions of the *Spry1* mutant kidneys associated with a normally draining ureter, suggesting that at least some cystic features of the *Spry1* mutant kidneys were due to an intrinsic alteration of the epithelium of the developing urinary system. In order to formally determine whether *Spry1* plays a more direct role in cystogenesis, it would be necessary delete this gene after E11.5 when normal ureteric bud induction had been allowed to take place.

We also noted qualitative changes in the renal epithelium of the *Spry1* mutant mice including cysts with multiple layers of cells and the loss of adhesion and E-cadherin expression. These may be attributable to chronic hyperstimulation of the ras/MAPK signaling pathway in *Spry1*$^{-/-}$ epithelial cells causing a partial epithelial–mesenchymal transition that is associated with the loss of E-cadherin and normal epithelial adhesion (reviewed in Liu, 2004). Thus, while our results provide evidence for the involvement of *Spry1* in cystogenesis, the mechanism by which epithelial cells are transformed to abnormal cystic epithelia is likely to be multi-factorial involving ureteric obstruction with accompanying pressure increases, hyperproliferation, abnormal differentiation, apoptosis and changes in adhesion, polarity and ion transport.

Our results confirm that *Spry1* is an important regulator of RET activation throughout the process of renal branching morphogenesis. One key mechanism to prevent RET activation in the trunks is by the restriction of high level RET expression and activity to the ureteric bud tips (Majumdar et al., 2003). Normally, the lower levels of RET present in the ureteric tree trunks may not be exposed to sufficient levels of GDNF to activate signaling (Fig. 8). We propose that in the absence of *Spry1*, there is sufficient activation of downstream signaling pathways in the trunks to induce *Wnt11* expression. WNT11 produced in these connecting segments in turn induces ectopic or elevated expression of *Gdnf* in the adjacent mesenchyme (Fig. 8). The GDNF/RET/Wnt11-positive feedback system may be imperative for orchestrating branching morphogenesis by focusing active GDNF/RET signaling to the tips of the ureteric buds. RET activation in the tips of the buds results in WNT11 expression that induces more GDNF expression locally, thus perpetuating the response. Such positive feedback systems are frequently employed during

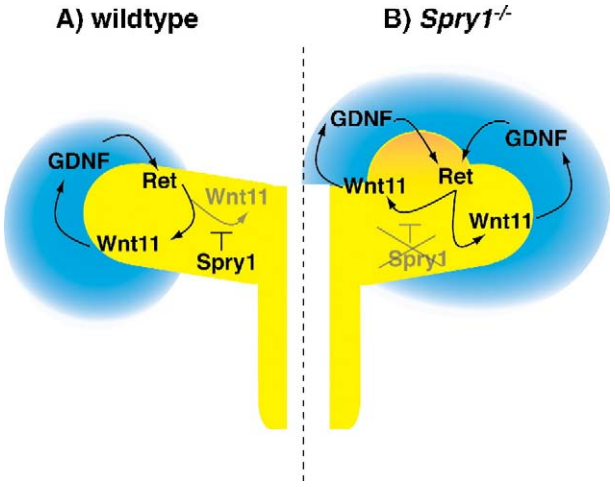


Fig. 8. Model of the genetic interplay between *Gdnf*, *Ret*, *Wnt11* and *Spry1* during ureteric bud morphogenesis. (A) Normally GDNF, expressed by metanephric mesenchyme near the tips of the ureteric tree, locally activates the receptor RET, resulting in *Wnt11* expression only in the tip cells. WNT11 in turn, maintains high levels of *Gdnf* expression in the mesenchyme surrounding the tips. We propose that Spry1 antagonizes RET signaling, thus providing a mechanism by which GDNF is prevented from activating RET signaling in the connecting segments that express low levels of RET, such that it is maintained below the threshold required for *Wnt11* expression. *Spry1* therefore functions to interrupt the positive feedback loop in ureteric stalks. (B) In the absence of the inhibitory activity of *Spry1*, low levels of RET activation in the UB stalk are sufficient to induce *Wnt11* expression. This ectopic *Wnt11* expression can in turn induce expression of *Gdnf* in the adjacent mesenchyme away from the tips that can activate yet more RET in these non-tip cells, resulting in ectopic branching of the ureteric epithelium.

organogenesis to focus proliferation at the tips of branching epithelial tubes, providing a driving force for morphogenesis. (Michael and Davies, 2004). Given the potentially disastrous consequences of deregulation of such a positive feedback mechanism, it is imperative that such a system be under strict negative regulation. Our studies indicate that *Spry1* is one gene that serves this function during kidney development.

The mechanism by which Sprouty proteins exert their function is only partially understood. Most studies support a role for Sprouty proteins as antagonists of the ras/MAPK pathway, which has been implicated in renal branching morphogenesis (Fisher et al., 2001). We show that reducing Spry1 levels in collecting duct cells results in hyperactivation of the ras/MAPK pathway in response to GDNF. To our surprise, the PI3K pathway was also hyperactive in these cells. Recent reports have indicated effects of Sprouty proteins on this pathway (de Alvaro et al., 2005; Edwin et al., 2006). Interestingly, this pathway has been implicated in ureteric bud formation and may also be employed during later branching morphogenesis (Tang et al., 2002).

GDNF is not the only factor regulating ureteric bud morphogenesis and kidney size. Genetic studies indicated that *Fgf7* and *Fgf10* also stimulating ureteric branching (Ohuchi et al., 2000; Qiao et al., 1999). In isolated ureteric bud culture, GDNF cannot induce branching morphogenesis in the absence of other soluble factors (Qiao et al., 2001; Shah et al., 2004). Since FGF and other growth factors signal through RTK

receptors, *Spry1* may also potentially regulate these pathways. Hence the phenotype of *Spry1*$^{-/-}$ kidneys may be the result of deregulation of a number of these signals (Chi et al., 2004). Indeed, we also observed increased mesenchymal *Fgf10* expression in *Spry1*$^{-/-}$ kidneys (not shown). Nevertheless, the striking rescue of the *Spry1*$^{-/-}$ phenotype by reducing *Gdnf* gene dosage, indicates that the interplay between *Gdnf* and *Spry1* is crucial for normal kidney development.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.051.

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