KIDNEY DEVELOPMENT: ROLES OF SPROUTY, WNT2B AND TYPE XVIII COLLAGEN IN THE URETERIC BUD MORPHOGENESIS

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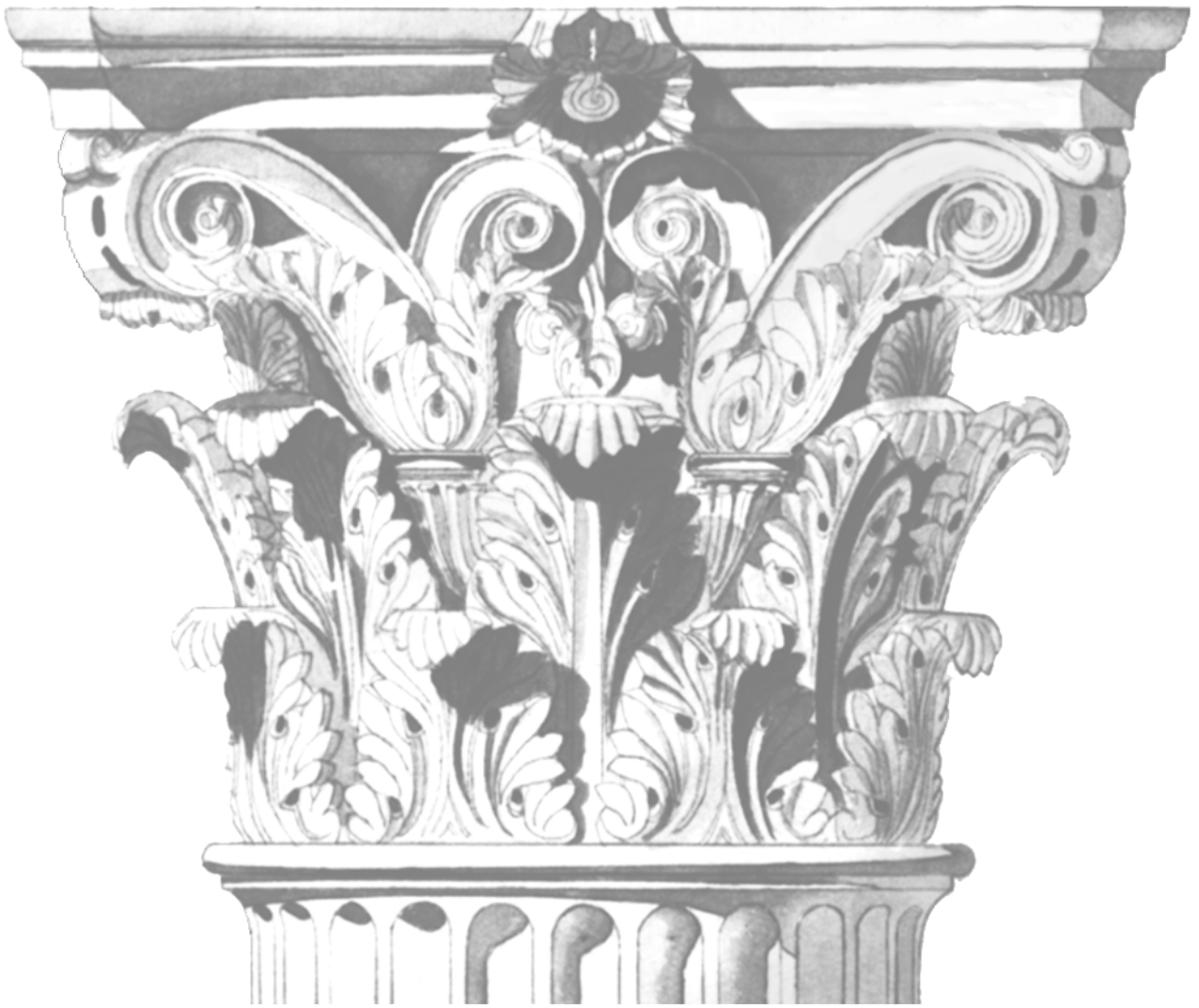

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2003

Abstract

The mammalian metanephric kidney develops through ureteric bud branching morphogenesis and tubule formation and involves secreted inductive signals and possibly their antagonists to regulate the process. *Sprouty (spry)* genes encode antagonists of *FGFs* and the *EGF* signalling pathways. To get an insight to potential developmental roles of the *spry* genes, the expression of *spry1*, *2* and *4* was analyzed in developing kidney. *Spry1* is expressed in the ureteric bud, and *spry2* and *4* in the ureteric bud, the kidney mesenchyme and the nephrons deriving from it suggesting developmental roles for the sprys in kidney development.

Spry function was addressed in vivo in the kidney by targeting *hspry2* expression to the ureteric bud with a *Pax2* promoter. *Hspry2* expression led to development of small, ectopic and cystic kidneys. Ureter branching was reduced and there was less glomeruli in a smaller kidney compared to the wild type controls. *Spry2* may antagonize signalling of *FGF2* and lead to changes in *FGFR1* and *FGFR3* expression. In organ culture ectopic *FGFs* restored ureteric branching of the *hSpry2* transgenic kidneys suggesting that hSpry2 may antagonize FGF signalling in embryonic kidney. In addition to changes in *FGFs*, *hspry2* expression also lead to downregulation of *GDNF* and *BMP4*. We conclude that the Sprouty-FGFs-FGFR signaling is important for kidney development.

Wnt2b is a recently identified member of the *Wnt* family of secreted growth factors, but its function in organogenesis is unknown. In the kidney *Wnt2b* is localized to the perinephric mesenchymal cells at the initiation of organogenesis. *Wnt2b* signalling supported ureteric bud growth and branching *in vitro*. Ureteric bud that was co-cultured with *Wnt2b* expressive cells or incubated with a known Wnt pathway regulator lithium, and then recombined with isolated kidney mesenchyme led to recovery of the expression of some ureteric epithelial marker genes and reconstitution of early kidney development. Hence, Wnt2b signalling is critical for induction of ureteric branching *in vitro*.

Type XVIII collagen is a matrix molecule and may be involved in *Wnt* signalling. Roles of type XVIII collagen in kidney and lung organogenesis was analysed. *Type XVIII* collagen expression correlated with the differences in epithelial branching in both of these organs and its expression in the epithelial tissue was mutually exclusive. In recombinants of ureteric bud and lung mesenchyme, *type XVIII* collagen expression pattern shifted from kidney to lung type and was accompanied by a shift in epithelial *Sonic Hedgehog (Shh)* expression and by ectopic lung *Surfactant Protein C* in the ureteric bud. Blocking of *type XVIII* collagen function prevented ureteric development with lung mesenchyme and associated with reduction in the expression of *Wnt2*.

Taken together, the findings suggest critical roles for *Sprouty2*, *Wnt2b* and *type XVIII* collagen in controlling pattern formation and the mode of ureteric bud branching in the embryonic kidney.

Keywords: FGFs signaling pathway, kidney, organogenesis, patterning

To Yanfeng and Lizhong

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Oulu, Finland, May 2002 Shaobing Zhang

Abbreviations

BM	basement membrane
BMP	bone morphogenetic protein
cDNA	complementary DNA
c-Cbl	cellular DNA sequences that code for Cbl
Cbl	casitas B-lineage lymphoma,
CRD	cysteine-rich domain
Dpc	days post-conception
Drk	*Drosophila* equivalent of mammalian Grb2
Dvl	dishevelled
E	embryonic.
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERKs	extracellular signal-regulated kinases
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Fz	frizzled
Gap1	GTPase-activating protein 1
GDNF	glial cell line-derived neurotrophic factor
HGF	hepatocyte growth factor
LB/KM	lung bud and kidney mesenchyme
LB/LM	lung bud and lung mesenchyme
LIF	leukaemia inhibitory factor
LRP	low-density lipoprotein receptor-related protein
MAPK	MAP Kinase
MAP	mitogen-activated protein
MEK	ERK kinase
MMP	matrix metalloproteinase
MVEC	human dermal endothelial cell
Pax	paired box-containing gene
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PFA	paraformaldehyde
PG	Proteoglycan
PKC	protein kinase C
RAR	Retinoic acid receptor
Ras	p21ras (encoded by Ras gene) G protein
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase PCR
sFRP	secreted frizzled-related protein
Shh	sonic hedgehog
SPC	surfactant protein C
SP/KM	spinal cord and kidney mesenchyme
Spry	sprouty
TB/LM	tracheal bud and lung mesenchyme
TCF	T cell factor
TGFβ	transforming growth factor beta
UB/KM	ureteric bud and kidney mesenchyme
UB/LM	ureteric bud and lung mesenchyme
UB/TM	ureteric bud and tracheal mesenchyme
VEGF	vascular endothelial growth factor

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Zhang S, Lin Y, Itaranta P, Yagi A & Vainio S (2001) Expression of *Sprouty* genes 1, 2 and 4 during mouse organogenesis. Mech Dev 109(2):367-70.

II Zhang S, Lin Y, Chi L, Vuolteenaho R, Kontusaari S & Vainio S (2002) Expression of *hSprouty2* in the metanephric kidney inhibits ureteric bud branching and leads to ectopic organogenesis. Submitted. Development.

III Lin Y, Liu A, Zhang S, Ruusunen T, Kreidberg J, Drummond I & Vainio S (2001) Induction of ureter branching as a response to Wnt2b signaling during early kidney organogenesis. Developmental Dynamics 222:26-39.

IV Lin Y, Zhang S, Rehn M, Itäranta P, Tuukkanen J, Heljäsvaara R, Peltoketo H, Pihlajaniemi T & Vainio S (2001) 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 128:1573-85.

Contents

1 Introduction

The formation of organised biological tissue structures has been one of the great sources of wonder for humankind. Organs are complex structures composed of differentiated cells. Most organs develop from two major tissues, epithelium and associated mesenchyme. The epithelial branching morphogenesis in parenchymal organs is crucial for the establishment of organ-specific forms and structures. There is evidence that a variety of specific soluble factors, including fibroblast growth factors (*FGFs*), epidermal growth factor receptor (*EGFR*) binding ligands, *Wnts*, transforming growth factor (*TGF*β), hedgehogs (*Shh*), transcription factors, distal effector molecules (such as extracellular matrix proteins, integrins, proteinases and their inhibitors) and genes regulating apoptosis and cell proliferation, are involved in the cell signalling that occurs in tissue interactions during organogenesis (Pohl *et al.* 2000, Slack *et al.* 2001). Several classes of antagonists that modulate growth factors have been also identified. The *spry* (sprouty) gene is a recently identified novel negative regulator of the *FGF* signalling pathway that functions in the *Drosophila* tracheal system (Hacohen *et al.* 1998). *Dspry* also inhibits other receptor tyrosine kinases (*RTKs*) such as the *EGF* receptor (Casci *et al.* 1999, Kramer *et al.* 1999, Reich *et al.* 1999). Four mammalian *spry* genes have been identified to date, based on their sequence similarity to *dspry* (Minowada *et al.* 1999). A conserved function between *dspry* and vertebrate *sprouties* has been implicated in lung organogenesis (Minowada *et al.* 1999, Tefft *et al.* 1999) and angiogenesis (Lee *et al.* 2001), but the expression and function of *spry* genes during organogenesis is not well characterized.

Wnt2b is a newly cloned gene of the *Wnt* family that may function as a haematopoietic growth factor and possess similar biological activity to the stem cell factor with respect to human haematopoietic progenitor cells (Van Den Berg *et al.* 1998). *Wnt2b* has recently been shown to be expressed transiently in the early developing chick limb, and it may induce ectopic limb development in the chick via sequential activation of *FGFs* (Kawakami *et al.* 2001). The function of *Wnt2b* in kidney development is not known, however. *Type XVIII* collagen, which is a matrix molecule, contains frizzled and endostatin domains (Rehn *et al.* 1994, Rehn & Pihlajaniemi 1995), of which the frizzles may function as parts of *Wnt* receptors and be involved in the process of *Wnt* signalling-mediated organogenesis. The embryonic lung and kidney have been proposed as useful

models for studying the molecular mechanisms of branching morphogenesis (Saxén, 1987, Hogan, 1999, Metzger & Krasnow, 1999). However, *type XVIII* collagen in the ureteric bud branching and the capacity of the lung mesenchyme to modify the nephrogenic pattern are not elucidated.

In this work we investigate the potential roles of *sprouty* (*spry*), *Wnt2b* and *Type XVIII* collagen in the development of these organs.

2 Review of the literature

2.1 Development of the mouse metanephric kidney

2.1.1 Kidney morphogenesis

Three successive sets of kidneys are formed during intrauterine life in mice: (1) the non-functional pronephros, (2) the mesonephros, which serves as a temporary excretory organ, and (3) the functional metanephros, which forms the permanent kidney. The development of the mouse metanephric kidney starts with the formation of a *Wolffian* duct-derived ureteric bud, which invades the nephric mesenchyme at around 11dpc in response to a signal from latter (Saxén 1987a). It then penetrates the metanephric blastema and starts to undergo repeated bifurcations to form the collecting duct, minor calyx, major calyx, renal pelvis and ureters. Signals from the branching ureteric bud tips in turn induce the loose kidney mesenchymal cells to condense, forming aggregates that epithelialize to give rise to the renal vesicles, which form first a comma-shaped body, then an s-shaped body and finally a functional nephron that includes the renal glomerulus, Bowman's capsule, proximal convoluted tubules, the loops of Henle and distal convoluted tubules that fuse with the collecting duct (Fig.1).

The process of nephrogenesis in the mouse ends 10 days after birth, and tissue interactions between the ureteric bud and mesenchyme play a key role throughout this period. Without the ureteric bud, there would be no tubule formation, as the bud emits the inductive signals necessary for tubule formation. Likewise, the bud would not arise from the nephric duct without the presence of the mesenchyme, nor would it continue to grow and branch (for reviews, see Sariola & Sainio 1997, Vainio *et al.* 1999).

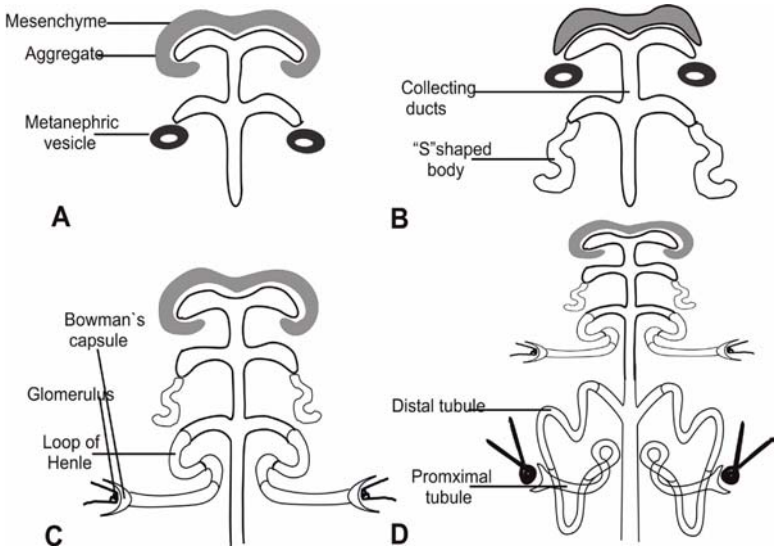

Fig. 1. The early developmental stages of kidney morphogenesis A. The metanephric mesenchyme condenses around the tip of the ureteric bud and forms metanephric visicles, then a comma-shaped body. B. The epithelialized body elongates and forms an S-shaped body. C. The S-shaped body fuses with the tip of the ureteric bud and the glomerulus expands. D. The metanephric tubules and the primordial nephrons become continuous with the collecting tubules to form the uriniferous tubules (modified from Moore KL and Persaud TVN, 1993).

2.1.2 Molecular mechanism of kidney organogenesis

The detailed molecular basis of the interaction between the epithelial and mesenchymal tissue components to form a functional kidney remains unclear (Pohl *et al.* 2000), but the complex process apparently involves sequentially activated genes and both contact-mediated and secreted signals. Genes such as transcription factors, growth factors, matrix molecules and regulators of apoptosis and cell proliferation are also involved (Saxén 1987, Vainio & Müller 1997, Pohl *et al.* 2000) (Fig. 2).

The mammalian kidney forms as a result of reciprocal interactions between the ureteric epithelium and the metanephric mesenchyme. Wilm`s tumour gene 1 (*WT1*), which encodes a transcription factor, may be necessary for the competence of the mesenchyme to form tubules. As a prospective metanephric mesenchyme is formed in *WT1* deficient mice but the cells of the metanephric blastema undergo apoptosis. Furthermore, the ureteric bud fails to grow from the Wolffian duct, and the inductive events that lead to formation of the metanephric kidney do not occur (Kreidberg *et al.* 1993). *Pax2*, another transcription factor, may be controlled by *WT1*, as its expression is reduced in the *WTI* mutant (G Ryan *et al.* 1995, Donovan *et al.* 1999). *Pax 2* is expressed in the metanephric mesenchyme and ureteric bud, and *Pax2* mutant embryos lack kidneys and ureters, pointing to an essential role in kidney development (Torres *et al.* 1995). The

ureteric bud does not grow and *GDNF* is not expressed in the uninduced metanephric mesenchyme of a *Pax2*-deficient kidney, which may suggest that *Pax 2* serves to specify the kidney mesenchyme by coordinating the position of outgrowth of the ureteric bud (Brophy *et al.* 2001). The expression of *GDNF* is also lost in the absence of another gene function, namely that of the transcriptional factor Eya1, but *Pax2* expression persists in the mesenchyme of *Eya1* null embryos and the ureteric bud fails to invade the mesenchyme (Abdelhak *et al.* 1997, Xu *et al.* 1999). The transcriptional factor *Emx2* is mainly expressed in the ureteric bud, and analysis of null mutants has demonstrated that *WT1* and *Pax2* expression are lost in the adjacent metanephric mesenchyme (Dressler *et al.* 1990, Kreidberg *et al.* 1993). The data indicate that *Emx2* lies upstream of *WT1* and *Pax2* in the signal transduction cascade in mesenchyme, followed by *Eya1* and then *GDNF*.

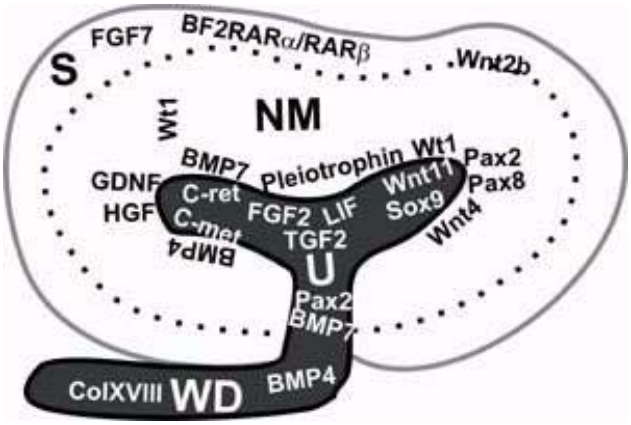

Fig. 2. Schematic presentation of the expression patterns of certain transcription factors, proto-oncogenes, growth factors, cell adhesion and *ECM* molecules in the early developing kidney. The dotted line outlines the nephrogenic mesenchyme (NM) and the stromal mesenchyme (S). For abbreviations, see page 7 and the text.

Growth factors are important modulators of epithelial and mesenchymal interaction, and most of them are expressed in secreted form. They bind to transmembrane receptors and activate signal transduction, which may lead to the activation or repression of specific genes and changes in cell behaviour. Cumulative data from organ cultures, gene targeting experiments and tissue separation and recombination experiments have suggested that a variety of soluble growth factor families, including *FGF*, *Wnt*, *TGF*-β and *hedgehog* are involved in specific tissue interactions and branching morphogenesis (Slack, 2001). The *FGF* and *Wnt* families will be discussed in detail in the next section.

The *TGF*-β superfamily contains *TGF*-β, *activin*, bone morphogenetic proteins (*BMPs*), the *Vg1* family, growth differentiation factors (*GDF*) and other proteins such as glial-derived neurotrophic factor (*GDNF*) and Müllerian inhibitory factor (MIS). One member of this superfamily, *GDNF*, is expressed in the kidney mesenchyme surrounding the ureteric bud (Hellmich *et al.* 1996, Suvanto *et al.* 1996) being directly bound to the bud tips, where its receptor, c-ret, is expressed and induces bud formation (Sainio *et al.* 1997). *GDNF*-deficient mice have no kidneys, as the ureteric bud fails to grow (Jing *et al.*

1996), while mice lacking *c-ret* or its co-receptor *GDNFR∝-1* have related phenotypes (Pichel *et al.* 1996, Schuchardt *et al.* 1994, 1996), strongly suggesting that the *GDNF-c-ret* signalling pathway plays an important role in the development of the renal epithelia. The failure of the ureteric bud to grow in *GDNF-/-* mice has been correlated with the absence of *Wnt11* expression in the bud tips. On the other hand, bone morphogenetic proteins (*BMPs*) and *FGFs* may form a regulatory network and have been proposed as prime candidates for mediating epithelial-mesenchymal interaction in many organ systems (Thesleff *et al.* 1995, Martin *et al.* 1998, Tickle *et al.* 1999). *BMP4* is thought to act as a regulator in metanephric kidney development (Martinez *et al.* 2002), as *BMP4* heterozygous null mutant mice display hypo/dysplastic kidneys, hydroureter, an ectopic ureterovesical (UV) junction and a double collecting system (Miyazaki *et al.* 2000). *In vitro BMP4* releasing beads also inhibit ureter branching and the expression of *Wnt11*, a target of *GDNF* signalling. Thus, where *GDNF* serves as an activator of ureteric development in early kidney morphogenesis, *BMP4* appears to be an inhibitor of the process and lead to down-regulation of *Wnt11* gene expression in the tips of the ureteric bud. Another member of the same family, *BMP7*, has been shown to play an essential role during ontogeny of the mammalian eye and kidney. The kidneys of *BMP7*-deficient mice show a gradual cessation of nephrogenesis, associated with a reduction in branching of the ureteric bud and loss of the metanephric mesenchyme via apoptosis (Dudley *et al.* 1995, Luo *et al.* 1995, Dudley & Robertson, 1997), suggesting that *BMP7* is essential for continuous growth and morphogenesis during the later stages of ureteric bud growth.

In addition to the *GDNF-c-ret* signalling pathway, that of *HGF-c-met* is also implicated in kidney development. Hepatocyte growth factor (*HGF*) is synthesised by the kidney mesenchyme and its receptor, *c-met*, is expressed in the ureteric bud tips. *HGF* regulates branching morphogenesis of epithelial cells derived from the Madin-Darby canine kidney (MDCK) in collagen-matrix cultures (Montesano *et al.* 1991). Mutant mice that are deficient in *HGF* (Schmidt *et al.* 1995) have no kidney phenotypes, however, suggesting that these molecules are not essential for kidney development. In addition, a soluble 18 kDa protein, pleiotrophin, which is able to induce isolated ureteric bud branching morphogenesis with *GDNF* has recently been identified in the conditioned medium of a metanephric mesenchyme cell line (Sakurai *et al.* 2001). Consistent with its role in ureteric bud morphogenesis, this factor was localized to the basement membrane of the developing ureteric bud, and hence it may act as an important mesenchymally derived factor regulating the branching morphogenesis of the ureteric bud.

As reciprocal tissue interaction between the epithelium and mesenchyme regulates kidney development, the ureteric bud also secretes specific factors for induction of the nephrogenic mesenchyme. A cell line derived from the rat ureteric bud is able to induce tubulogenesis in the mesenchyme as well (Barasch *et al.* 1996), and still more interestingly, a specific soluble leukaemia inhibitory factor (*LIF*), identified from this cell line by the same approach, coordinates with *FGF2* and *TGF-2* to cause epithelial conversion and tubular formation in the rat metanephric mesenchyme (Barasch *et al.* 1999, Plisov *et al.* 2001).

The binding patterns of epidermal growth factors (*EGFs*) in various tissues suggest that these may play a role in organogenesis as stimulators of epithelial proliferation during initial epithelial bud formation and during subsequent branching morphogenesis. There is currently no direct evidence, however, that *EGFs* play key roles in the developing kidney.

It has been proposed that the renal stroma may be an important signalling centre for metanephric kidney development, based on the finding that inactivation of the stromal-specific winged-helix transcription factor *Fox2* (*BF2*) results in impaired collecting duct development and nephrogenesis (Hatini *et al.* 1996). *RAR*α and β*2*, which are co-localized in the stromal mesenchyme, are involved in the control of ureteric bud branching morphogenesis. Altered stromal cell patterning and growth defects in the ureteric bud are observed in mice carrying heterozygous mutations for the *RAR*α and β*2* receptor genes, and double knock-out of *RAR*α and β*2* receptors results in down-regulation of the ureteric bud-specific markers, such as *c-ret* and *Wnt11*. Interestingly, forced expression of *ret* in the ureteric buds of mice that are deficient for both *RAR*α and β*2* is sufficient to rescue renal development (Mendelsohn *et al.* 1999), suggesting that there is a reciprocal signalling loop between the ureteric bud epithelium and the stromal mesenchyme which depends on *ret* and vitamin *A* (Batourina *et al.* 2001).

In conclusion, transcription factors such as *WT1*, *Pax2* and *Eya1* in the intermediate mesoderm are required for the formation of the metanephrogenic mesenchyme, and once it has formed *GDNF* from the mesenchyme binds directly to c-ret in the ureteric tips and induces bud initiation and growth. *BMP4* is also involved in the process of bud initiation and growth as an inhibitor, and *BMP7* plays major role at a later stage to maintaining the continuity of bud development. In turn, factors in the ureteric bud such as *Emx2* via *Wnt* signals (*Wnt6*), *c-ret*, *Wnt11*, *BMP4* and *BMP7* stimulate mesenchymal differentiation and epithelization at different stages, and other factors such as *LIF* and *TGF2* cooperate with these to induce epithelial conversion and tubular formation in the metanephric mesenchyme. There is a reciprocal signalling loop between the ureteric bud epithelium and the stromal mesenchyme which depends on *ret* and vitamin *A*. Many genes may contribute to the development of either a single tissue (the epithelium or mesenchyme) or of both.

2.2 Fibroblast Growth Factors, their receptors and organogenesis

2.2.1 Their biochemical activities, and the Ras/MAPK signalling pathway

There are 22 members of the fibroblast growth factor (*FGF*) family in vertebrates, ranging in molecular mass from 17 to 34kDa and sharing 13-71% amino-acid identity (Ornitz *et al.* 2001). The defining features of the family are a high affinity for heparin and heparin-like glycosaminoglycans (*HLGAGs*) and a central core of 140 amino acids that is highly homologous between the family members (Burgess & Maciag 1989, Powers

et al. 2000). The cloning of the signal-transducing receptors for *FGFs* has revealed a tyrosine kinase gene family with at least four members. These four cell surface *FGF* receptors (*FGFRs*) bind members of the *FGF* family with varying affinity（Partanen *et al.* 1993, Ornitz *et al.* 2001). The *FGFs* and relevant information on their specific receptors are listed in Table1. *FGFRs* contain an extracellular ligand binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. Upon reception of the extracellular signals (*FGFs*), these receptor tyrosine kinases (*RTKs*) activate the *Ras/MAPK* signalling pathway. Many of the molecular components, such as *Ras*, *GTPase*, *Raf* kinase and *MAPK* are shared among different *RTKs* (reviewed by Schlessinger, 1993). *Ras* directly interacts with and activates *Raf*, which in turn phosphorylates and activates *MEK*, which then phosphorylates and activates the *MAP* kinases, including *ERK1* and ERK2 (Sasaki *et al.* 2001). *ERK* is an extracellular-signal-regulated kinase that can enter the nucleus and phosphorylate certain transcription factors like *ELK-1* within the responding cell (Figure. 3).

Table 1. Characteristics of the members of the FGF family (modified from Powers et al. 2000).

Name	Synonym(s)	Signalling through high-affinity receptors
FGF1	Acidic FGF, aFGF	FGFR1,IIIb & IIIc; FGFR2, IIIb &IIIc; FGFR3, IIIb &IIIc; FGFR4
FGF2	Basic FGF, bFGF	FGFR1, IIIb & IIIc; FGFR2, IIIc; FGFR3, IIIc; FGFR4
FGF3	Int-2	FGFR1, IIIb; FGFR2, IIIb
FGF4	kFGF,Kaposi FGF,hst-1	FGFR1, IIIc; FGFR2, IIIc; FGFR3, IIIc; FGFR4
FGF5		FGFR1, IIIc; FGFR2, IIIc
FGF6	hst-2	FGFR1, IIIc; FGFR2, IIIc; FGFR4
FGF7	KGF	FGFR2, IIIb
FGF8	AIGF	FGFR1; FGFR2, IIIc; FGFR3, IIIc; FGFR4
FGF9	GAF	FGFR2, IIIc; FGFR3, IIIb & IIIc; FGFR4
FGF10	kFGF-2	FGFR1, IIIb; FGFR2, IIIb
FGF11-14	FHFs	Unknown
FGF15		Unknown
FGF16-19		FGFR1, IIIc; FGFR2, IIIc
FGF20	XFGF-20	Unknown

2.2.2 The roles of FGFs and FGFRs in vertebrate development

FGFs are vital intercellular signalling molecules that regulate numerous processes in embryogenesis and organogenesis, including kidney development (reviewed by Basilico & Moscatelli 1992, Szebenyi & Fallon 1999). *FGF* signaling is required for cell proliferation/survival at the time of mouse embryonic implantation (embryonic day E 4.0) (Feldman *et al.* 1995, Arman *et al.* 1998) and for cell migration during gastrulation (beginning at ~6.5) (Ciruna *et al.* 1997, Sun *et al.* 1999). *FGF4* homozygous embryos die

at E6.5 as a consequence of impaired growth of inner cell mass. *Fgf5* expression increases dramatically in the pluripotent embryonic ectoderm just prior to gastrulation, suggesting that *FGF5* might play a regulatory role in gastrulation (Hebert *et al.* 1991).

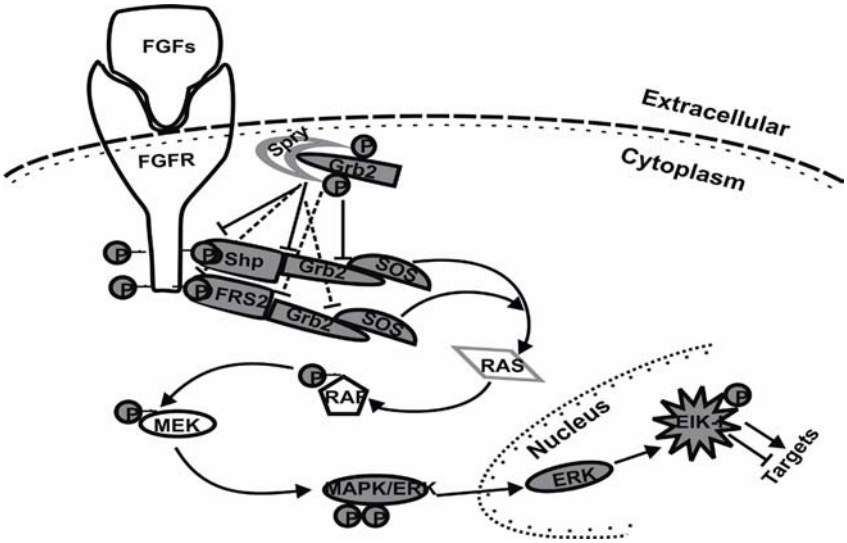

Fig. 3. The Ras/MAPK signalling pathway. The binding of the *FGF* to *FGFR* causes the autophosphorylation and activation of the *RTK*. The *RTK* binds to an adaptor protein *Grb2* or *Grb2-Sos* complex via its docking protein *Shp* or *FRS2*. The *Sos* then activates the *Ras*, which in turn phosphorylates a series of *MAP* kinases (*Raf*, *MEK*, *ERK*). *ERK* enters nucleus phosphorylates and activates transcription factor like *ELK-1*, which regulates its targets. *Spry* will be described later.

FGFs regulate the development of the brain, teeth, limbs, lungs, kidneys and many other organs at the later stages of embryogenesis (reviewed by Goldfarb, 1996, Szebenyi and Fallon, 1999, Qiao *et al*. 2001). Most of FGFs are expressed in the various stages of brain development, although perinatal *FGF6* expression in mice was restricted to the central nervous system and skeletal muscles, with an intense signal in the developing cerebrum of embryos and of 5-day-old neonates. The results suggest the involvement of these growth factors in the regulation of a variety of developmental processes in the brain (Ozawa *et al*. 1996). Tooth morphogenesis is regulated by epithelial-mesenchymal interactions and involves conserved signalling pathways shared with many other developmental processes. *FGF* signals exhibit striking expression in the dental epithelium at specific stages of tooth development (for a review, see Jernvall and Thesleff, 2000). *FGF4* was the first member of the *FGFs* to be implicated in tooth development (Niswander & Martin 1992), but *FGF9* was subsequently found to be expressed in the enamel knot. There is evidence that *FGF8* acts as an early epithelium signal during tooth initiation (Jernvall &Thesleff 2000, Peters & Balling 1999, Tucker & Sharpe 1999), while functional studies of *FGF3*, *FGF7* and *FGF10* in tooth development have pointed to the existence of regulatory signalling cascades between epithelial and mesenchymal FGFs

(Kettunen *et al.* 2000). Many targets of growth factors have been identified, and mutations in several genes within the signalling networks are known to cause defective tooth formation in both humans and mice (Thesleff *et al.* 2002).

FGF signalling has been studied extensively in vertebrate limbs, where it is thought to provide the signals that induce limb formation and maintain the initial outgrowth of the limb bud (reviewed by Martin, 1998). Mice overexpressing *FGF2* has short limb bones (Coffin *et al.* 1995), and limb bud formation was initiated in *FGF10-/-* embryos but no outgrowth of the limb buds occurred (Sekine *et al.* 1999).

Both in vivo and in vitro experiments support the importance of *FGF* signalling for lung morphogenesis. *FGF* polypeptides, including *FGF1*, *FGF2*, *FGF7*, *FGF9*, *FGF10* and *FGF18*, are expressed in the developing lung (Clark *et al.* 2001), and various *FGF* polypeptides are expressed in the lung, with distinct temporospatial patterns of expression. *FGF-7* (also termed keratinocyte growth factor), for example, is expressed in the foetal lung mesenchyme so that ectopic expression of *FGF7* in the developing lung *in vivo*, or the application of *FGF7* to lung explants *in vitro*, disrupted branching morphogenesis and enhanced epithelial cell proliferation, causing cystadenomatoid malformations (Simonet *et al*. 1995, Zhou *et al.* 1996). *FGF10* is expressed dynamically in the mesenchyme adjacent to the distal buds from the earliest stages of lung development onwards (Bellusci *et al.* 1997), and its spatially restricted expression in the mesenchyme induces cell shape remodelling, cell migration and proliferation of the adjacent epithelial cells (Park *et al.* 1998, Weaver *et al.* 2000). Since *FGF10-/-* mice died at birth due to a lack of lung development, *FGF10* evidently serves as an essential regulator of lung and limb formation (Sekine *et al.* 1999).

The *FGF* signalling pathway also plays an essential role during kidney organogenesis. The expression patterns of *FGFs* showed that all of them from *FGF1* to *FGF10* except for *FGF6* are expressed in the developing rat kidney, suggesting an important role in kidney development (Cancilla *et al.* 2001). Selected *FGFs* and *FGFR* that are expressed in the embryonic kidney are shown in Table2. It has been reported that the renal mesenchyme secretes one or more *FGF* family members, e.g. *FGF2* and/or *FGF7,* in order to regulate ureteric bud growth (Drummond *et al.* 1998). In situ hybridization and immunocytochemistry performed on samples of human foetal kidney demonstrated *FGF2* expression in the epithelial cells of the branching ureteric bud epithelium, S-shaped bodies, proximal tubule epithelium and parietal epithelium of the glomerulus (Drummond *et al.* 1998). Furthermore, *FGF2* is one of the limited number of factors that induce ureteric bud cells to undergo morphogenesis (Sakurai *et al.* 1997), and it can mimic some effects of established inductor tissues for the metanephrogenic mesenchyme and cause the up-regulation of two genes that appear to be required for renal morphogenesis, *WT1* and *c-met*, as discussed by Perantoni *et al.* (1995). *TGF*β*-2*, *LIF* and *FGF2* cooperate to regulate nephrogenesis through a common *Wnt*-dependent mechanism (Plisov *et al.* 2001), while *KGF* (*FGF7*) is expressed in stromal cells adjacent to the trunk portions of the ureteric bud and the collecting duct branches, and *FGF10* is expressed in the mesenchyme.

More recent research has shown that several *FGFs*, including *FGF1*, *FGF2*, *FGF7* and *FGF10*, in combination with *GDNF* and *BSN-CM* (BSN cell-conditioned medium), were found to affect the growth and branching of the isolated ureteric bud, but *FGF1* and *FGF10* appear to be more important for branching and branch elongation, and may thus

play a role in determining the number of nephrons and their patterning in the developing kidney (Qiao *et al.* 2001). Transgenic studies have also suggested a role for ligands of *FGFR2* (IIIb) group of proteins, which includes *FGF1*, *FGF3*, *FGF7* and *FGF10*, in kidney development (Celli *et al.* 1998). Only null mutations of *FGF7* and *FGF10* have been found to have any effect, albeit variable, on kidney development, however (Ohuchi *et al.* 2000, Qiao *et al.* 1999). The kidneys of *FGF7* null embryos are markedly smaller due to reduced growth of the ureteric bud and collecting system (Qiao *et al.* 1999). Hence, both *in vivo* and *in vitro* experiments suggest a role for *FGFs* in different stages of kidney development.

Like the FGFs, FGFRs also play important roles during embryogenesis and organogenesis. The extracellular region of the FGFR contains three immunoglobulin-like (Ig-like) domains (IgI, IgII and IgIII). Three alternative versions of IgIII domains (referred to as domains IIIa, IIIb and IIIc) exist in FGFRs1-3. The IIIb splice forms of *FGFR1* and *FGFR2* are expressed in the dental epithelium during tooth morphogenesis, while the *IIIc* splice form of *FGFR1* is expressed both in the epithelium and mesenchyme and *FGFR2 IIIc* is confined to the mesenchymal cells of the dental follicle. Both splice forms of *FGFR3* are expressed in the dental papilla mesenchyme, but *FGFR4* is not expressed in the developing teeth at all (Kettunen *et al.* 1998).

Table 2. Expression of selected FGFs and FGFRs in kidney development.

Gene	Epithelium	mesenchyme		Reference
		Nephrogenic mesenchyme	Stromal mesenchyme	
FGF2	tips and trunk	S-shaped body; proximal tubule; epithelium; parietal epithelium of glomeruli		Drummond1998
FGF7			Stromal cells	Qiao 1999
FGF8		Mesenchymal condensates tubules		Zhang 2002
FGF10		Weak signal in mesenchyme	Stromal cells	Zhang 2002
FGFR1	Ureteric bud	Condensates		Cancilla 1999
FGFR2	Ureteric bud			Cancilla 1999
FGFR3	Ureteric bud	Condensates		Cancilla 1999
FGFR4	Ureteric bud			Cancilla 1999

The similar expression patterns obtained for *FGFR4* and its ligand *FGF6* suggest that these play a significant role as a ligand-receptor system in the maturation of the nervous system (Ozawa *et al.* 1996). Transgenic mice that are hemizygous for the mutant human *FGFR3* gene display disproportionate dwarfism, with skeletal phenotypes remarkably similar to those of human achondroplasia, while mice that are homozygous for the transgene suffer from a profound delay in skeletal development and die at birth, being similar in this respect to humans who are homozygous for the achondroplasia mutant gene (Segev *et al.* 2000). Mice with loss-of-function mutations in *FGFR3* have abnormally long limb bones, suggesting that *FGFR3* plays a role in bone development (Colvin *et al.* 1996, Deng *et al.* 1996).

The *FGFR2-IIIb* splice variant is expressed at a high level in the epithelial cells of the lung buds, probably binding the *FGF7* and *FGF10* expressed by mesenchymal cells. The expression of a dominant negative FGFR2 in the lung bud epithelium blocks branching morphogenesis and epithelial differentiation of the mouse lung (Peters *et al.* 1993). The receptors were localized to distinct, overlapping nephron segments in the developing kidney, and all the nephron and collecting duct epithelia contained at least one *FGFR*. *FGFR1* and *FGFR3* were localized to the glomeruli, *FGFR3* to the proximal tubules and *FGFR1* to the thin limbs. *FGFR1* to *FGFR3* were localized to the distal straight tubules, and *FGFR1* and *FGFR3* to the distal convoluted tubules, and also to the medullary collecting ducts (Cancilla *et al.* 2001). *FGFR3* transcripts in the chick were mostly lost from the kidney at later stages, while *FGFR2* expression persisted in the nephric duct (Walshe *et al.* 2000). Embryos with targeted deletion of *FGFR1* die of growth and patterning defects shortly after gastrulation (Yamaguchi *et al.* 1994). Genetic analysis of the Apert syndrome, which results from mutations in the *KGF* (*FGF7*) receptor *FGFR2*, also demonstrated that defects in signal transduction mediated by *FGFR2* can result in abnormalities in the growth and maturation of the ureteric bud (Wilkie *et al.* 1995).

2.3 Sprouty genes and their roles in organogenesis

2.3.1 Sprouties in signal transduction

The *spry* gene was initially identified as an inhibitor of the *FGF* signalling pathway in the *Drosophila* tracheal system (Hacohen *et al.* 1998), and subsequent reports indicated that *spry* can also inhibit other receptor tyrosine kinases (*RTKs*) such as the *EGF* receptor, Torso and Sevenless (Casci *et al.* 1999). A single *EGF* receptor homologue encoded by the *EGFR* gene and two *FGF* receptor homologues encoded by *breathless* and *heartless* have been identified in *Drosophila* (Beiman *et al.* 1996, Gisselbrecht *et al.* 1996, Glazer & Shilo 1991, Klämbt *et al.* 1992, Livneh *et al.* 1985). In contrast to an earlier proposal that *spry* is an extracellular protein (Hacohen *et al.* 1998), biochemical analysis suggested that it is an intracellular protein with a highly conserved cysteine-rich region that is tightly associated with the inner surface of the plasma membrane. *Spry* binds to two intracellular components of the *Ras* pathway, *Drk* and *Gap1*, which indicates that *spry* is a widespread inhibitor of *Ras* pathway signal transduction (Casci 1999).

Low level *spry* enhances the phenotype of constitutive *EGFR,* but has no effect on the constitutive *Ras1* or *Raf* phenotypes. Overexpression of *spry* is able to rescue the phenotype caused by the expression of constitutive *EGFR*. These interactions indicate that *spry* acts downstream of (or parallel to) *EGFR*, but upstream of *Ras1* and *Raf* (Casci, 1999). Ectopic *spry* eliminates the normal accumulation of *dpERK* (double phosphorylated MAP kinase) in the embryo and wing discs, demonstrating that the intersection point is at the *MAP* kinase kinase (MEK) stage, or upstream of it (Reich, 1999). Due to lethality resulting from the ectopic expression of activated *Ras* or *Raf*, it was difficult to determine the intersection point any more precisely, but the capacity of

ectopic *spry* to reverse the lethality induced by activated *Raf* suggests that it can inhibit *Raf* itself or components downstream of *Raf*. Similarly, *spry* expression in the ovary is capable of eliminating phenotypes induced by activated *Raf* (Reich *et al.* 1999).

Although three human genes have been identified to date that bear a sequence similarity to *Drosophila spry* (Hacohen *et al.* 1998), the function of mammalian *spry* proteins has not yet been elucidated. The primary sequences of the mammalian *spry* gene products share a conserved cysteine-rich C-terminal domain with their *Drosophila* counterpart, but the N-terminal regions do not exhibit any great degree of homology. The *hspry2* sequence encodes a 315-residue (35 kD) polypeptide and contains a cysteine-rich domain which is highly conserved in relation to *spry* (51% identity, with 21 of the 22 *spry* cysteines conserved) and two additional short stretches possessing a similarity to *spry* in the N-terminal region but no predicted signal peptide. *Hspry1* and *hspry3* also show strong conservation of the cysteine-rich domain, with 51%-70% identity to other family members in the available sequences (Hacohen 1998).

Data from Yigzaw *et al.* (2001) show that *hspry2* inhibits the actions of a number of growth factors and that its C-terminus, which is homologous among the various *spry* isoforms, is important for mediating its biological activity. A short sequence in the N-terminus of *hspry2* was found to bind directly to the ring finger domain of *c-Cbl*, suggesting that one function of *hspry2* in signalling processes downstream of *RTKs* may be to modulate *c-Cbl* (Wong *et al.* 2001). *Spry2* mRNA is transiently upregulated in primary human dermal endothelial cells (*MVEC*) in response to *FGF2,* while overexpression of *spry2* in A375 cells leads to the secretion of a soluble factor that inhibits *FGF2*- but not *VEGF*-stimulated proliferation of *MVEC*. ìPull downî and coimmunoprecipitation experiments demonstrated that *spry2* protein binds the intracellular adaptor protein *GRB2*, the human homologue of *Drk*, indicating an intracellular localization (Glienke *et al.* 2000). It may be speculated from these results that *spry* may have a similar function in vertebrate development to that observed in *Drosophila melanogaster*, as an *FGF* and *EGF* antagonist inhibiting the *Ras/MAP* signalling pathway. Indeed, a number of studies of the expression pattern of *spry* during mouse embryogenesis and organogenesis support this possibility.

The question of where a spry acts in the *Ras* signal transduction pathway is obscure, although spry genes have been extensively studied in the past years. Most resent data from Hanafusa (2002) finally found a solution to answer this question. According to his report that *spry* is translocated and phosphorylated to the plasma membrane in response to *FGF* or *EGF* stimulation. Then, *spry* binds to the adaptor protein *Grb2* and inhibit the recruitment of the *Grb2-Sos* complex either to *FGFR* and *EGFR*, or to docking adaptor protein *FRS2* and *Shp2*. Thus, *spry* blocks Ras/MAPK signaling pathway, see also Figure3.

2.3.2 Spry genes and their roles in vertebrate development

Four potential mouse homologues of *spry* have been identified. One of them, *spry4*, exhibits a very restricted expression pattern. *Spry4* is widely expressed in the distal mesenchyme of the developing lung, but not in the epithelium or trachea. Strong expression is detected in the mesenchyme of the accessory lobe of the lung, whereas *Fgf10* is expressed in discrete areas of the distal mesenchyme. Strong expression is observed in the distal mesenchyme of the median, accessory and light caudal lobes and in the lateral and distal region of the left lobe at 11.5 dpc, but no signal is detected in the trachea. At 12.5 dpc, *FGF10* expression is restricted to the distal mesenchyme between buds. In conclusion, *spry4* and *FGF10* have overlapping but not identical expression patterns (de Maximy 1999). Mouse *spry2* and *spry4* RNAs are detected in the embryo and in many adult tissues, including the heart, brain, lung, kidney and skeletal muscle. Whole-mount in situ hybridisation analysis has demonstrated that *spry1, spry2* and *spry4 a*re expressed in similar, highly localised domains in the mouse embryo at E8.5-E9.5. In limb development, *spry* overexpression caused a reduction in limb bud outgrowth and an associated reduction in *FGF* signalling from the apical ectodermal ridge. At the later stages of development there was a dramatic reduction in the length of the skeletal element in the infected limbs, due to the inhibition of chondrocyte differentiation. The results suggest that the vertebrate *spry* protein functions as an *FGF*-induced feedback inhibitor and suggest a possible role for *spry* genes in the pathogenesis of a specific human syndrome caused by activating mutations in *FGFR3 (*Minowada *et al. 1999)* The use of an antisense oligonucleotide strategy to reduce the expression of *mspry2* by 96%, in E11.5 murine embryonic lungs cultured for 4 days apparently resulted in a 72% increase in embryonic lung branching morphogenesis and a significant increase in the expression of the lung epithelial marker genes *SP-C, SP-B* and *SP-A* (Tefft *et al.* 1999). *Mspry2* was expressed in a domain that was restricted in time and space, adjacent to that of *FGF10* in the peripheral mesenchyme, and *FGF10* beads upregulated its expression in the adjacent epithelium in embryonic lung explant culture. Lung cultures treated with exogenous *FGF10* showed greater branching and higher levels of *mspry2* mRNA, while conversely *FGF10* antisense oligonucleotides reduced branching and lower the *mspry2* mRNA levels. Treatment with exogenous *FGF10* or antisense *FGF10* did not alter the *Shh* or *FGFR2* mRNA levels in the lungs, however. Overexpression of *mspry2* in the peripheral lung epithelium resulted in a low level of branching, lung lobe edges that were abnormal in appearance and inhibition of epithelial proliferation. These results indicated that *mspry2* functions as a negative regulator of embryonic lung morphogenesis and growth (Mailleux *et al.* 2001).

Chick *spry1* and *spry2* have also been identified (Chambers *et al.* 2000) and their genes have been found to display similar but distinctive expression patterns at comparable developmental stages, HH9-15. In situ hybridization performed on whole chick embryos showed chick *spry2* to be expressed initially within the isthmus and rhombomere 1, spatially and temporally coincident with *FGF8* expression, whereas this domain was more extensive than that of *FGF8* at the later stages. Introduction of ligand-coated beads into either the midbrain or hindbrain region revealed that *spry2* can be

rapidly induced by *FGF8*. These data suggest that *spry2* participates in a negative feedback regulatory loop to modulate the patterning activity of *FGF8* in the isthmus (Chambers, 2000).

Xenopus spry2 is expressed in a similar pattern to the known *FGFs* and is dependent on the *FGF/Ras/MAPK* pathway for its expression. It is expressed in a dynamic pattern throughout gastrulation and brain and neural development in a manner resembling that of *xFGF8*, but was not expressed at all in the absence of *FGF* signalling. The shortened A-P axis produced after mis-expression of *xspry2* was consistent with a role for *xspry2* in inhibiting *FGFR*-dependent signalling. Blocking the *FGF* pathway in early *Xenopus* embryos inhibits mesoderm induction and results in truncation of the anterior-posterior axis (Nutt *et al.* 2001). It has been shown using *xenopus* oocytes that *xspry2* is an intracellular antagonist of *FGF*-dependent calcium signalling. These results provide evidence for at least two distinct *FGF*-dependent signalling transduction pathways: a *spry*-insensitive *Ras/MAPK* pathway required for the transcription of most mesodermal genes, and a *spry*-sensitive pathway required for coordination of cellular morphogenesis (Nutt, 2001).

In looking for novel factors involved in regulation of the fibroblast growth factor (*FGF*) signalling pathway, a *Zebrafish spry4* gene was isolated, based on its extensive similarities with the expression patterns of both *FGF8* and *FGF3*. Gain and loss-of-function experiments have demonstrated that these growth factors act *in vivo* to induce the expression of *spry4*, which in turn can inhibit their activity. When overexpressed at low doses, *spry4* induces loss of cerebellum and reduction in size of the otic vesicle, thereby mimicking the *FGF8*-/- cerebellar mutant phenotype. Injections of high doses of *spry4* cause ventralization of the embryo, a phenotype opposite to the dorsalization induced by overexpression of *FGF8* or *FGF3*. Conversely, inhibition of *spry4* function through injection of antisense morpholino oligonucleotide leads to a weak dorsalization of the embryo, the phenotype expected for an upregulation of the *FGF8* or *FGF3* signalling pathway. Finally, this analysis reveals that *spry4* interferes with *FGF* signalling downstream of the *FGF* receptor 1 (*FGFR1*) and downregulates *BMP*4 expression (Furthauer *et al.* 2001).

Considering the role of mammalian *spry* homologues in angiogenesis, it has been shown that *spry* may regulate angiogenesis in normal and disease processes by modulating signalling by endothelial tyrosine kinases. The ability of constitutively activated mutant RasL61 to reverse *spry4* inhibition of *MAPK* phosphorylation suggests that *spry* inhibits receptor tyrosine kinase signalling upstream of *Ras* (Lee *et al.* 2001).

2.4 The Wnt family in kidney organogenesis

Together with other families of secreted factors such as *FGF*, *TGF*-β and *hedgehog* protein, *Wnt* proteins are implicated in a wide variety of biological processes such as cell differentiation, cell polarity, cell migration, cell proliferation and tumorigenesis (Wodarz A & Nusse 1998, Miller *et al.* 1999). The 18 *Wnt* genes identified in the mouse so far encode secreted glycoproteins that bind to the cell surface and the extracellular matrix

(Hays *et al.* 1997, Pfeiffer *et al.* 2000). The receptors of *Wnts* are members of the *Fz f*amily, containing a cysteine-rich domain (*CRD)* (Vinson *et al.* 1989, Leyns *et al.* 1997). Vertebrate *Wnts* have been divided into two functional groups by reference to their biological activities in specific assays. Ectopic expression of one group (e.g. *Wnt1, 3A,* 8, and *8B)* activates the function of Frizzled receptors through the canonical *Wnt/*β-catenin signalling pathway in order to stabilise β-catenin, which then functions in the nucleus to activate the expression of specific target genes (Figure 4). Lithium mimic Wnt signaling by direct inhibition of GSK-3, leading to accumulation of cytoplasmic β-catenin (Klein &Melton, 1996, Hedgepeth *et al.* 1997). The other *Wnts* (e.g. *Wnt4*, *5A* and *11*) activate the function of Frizzled receptors through a non-canonical *Wnt* signalling pathway named the *Wnt/*Ca^{2+} pathway in order to stimulate intracellular Ca^{2+} release and activate two kinases, *CamKII* and *PKC*, in a *G*-protein-dependent manner, see Figure 4 also (Kuhl *et al.* 2000).

Wnt proteins play several roles in the development of the urogenital organ. *Wnt4* is expressed in early condensate and pretubular aggregates preceding tubulogenesis, so that its expression suggests a role in the control of kidney tubulogenesis. Indeed, *Wnt4* mutants have severely hypoplastic kidneys, which implie that *Wnt4* is necessary for tubulogenesis, and especially for mesenchymal-epithelial transformation (Stark *et al.* 1994). In the absence of *Wnt4* the metanephric mesenchyme condenses normally and the early induction markers *WT1*, *Pax2* and *N-myc* are induced, but the mesenchyme fails to generate pretubular aggregates or to undergo subsequent tubulogenesis. *Wnt4* is involved in the transition of the tubular mesenchyme to the epithelium and acts downstream of the initial inductive events by controlling cell adhesion, possibly via cadherins expressed between induced pretubular cells (for reviews, see Vainio *et al.* 1999, Uusitalo *et al.* 1999, Kuure *et al.* 2000), and is required for tubulogenesis both *in vivo* and *in vitro* (Stark *et al.* 1994, Kispert *et al.* 1998). Cell lines expressing *Wnt1*, *Wnt4*, *Wnt6* and *Wnt7b* can induce tubule formation in the kidney mesenchyme (Herzlinger *et al.* 1994, Kispert *et al.* 1998, Itäranta *et al.* 2002). *Wnt7b* and *Wnt6* are both expressed in the ureteric bud, but only *Wnt6* is expressed in the branching tips, making it a natural candidate for a ureter epithelium-derived signal that leads to mesenchymal tubule formation. There are also another two *Wnts* that are expressed in the developing kidney, *Wnt11* and *Wnt15*. *Wnt11* expression is induced at the extreme tips of the ureteric bud by contact with the metanephric mesenchyme (Kispert *et al.* 1996), and is well positioned to be an inducer of tubulogenesis. It is inactive in this capacity, however, when expressed in cells co-cultured with isolated metanephric mesenchymes. *Wnt15* is also expressed at the tips of the ureteric bud and collecting system, but its function is unknown.

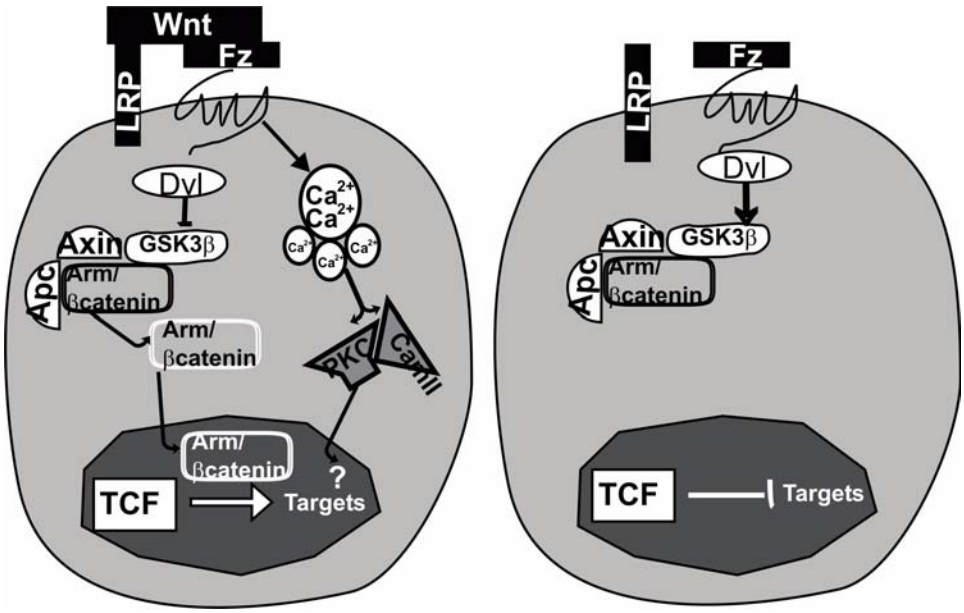

Fig. 4. The *Wnt* signalling pathway. (Left) *Wnt*/β-*catenin* pathway: *Wnt* proteins bind to receptors (*Frizzles*) and a co-receptor (*LRP*) on the cell surface through a cytoplasmic relay component Dishevelled (*Dvl*), which inhibits the function of a complex containing *GSK-3*, *Axin* and *APC*, resulting in an accumulation of β-*catenin* in the cytoplasm. The β-*catenin* then enters the nucleus and forms a complex with *TCF* to activate the transcription of *Wnt* target genes. Wnt/Ca^{2+} pathway: *Wnt* proteins bind to *Frizzles,* which stimulates the release of intracellular Ca^{2+} and activates PKC and CamII kinases. The cellular responses are not known. (Right) *Wnt*/β-*catenin* pathway: In the absence of *Wnt*, *Dvl* activates the function of a complex containing *GSK-3*, Axin and *APC*, resulting in the degradation of β-catenin in the cytoplasm. This means that β-catenin does not enter the nucleus or form a complex with *TCF* to activate the transcription of *Wnt* target genes.

Wnt2b (*Wnt13*) is expressed in the embryonic mesoderm during gastrulation, and transcripts are detected at later stages in the dorsal midline of the diencephalon and mesencephalon, the heart primordia, the periphery of the lung bud and the otic and optic vesicles. These data suggest that *Wnt2b* might partially overlap in function with other *Wnt* genes involved in the cell signalling mechanisms controlling mesoderm specification during gastrulation and some aspects of brain, heart and lung formation (Zakin *et al.* 1998). The expression pattern and function of *Wnt2b* in kidney development is not yet well known, however.

2.5 Type XVIII collagen in kidney organogenesis

The extracellular matrix (ECM), consisting of collagens, elastin, proteoglycans (PG) and a variety of specialized glycoproteins such as fibronectin and laminin, is an important component in morphogenesis. The collagen superfamily includes 24 proteins formally defined as collagens and several less well-characterised ones (for a review, see Myllyharju & Kivirikko, 2001). All collagen molecules consist of three polypeptide chains, called α chains that are wrapped around each other into a triple helix. Every third amino acid in each chain is glycine, and thus the sequence of an α chain in a protein can be expressed as (Gly-X-Y), where X and Y represent amino acids other than glycine and vary according to the collagen type and domain. Collagen plays a dominant role in maintaining the structural integrity of various tissues and organs and also has a number of other important, newly identified functions. Collagens also mediate epithelial-mesenchymal interactions during organogenesis, e.g. branching morphogenesis.

Type XVIII collagen is a component of the basement membrane (BM) zone characterized by the occurrence of short and long N-terminal variants, see also Figure 5 (Rehn *et al.* 1994, Saarela *et al.* 1998a, Saarela, 1998b). The variant polypeptide forms are transcribed from two widely separated promoters (Rehn *et al.* 1996). *Type XVIII* collagen may play roles in morphogenesis and tumorigenesis, since its longest variant contains a *Fz* domain homologous to the ligand-binding domain of frizzled molecules which act as part of the Wnt receptors (Rehn & Pihlajaniemi, 1995, Rehn *et al.* 1998, for a review, see Wodarz & Nusse, 1998), and its C terminus contains a proteolytic anti-angiogenic peptide with tumor-suppressing activity known as endostatin (O'Reilly *et al.* 1997, Dhanabal *et al.* 1999a,b, for reviews, see also Cao *et al.* 2001, Myllyharju & Kivirikko, 2001).

The *Fz* domain is an interesting motif, as it shows 26-27% identity and 50-51% homology with the cysteine-rich domain (*CRD*) found in each of the three previously characterized ´frizzled` proteins, namely the rat *Fz* proteins 1 and 2 and the *Drosophila Fz* protein (for a review, see Pihlajaniemi & Rehn, 1995). As the extracellular part of the *Fz* protein in *Drosophila* is a tissue polarity receptor for the wingless family of signalling molecules the corresponding *Fz* domain in *type XVIII* collagen may also possess ligand-binding properties (Pihlajaniemi & Rehn, 1995, Rehn *et al.* 1998). The endostatin fragment of *type XVIII* collagen also has other interesting features. It has been shown to inhibit hepatocyte growth factor *(HGF)* and *EGF*-dependent ureter branching morphogenesis when present in excess, whereas blocking of its function has been shown to enhance the outgrowth and branching of renal epithelial cells or the isolated ureteric bud *in vitro* (Karihalloo *et al.* 2001). Hence the presence of endostatin at the tips of the ureter bud may play a role in the regulation of UB arborization. Mice lacking collagen *type XVIII* and its proteolytically derived product endostatin show delayed regression of blood vessels in the vitreous body along the surface of the retina after birth and abnormal outgrowth of the retinal vessels or a lack of these vessels. This suggests that collagen *XVIII*/endostatin is critical for normal blood vessel formation in the eye (Fukai *et al.* 2002).

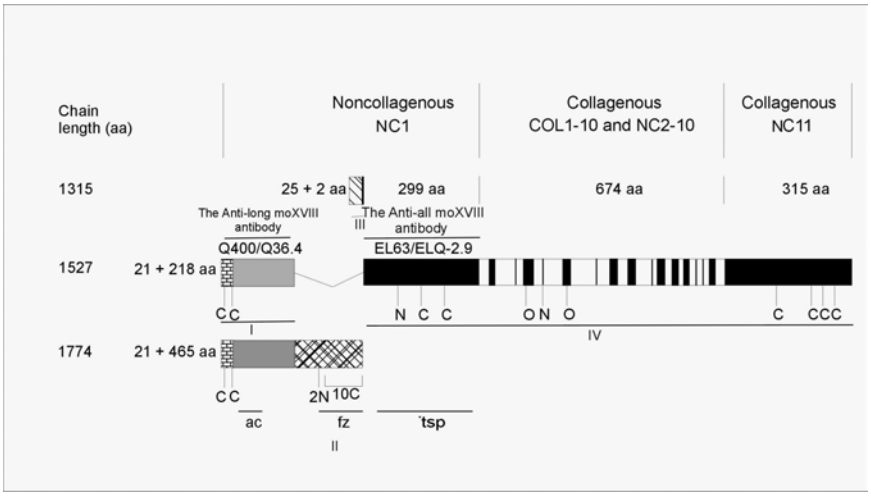

Fig. 5. Schematic structures of the full-length variant polypeptides of the mouse 1 (XVIII) collagen chains. The collagenous sequences are shown in white, the non-collagenous domains common to all variants are shown in black, the non-collagenous sequences common to both long variant NC1 domain portions are shown in gray, and the non-collagenous sequence unique to NC1-764 variant is shown by cross-hatching. The putative signal peptide 1 is indicated with brisk hatching, and the putative signal peptides 2 are indicated with right hatching. The lengths of the amino acid sequences (aa) specific to each variant are given, as is also the length of the common regions. C, cysteine residue; 10C, cluster of 10 cysteine residues; N, potential N-glycosylation site; 2N, two adjacent N-glycosylation sites; O, potential O-linked glycosylation site; Fz and tsp, Fz and thrombospondin sequence motifs, respectively; ac, acidic domain. The cDNA fragments, named ELQ-2.9, used for the production and purification of the anti-all mo XVIII antibody, and Q36.4, used for the production and purification of the anti-long moXVIII, are shown above in the schematic structure. (After Pihlajaniemi & Rehn 1995, Saarela 1998).

Type XVIII collagen is abundant in the retina, epidermis, pia, cardia, striated muscle, kidney, blood vessels and lung. Positive staining was notable in association with the blood vessels, but also in the BM zone of the skin and faintly in Bowman's capsule in the kidney (Muragaki *et al.* 1995). *Type XVIII* collagen resides in the majority of the BM zones of many human and mouse tissue and organs (Saarela *et al.* 1998a, Saarela *et al.* 1998b), and its protein is already visible at E9 in some BM-like structures such as liver sinusoids. The staining pattern in embryos was more restricted when an antibody recognizing the two longer variants was used, since these variants were found to reside only in a limited number of BM zones, e.g. in the kidney, skin and liver sinusoids. The antibody recognizing all *type XVIII* collagen variants, however, stained most of the BM zones of the central and peripheral nervous system, the gastrointestinal system and the cardiovascular system and some of those in the respiratory system. The short variant is significantly expressed in the tissues where the long variant is also present, except in the liver sinusoids. There are also variant-specific differences in the distribution of *type XVIII* collagen during mouse development. The short variant was found to be expressed evenly throughout the course of development, while expression of the long variant became stronger in the interval E11-15 (Saarela, 1998a).

3 Aims of the research

The kidney is an excellent model for studying the mechanisms of the reciprocal signalling interactions between epithelial and mesenchymal tissues during organogenesis. The *FGF* signalling pathway plays an important role in kidney development, and the *spry* genes are a recently identified antagonist of this pathway. The expression and function of *spry* genes during organogenesis has not been well characterized, however. *Wnt2b* has recently been shown to be expressed transiently in the early developing chick limb, where it may induce ectopic development by sequential activation of *FGFs* (Kawakami *et al.* 2001). *Type XVIII* collagen contains frizzled and endostatin domains (Rehn *et al.* 1994, Rehn & Pihlajaniemi, 1994), the Frizzles being capable of functioning as parts of *Wnt* receptors and being involved in the process of *Wnt* signalling-mediated organogenesis. The functions of *type XVIII* collagen and *Wnt2b* in kidney development are not clear, however.

Thus the main aims of this thesis are:

1. to investigate the expression of *spry* genes during mouse organogenesis
2. to study the functions of *spry* genes by generating transgenic mice
3. to study the functions of *Wnt2b* during kidney organogenesis
4. to explore the functions of *type XVIII* collagen during ureteric bud branching morphogenesis.

4 Material and methods

The primers used to screen the transgenic mice spanned the junction of the rabbit β-*globin* and *hspry2* cDNA. The β-*globin* primer P1 (universe 5`GAGTCCAAACCGGGCCCCTCTGC3`) and the *hspry2* primer P2 (reverse 5`CGAGGAGCAGGCTTGAGCCCAGG3´) amplified the expected 300bp band (data not shown).

4.1 Mouse strains and cell lines (I-IV)

The CD-1, Rosa-26 (Soriano, 1999) and green fluorescent protein (*GFP*) (Hadjantonakis *et al.* 1998) mouse lines, 3T3 cells expressing *Wnt2b*, *Wnt4* or *Wnt6* and untransfected cell lines were used for the experiments. The transgenic construct was injected into (BLxDBA2) F2 zygotes, and the transgenic mice were backcrossed to the C57BL/6 strain.

4.2 Organ culture methods (I-IV)

4.2.1 Normal organ culture and conditional organ culture (I-IV)

Organ culture was performed according to Vainio *et al.* (1993). The dissected organ rudiments were placed directly on slices of Nuclepore filter (pore size 0.1 mm, Costar) supported by a stainless steel grid. The culture medium, *i.e.* Dulbecco's modified Eagle's medium (MEM, Gibco), was supplemented with 20% foetal bovine serum (FBS) (Gibco) and penicillin/streptomycin (GibcoBRL). Human growth factors, recombinant *FGF2*, *FGF7* and *FGF10* and *GDNF* were applied to the medium at different concentrations to rescue the transgenic kidney. The samples were incubated in 5% CO2 in humidified air at

37°C, and the medium was changed every other day. In the assay of *Wnt2b* function, an isolated ureteric bud was placed on top of a monolayer of *Wnt2b*, *Wnt6* and *Wnt4* cell lines. Untransfected cells and normal culture media were used as controls.

4.2.2 Hanging drop assays and agarose bead experiments (III-IV)

The hanging drop assay was conducted according to Vainio *et al.* (1992). The isolated ureter buds were washed before and after incubation in a hanging drop with 30-35 µl of culture medium. The *GDNF* concentration in the hanging drop was 100ng/ml. Bovine serum albumin was used as a control, and cell lines expressing *Wnt4* or *Wnt6* and untransfected NIH 3T3 cells served as controls in the *Wnt2b* cell line hanging drop experiment. The subculture time was 2 to 4 hours. The bud was washed extensively to remove the cells and recombined with kidney mesenchyme that had been separated from it. The agarose beads containing *GDNF*, *FGF2*, *FGF7*, *FGF10* and BSA were generated using affi-gel blue agarose beads (100-200 mesh, 75-150 nm diameter; Bio-Rad, UK) (Vainio *et al.* 1993).

4.2.3 Tissue recombination (III-IV)

The tissue separation and recombination experiments were mainly performed as described in previous publications (Vainio *et al.* 1993, Shannon *et al.* 1994). Most of the tissue separation and recombination experiments were carried out on E11.5 mouse embryos if not otherwise indicated. The organ rudiments were incubated for two minutes in 3% pancreatin/trypsin (GibcoBRL, UK) in Tyrode's solution and separated out microsurgically into culture medium at room temperature. The tissues were then kept on ice until used. Several heterotypic and homotypic recombinations were carried out, such as ureteric bud and lung mesenchyme (UB/LM), lung bud and kidney mesenchyme (LB/KM), ureteric bud and kidney mesenchyme (UB/KM), and lung bud and lung mesenchyme (LB/LM). Additional combinations included tracheal bud and lung mesenchyme (TB/LM), lung bud and tracheal mesenchyme (LB/TM), and spinal cord and kidney mesenchyme (SP/KM). One lung mesenchyme and one ureteric bud were used for each UB/LM recombinant, whereas for the recombinations involving left or right lung mesenchymes (UB/LLM or UB/RLM), two left lung mesenchymes or right lung mesenchymes were used with each ureteric bud. Likewise, three or four kidney mesenchymes were used with one lung bud in LB/KM and two lung mesenchymes for one lung bud in LB/LM. For the UB/TM recombination three tracheal mesenchymes were used for one ureteric bud, and for UB/KM two kidney mesenchymes were used for one ureteric bud. *GDNF* (PeproTech, USA) was always added to the culture medium in the UB/KM recombination.

4.3 Production of transgenic mice (II)

4.3.1 Transgenic constructs (II)

The *Pax2* promoter, containing a 4.3KB BamHI-NotI fragment that included the 5`leader but not the ATG or 3.6KB of the 5`sequence (Ryan *et al.* 1995) was cloned to pBS (Stratagene)***. The plasmid pIRES2-EGFP, containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), the enhanced green fluorescent protein (EGFP) coding region and the SV40 early mRNA polyadenylation signal, was purchased from Clontech. A 0.64KB BamHI-EcoRI fragment of the rabbit -*globin* gene that contains parts of exons 2 and 3 and intron 2 (Sasaki *et al.* 1994) provided the 5`splice acceptor and donor site for the construct. The *hSpry2* full-length cDNA containing EcoRI and BamHI was amplified from dbEST (R552589) by PCR using the 5`-universe primer GAATTCATGGAGGCCAGAGCTCAGAGTG and the 3`-reverse primer CGCGGATCCCTATGTTGGTTTTTCAAAGTTCC.

To generate the Pax2/hspry2GFP construct, a 1KB BamHI-XbaI fragment containing the polylinker BamHI-SalI-XbaI from PQE31 (QIAGEN) was cloned into the pBS vector. The PCR product of *hspry2* cDNA was first inserted at the EcoRI and BamHI sites of IRES2-EGFP, after which a fragment consisting of *hspry2*, IRESGFP and SV40polyA was excised from the pIRES2-EGFP vector by cutting with EcoRI and SspI, and ligated into modified pBS at EcoRI and SmaI. The rabbit β-*globin* was excised with BamHI and EcoRV, blunted and inserted downstream of the *Pax2* promoter, which was cut at NotI and blunted. The orientation was confirmed by sequencing. The whole fragment of the *Pax2* promoter flanking β-*globin* was excised from the vector with EcoRI and ligated at the EcoRI site upstream of the *hspry2* cDNA. The orientation was again confirmed by sequencing. The transgenic construct was excised from the vector with SalI.

4.3.2 Generation of transgenic mice (II)

The pronuclear microinjections of the transgene into mouse eggs were performed essentially as described by Hogan *et al.* (1994), the purified insert fragment being diluted to a concentration of 5.0ng/ul for the injections. Potential transgenic founders were screened by PCR and confirmed by expression of the GFP (green fluorescence protein) in the offspring.

The primers used to screen the transgenic mice spanned the junction of the rabbit β-globin and hspry2 cDNA. The β-globin primer P1 (universe 5`GAGTCCAAACCGGGCCCCTCTGC3`) and the hspry2 primer P2 (reverse 5`CGAGGAGCAGGCTTGAGCCCAGG3´) amplified the expected 300bp band (data not shown).

4.4 Whole-mount and section mRNA in situ hybridization (I-IV)

Details on the preparation of the probes and the methods used for whole-mount, non-radioactive and radioactive *in situ* hybridization are provided in the separate articles. The double staining for mRNA and protein was also based on the protocol described by Parr *et al.* (1993), with some modifications. The β-galactosidase stainings of cultured whole-mount samples were performed according to Nonchev *et al.* (1996) and those for the section samples according to Bobola *et al.* (1995).

4.5 Whole-mount and section immunostaining (II-IV)

The ELQ and Q36.4 antibodies against type XVIII collagen are described by Saarela *et al.* (1998). Both were used for immunostaining at a concentration of 20 µg/ml. The TROMA-I antibody against cytokeratin Endo A was from the Developmental Studies Hybridoma Bank (USA), and the antibody against mouse pulmonary surfactant protein C (*SP-C*), m-20, was from Santa Cruz Biotechnology, Inc. (USA). The antibody against the brush-border antigen was from Dr. Ekblom (Ekblom *et al.* 1980b). The secondary antibodies used were peroxidase-conjugated donkey anti-rabbit IgG and donkey anti-rat IgG, TRITC or FITC-conjugated donkey anti-rabbit IgG and Biotin SP-conjugated mouse anti-goat IgG (Jackson Immuno Research Laboratories, Inc, USA). The normal bovine serum was also from Jackson Immuno Research Laboratories, Inc, and the normal goat serum from DAKO (Denmark). The whole-mount immunostaining was performed according to Marti *et al.* (1995). Diaminobenzidine (DAB) and aminoethylacarbazole (AEC) (ZYMED, USA) were used as substrates for the peroxidase-conjugated secondary antibodies. The single or double labelling for whole-mount immunofluorescence was according to Sainio *et al.* (1997). The β-galactosidase / antibody double staining was accomplished by performing the β-galactosidase staining first, followed by whole-mount immunostaining. The samples for immunohistochemistry were fixed overnight in 4% paraformaldehyde (PFA), after which 8 µm paraffin-embedded serial sections were cut.

4.6 RNA isolation and RT-PCR (III)

For *Wnt-2b* RT-PCR, embryonic lung and kidney at E11.5, E12.5 and E13.5 were dissected in Dulbecco's PBS and stored in RNAlater™ Tissue Storage (Ambion) until used for RNA isolation. Total RNA was isolated with the RNAeasy Mini Isolation Kit® (Qiagen), and RT-PCR was performed with the Robust RT-PCR kit (Finnzymes), both according to the manufacturers' instructions

4.7 Cell proliferation and apoptosis assays (II-IV)

Apoptosis was visualized using an *in situ* cell detection kit (Boehringer Mannheim). Bromodeoxyuridine labelling and detection of the explants was carried out using a cell proliferation kit (RPN 20, Amersham Life Science, UK) according to Sainio *et al.* (1997).

4.8 Photography and image analysis (I-IV)

The photographing of the whole-mount and section *in situ* samples is described in the separate articles. A Leitz confocal microscope (Leica, Germany) was used for monitoring fluorescence in the explants. For the time-lapse studies, the GFP-UB/KM and GFP-UB/LM recombinant samples were photographed every 24 hours. Digital photomicrography was used to acquire images for analysis using the Scion image analysis software. The composites were generated using the Adobe Photoshop v5.0 program.

5 Results

5.1 Expression pattern of spry genes during mouse embryogenesis (I)

To determine how *spry* genes function in mouse development, we first studied their expression pattern during embryogenesis. Whole-mount and section *in situ* hybridization demonstrated that *spry* genes *1*, *2* and *4* are expressed in several developing organs of the craniofacial area and trunk, including the brain, cochlea, nasal organs, teeth, salivary gland, lungs, digestive tract, kidneys and limb buds. They were expressed in the neopallial cortex, cranial flexure and primordium of the cerebellum at E12.5 and E14.5, but *spry1* was expressed in the midbrain region only at E14.5, while *spry2* and *4* were expressed in this region at E12.5 and 14.5. Viewed in more detail, *spry1* and *2* were also expressed in the optic recess of the diencephalons and in the nasal epithelium at E12.5, whereas *spry4* was expressed in the nasal mesenchyme. At 14.5, *spry1* and *2* were additionally expressed in Rathkeís pouch, the nasal epithelium and the epithelium of the follicles of the vibrissae, while *spry4* was localized to the mesenchymal cells and neuronal cells in these regions. In addition to the brain, *spry1*, *2* and *4* were also expressed in the cranial ganglia, e.g. the trigeminal ganglion. *Spry1* and *2* were also expressed in the epithelium of the cochlea at E14.5, while conversely, *spry4* exhibited a mesenchymal expression pattern. Expression of *spry1*, *2* and *4* was also detected in the epithelium of the developing salivary gland at E12.5 and 14.5, but *spry4* was more restricted to the distal epithelium during branching. *Spry1* and *2* were also detected in the epithelial cells of the embryonic teeth at E12.5 and 14.5, whereas *spry4* was detected in the dental mesenchymal cells at all stages studied.

Regarding the trunk and the primordia of internal organs, a *spry1* transcript was found throughout the epithelium of the lung, but expression was higher in the distal epithelial tips than in the stalk region at E11.5, 12.5 and 14.5. Expression of *spry2* was limited to the distal leading edge of the branching tips in the lung at these stages, while that of *spry4* was intense in the distal tip region of the branching epithelium at E11.5 and it was also localized to the mesenchymal cells adjacent to epithelial tips, but no signal was detected in the presumptive bronchi or the trachea. In the embryonic digestive tract, all three spry

genes were localized to the stomach at E11.5 and to the serosa and submucosa of the gut at E12.5 and 14.5. *Spry1* was prominent in the ureteric bud of the kidney, whereas *spry2* and *4* were expressed in both the ureteric bud and the kidney mesenchyme and glomerular nephron (for details, see Table 3). The expression profiles suggest roles for these *spry* genes in the epithelial-mesenchymal interactions that govern organogenesis.

Table 3. Expression of three spry genes in the developing kidney

gene	Epithelium	Mesenchyme
Sprouty1	E11.5 in the tips, weakly in the Wolffian duct; E12.5, E14.5 only in the tips	
Sprouty2	E11.5 in the tips and the Wolffian duct; E12.5, E14.5 in the tips and collecting ducts	E12.5 mesenchyme adjacent to tips; E14.5 also in the glomerulus
Sprouty4	E11.5 only in the tips	E11.5 and E12.5 in the mesenchyme adjacent to tips; E14.5 also in the glomerulus

5.2 *Sprouty* signalling is involved in coordination of mouse kidney development (II)

To address the function of *spry* in ureteric bud branching and possibly in the control of tubule induction *in vivo*, we expressed the human *spry2* gene in ureteric bud tissue with a *Pax2* promoter.

As judged from *hspry2* mRNA expression and *GFP* expression, a 4.3kb *Pax2* promoter is sufficient to direct transgene expression into the branching tips of the ureteric bud throughout renal development. The DNA construct generated for this purpose contained a green fluorescent protein (GFP), the expression of which in the *Pax2*hSpry2 kidney in three independent transgenic mouse lines (TG1, TG2 and TG3, marked as *Pax2*hSpry2) was initiated in the ureteric bud at E10.75, persisted there throughout kidney development *in vivo* and was also analysed in an *in vitro* organ culture system. *Hspry2* was simultaneously driven by the *Pax2* promoter in the kidneys of the *Pax2*hSpry2 embryos.

Overexpression of *spry* in *Pax2*hSpry2 embryos led to small, ectopic and cystic kidney phenotypes. Kidneys from all three lines were smaller in appearance than those of their wild-type littermates. This feature appeared at E12.5, but was more marked at E15.5, when the transgenic kidneys had visibly less ureteric bud branches and nephron material. The thickness of the cortical layer was reduced by 18.3%, being 0.306± 0.035 μm in the wild type and 0.25± 0.039 μm in the transgene kidney, and the cortical architecture of the kidney was also disorganized. The transgenic kidneys were 17% smaller in volume than the wild-type kidneys and the number of glomeruli 42% less.

In order to reveal the mechanisms involved in the reduction in size in the *Pax2*hSpry2 kidneys, changes in apoptosis and cell proliferation were analysed. Consistent with the reduced size of the *Pax2*hSpry2 kidneys, apoptosis was enhanced in both the epithelium and the mesenchyme, with an associated overall reduction in cell proliferation.

In addition to the small size of the kidneys, we also detected other particularly striking features of the *Pax2*hSpry2 kidneys in all three transgenic lines, including the formation of ectopic kidney lobes (9.68%) and cystic kidneys (4.84%) at different stages. The ectopic ureteric bud was also a hydroureter and began ectopic budding from the stalk region in *in vitro* culture. The phenotypes obtained were mostly unilateral. Serial histological sections indicated that the ectopic kidney was derived from an involuted mesenchyme that gradually separated the kidney into lobes. The kidneys that had cysts possessed either a single cyst or multiple cysts with a dilated transparent ureter. Cysts were also found within the ectopic kidney lobes.

Spry2 overexpression in the $Pax2^{hSpry2}$ kidneys led to downregulation of the expression of the *FGFs* gene. *FGF2* is expressed in the epithelial cells of the branching ureteric bud, nephron precursors ("S-shaped bodies"), the proximal tubule epithelium and the parietal epithelium of the glomerulus, but its expression in the ureteric bud epithelium and mesenchymal cells was down-regulated in the transgenic kidney, although some persisted in the remaining developing glomeruli.

FGF7 is expressed in the wild-type stromal cells, and in the mesenchymal cells of the cortical region, but was down-regulated in the cortical mesenchymal cells and stromal cells of the $Pax2^{hSpry2}$ kidney, although the changes did not occur throughout the kidney and areas of expression similar to the wild type were also observed. *FGF7* was also expressed ectopically in the interstitial cells of the medulla of the $Pax2^{hSpry2}$ kidneys.

FGF8 is normally expressed in the early mesenchymal condensates and developing tubules of the wild-type kidney, but expression was reduced in the transgenic kidney. *FGF9* and *FGF 10* are expressed in the cortical mesenchymal cells in the wild type, and no changes in their gene expression were observed in the $Pax2^{hSpry2}$ kidneys.

Expression of *hspry2* in the ureteric bud led to changes in the mesenchymal expression of *FGF* receptors. Expression of four *FGF* receptors was analysed in transgenic mice and their wild-type control littermates at E15.5 and E17.5. *FGFR1*, which is normally expressed in the metanephric mesenchymal cells adjacent to the branching ureteric tips and in tubules during their development, while no signal is detected in the ureteric bud epithelium, was down-regulated in the mesenchymal cells of the kidneys of the $Pax2^{hSpry2}$ transgenic mice.

Consistent with previous data on the chick kidney (Walshe and Mason, 2000), *FGFR3* was weakly expressed in the stromal cells of the medulla and in the mesenchymal cells surrounding the pelvic epithelium at E17.5. Interestingly, its expression was up-regulated at these sites in the kidneys of the $Pax2^{hSpry2}$ embryos. No changes in the expression of the *FGFR2* and *FGFR4* genes were detected between the wild-type and transgenic kidneys.

Hspry2 expression altered mesenchymal and stromal gene expression. Histological analysis revealed reduced ureter branching, a reduced number of glomeruli and weakened cortical organization in the kidneys of the $Pax2^{hSpry2}$ embryos, suggesting changes in the expression of genes implicated in kidney development.

Pax2 is normally expressed in the ureteric bud, the pretubular mesenchymal cells and the epithelial tubules that derive from these cells, whereas its expression was reduced in the collecting duct of the transgenic kidney. Expression of *GDNF*, another mesenchymal factor that lies downstream of *Pax2*, was down-regulated in the embryonic $Pax2^{hSpry2}$ kidneys relative to the wild type, but its receptor, *C-ret*, was unchanged. Further *in vitro*

organ culture experiments also demonstrated a down-regulation of *GDNF* expression in the *Pax2*^{hSpry2} kidneys as compared with the wild type *Wnt11* expression, which is a target for *GDNF* signalling (Peppicelli *et al.* 1997). Wnt11 was detected in lesser intense amounts in the ureteric tips, which is consistent with reduced *GDNF* expression. *BMP4* gene expression, on the other hand, remained in the pretubular aggregates of the *Pax2*^{hSpry2} kidneys but was below the level of detection in other areas. *FoxB2*, which is normally expressed in stromal cells, showed reduced expression in the stromal component of the *Pax2*^{hSpry2} embryonic kidneys, and a clear reduction in *FoxB2* staining in the cultured kidneys supported the conclusion that expression of this gene was reduced in *Pax2*^{hSpry2} kidneys relative to the wild type.

Pax2^{hSpry2} kidneys *in vitro* have less branches and tubules than the wild type and produce ectopic ureteric budding. When E11.5 kidney explants from transgenic or wild-type embryos were isolated and cultured for various periods before staining with Troma-I antibody to identify the ureteric bud, comparison of the number of branching tips between the transgenic and wild-type kidneys revealed a 64.66% reduction in the transgenic kidneys at 48 hrs of culture, a 47.5% reduction at 96 hrs and an 80% reduction at 144 hrs. Associated with this reduction in the number of ureteric tips was a reduction in the number of tubules, as evaluated by counting the tubules that were reactive to the Brush-border (BB) antigen at all the time points analysed.

FGF and *GDNF* signalling can partially restore ureteric branching in *hSpry2* kidneys. The *in vitro* rescue results indicated that both *FGF7* and *FGF10* promoted branching of the ureteric bud in the transgenic kidney by comparison with the situation in the non-treated transgenic littermates, whereas *FGF2* appeared to inhibit it. No evidence of synergistic effects was seen. In addition to *FGF7* and *FGF10* signalling, *GDNF* also increased the number of branching tips in the transgenic kidney comparing with the non-treated transgenic control kidneys. Interestingly, *FGF7* and *GDNF* induced several ectopic buds on the main trunk of the Wolffian duct in the *Pax2*^{hSpry2} embryonic kidneys, but not in the wild-type controls. *FGF7* and *FGF10* gave rise to 57.4% and 21.9% restorations of the number of branching tips in the transgenic kidneys relative to the controls, whereas *GDNF* restored the number of tips by 63.9%. When the transgenic explants were treated with *FGF2* alone, the number of tips decreased by 40% relative to the untreated control transgenic kidneys.

5.3 Wnt2b promotes ureteric bud survival and growth (III)

To explore the potential role of *Wnt2b* during organogenesis, its expression was examined in mouse embryos at E11.5-15.5. It was found to be expressed in several organs or tissues. Weak expression was seen in the perinephric cells of the kidney and in the mesenchymal cells close to the stalk of the ureter, the area concerned being close to the site of *Fox2*, a marker of presumed stromal cells.

The expression of *Wnt2b* in the mesenchyme and its lack of activity in kidney tubule induction assays suggested that it might alternatively function as a reciprocal signal to control the growth and branching of the epithelial bud. To test this option, we cultured

isolated E11.5 kidney ureteric buds on a monolayer of cells expressing *Wnt2b*, *Wnt4*, *Wnt6* and 3T3 cell. Only *Wnt2b* cells promoted their survival and growth. On the other hand, the development of explants preincubated with normal medium, media containing lithium, NIH 3T3 cells, and cells expressing *Wnt2b* or *Wnt4*, respectively, were monitored after 96 hours in culture and the success rate of the initiated morphogenesis, *i.e.* tubulogenesis and ureteric branching, was scored.

Preincubation of the ureter with cells expressing *Wnt2b* and with lithium led to a remarkable recovery, reconstitution of *in vitro* kidney development and normal expression patterns for *C-ret*, *Wnt11* and *Sox9*. *Wnt4* signalling did not support the reconstitution of kidney organogenesis via signalling to the ureteric bud, but rather restrained it.

5.4 Type XVIII collagen is essential for lung development and ureteric bud repatterning

At the initiation of lung and kidney organogenesis (E10.5), when the epithelial bud grows into the mesenchyme, *type XVIII collagen* mRNA is uniformly present in the epithelial bud of both organs. Expression then became localized to the lung epithelial tips during the formation of the first branches and was lost from the stalk area, while an opposite pattern was observed in the kidney, where both *type XVIII* collagen protein and mRNA were lost from the branching ureter tips.

In heterotypic recombinants between the lung mesenchyme and the ureteric bud, *type XVIII* collagen expression disappeared from the stalk and was instead localized in the epithelial tip cells, accompanied by a shift in signalling molecule sonic hedgehog expression in the epithelium. Moreover, the respecification of *type XVIII* collagen in the heterotypic tissue recombinants was accompanied by changes in the branching pattern. This was observed with both antibodies ELQ and Q36.4, of which the latter specifically recognizes only the two longest *type XVIII* collagen variants. Interestingly, even the long variants were respecified to a lung-type location in the epithelial tip cells, even though these two isoforms are not normally expressed in the early embryonic lung (data not shown). *SP-C*, a lung-specific marker, was also induced in the ureteric bud. Lung samples treated with the anti-all antibody ELQ resulted in an average decrease of 34% in the number of tips and a marked reduction in *Wnt2* expression in the lung mesenchyme, whereas no difference was detected in the expression of *FGF10* or *Shh*, suggesting a regulatory interaction between *type XVIII* collagen and *Wnt2b*.

6 Discussion

6.1 *Spry* plays a functional role in kidney organogenesis

It is evident that three *mspry* genes are expressed in the developing kidney (Zhang *et al.* 2001). *Spry1* is prominent in the ureteric bud, whereas *spry2* and *4* are expressed in both the ureteric bud and the kidney mesenchyme and glomeruli derived from it. Tables 1 and 3 summarize the expression patterns of selected *FGFs* and three *spry* genes in the developing kidney, providing data that raise the possibility that these *spry* genes may act as antagonists of the signalling pathway of *FGFs* during kidney organogenesis.

The overexpression of *spry2* resulted in the down-regulation of several *FGF* signals, like *FGF2*, *FGF7* and its receptor *FGFR1*. Simultaneously, the other important factors, such as *Pax2*, *GDNF*, *BMP4* and *Fox2* were also down-regulated. The addition of *FGF7*, *FGF10* and *GDNF* to the culture medium, but not *FGF2*, restored kidney development and rescued 57.35%, 21.85% and 63.89% of the branches of the ureteric tips in the transgenic kidneys compared with the wild type.

To determine whether *spry* genes play the roles of antagonists of the *FGF* and *EGF* signalling pathways in the developing kidney, we generated *spry2* transgenic mice in which full-length human *spry2* cDNA was mis-expressed into ureteric buds under the control of a 4.3kb *Pax2* promoter. Overexpression of *hspry2* in the ureteric bud leads to a small kidney, a double pelvis with dual ureters and a cystic kidney. These three phenotypes may share a similar mechanism that high level *spry* inhibits and disrupts the ureteric bud elongation and branch at an early stage. A short or split ureteric bud does not fuse with epithelialized renal vesicles properly and the urine does not drain into the collecting duct. As a consequence a small kidney, duplication of the renal pelvis and ureter or a cystic kidney could occur.

The molecular mechanism behind the *hspry2* phenotypes observed in the transgenic kidneys is not well understood at the moment. As *spry* is known to be the inhibitor of the *FGF* signalling pathway in lung development, we also thought that *spry2* may play a negative role in that of the *FGFs* in the development of the mouse kidney as well. During normal development, signals from the ureteric bud are required for induction of the nephrogenic mesenchyme, and these cells in turn signal to the adjacent stromal progenitor

cells, promoting their survival, growth and differentiation. In a reciprocal manner, signals from the mesenchyme also induce ureteric bud growth and elongation. We propose that overexpression of *spry2* in the ureteric bud may first lead to a local change of signal in the ureteric bud. Basic fibroblast growth factor (*FGF2*), one of the *FGF* signals, is mitogenic for most cells and exerts intracrine, autocrine and paracrine effects on epithelial and mesenchymal cells (Coutts *et al.* 1995). In *Pax2*^{hSpry2} kidneys *FGF2* was down-regulated in both the ureteric buds and mesenchyme. Hence, *FGF2* supplies candidate signals for mediating the effects of *hspry2* to the ureteric bud and the kidney mesenchyme. Among the high-affinity receptors for *FGF2*, namely *FGFR1*, *FGFR2* and *FGFR3*, the expression of *FGFR1* in the mesenchyme was also down-regulated, but *FGFR2* in the ureteric bud showed no change, and interestingly, *FGFR3* showed up-regulation in the transgenic kidney at E17.5, although its expression is lost in later kidney development. The mutation of *FGFR3* enhances chondrocyte proliferation during early embryonic skeletal development, but the activation of *FGFR3* reduced proliferation in postnatal-onset dwarf mice, suggesting that signalling through *FGFR3* both promotes and inhibits chondrocyte proliferation depending on the point in time during development (Iwata *et al.* 2000). Consistent with the findings regarding skeletal development, we conclude that *spry* may inhibit *FGF2* via the activation of *FGFR3* signalling in the later stages of kidney development. Overexpression of *spry2* in the ureteric bud of the transgenic kidney causes a local reduction in *FGF2*, where upon it may exert its mainly paracrine effects on the mesenchyme.

We conclude that misexpression of *hspry2* may directly antagonize *FGF2* in the ureteric bud; then indirectly from ureteric bud signals mesenchyme inhibiting *FGF2* expression via *FGF2* mediation; again from mesenchyme signals stromal cells inhibiting expression of *FGF7*, *FGFR1* and activiating *FGFR3* via *FGF2* medication.

Another mediator is *Pax2*, the expression of which was specifically reduced in the epithelial ureteric bud, as this may contribute to diminish branching by downregulating the expression of its target genes, possibly also involving *FGF2*. *GDNF* is not expressed in *Pax2* mutant mice, suggesting that *GDNF* is downstream of *Pax2* (Brophy *et al.* 2001). We might speculate that misexpression of *hspry2* directly downregulates *Pax2* expression in ureteric bud via unknown mechanism; then lost of *Pax2* expression in epithelial cells mediates *GDNF* expression in adjecent mesenchymal cells.

FGF2 is a growth-promoting factor for mesenchymal cells and can prevent their apoptosis. Extracts of pituitary gland with *FGF2* can induce tubulogenesis in the kidney mesenchyme, and *FGF2* may also be critical for kidney vessel formation (Perantoni *et al.* 1995, Barasch *et al.* 1997, Drummond *et al.* 1998, Kloth *et al.* 1998). Hence, by reducing the expression of *FGF2*, *spry2* would apparently lead to the diminished mesenchymal cell proliferation and increased apoptosis observed in the transgenic kidneys, possibly accompanying reduced expression of some tubular markers. *Pax2* suppresses apoptosis in the renal collecting duct cells (Torban *et al.* 2000), and the loss expression of *Pax2* in collecting ducts may explain the reduction in cell proliferation and increased apoptosis in the collecting ducts in *Pax2*^{hSpry2} kidneys as occurring secondarily in the mesenchyme via changes in ureteric bud signalling.

We observed a double pelvis and dual ureters in *Pax2*^{hSpry2} kidneys *in vivo*, and *FGF7* and *GDNF* also promoted ectopic ureteric budding *in vitro*, whereas the controls did not show this effect. Hence, the data suggest that *hspry2* may modulate the expression of

factors that control the patterning of the ureteric bud and *Wolffian* duct, and that *spry2* starts to function at a very early stage in budding. Similar ectopic bud formation to that observed in *hspry2* transgenic kidneys has been reported in *Bmp4 +/-* and *Foxc1* knock-outs (Kume *et al.* 2000, Miyazaki *et al.* 2000). In the case of *Bmp4 +/-* knock-outs, the kidney phenotypes mimic human congenital anomalies of the kidney and urinary tract (CAKUT) and include defects such as hypo/dysplastic kidneys, hydroureter, an ectopic uterovesical junction and a double collecting system (Miyazaki *et al.* 2000), phenotypes that are mostly recapitulated by transgenic *hspry2* kidneys. This may suggest that *BMP4* and *hspry2* contribute to similar processes during kidney development. *Spry2* expression may function via the Fox genes, for example, to alter the proportions of growth factors such as *BMP4* and *GDNF*, the inhibitory and stimulatory factors for bud formation, and may thereby lead to ectopic bud formation.

Taken together, the *in vivo* analysis points to that *spry2* play a critical role during kidney development.

6.2 *Wnt2b* regulates ureteric bud branching and kidney organogenesis

Wnt2b is expressed in kidney organogenesis in the perinephric mesenchyme at E11.5, i.e. in close proximity to the presumed stroma marked by BF-2 expression. Analyses of the function of the stromal genes BF-2 (Hatini *et al.* 1996), RARα and RARβ2 (Mendelsohn *et al.* 1999) have suggested that kidney stromal cells may synthesize factors that influence kidney tubulogenesis. Such stromal signals are also expected to regulate ureteric bud development, because ureteric branching is affected in BF-2 knockout mice and in the RARα/RARβ2 double mutant (Mendelsohn *et al.* 1999). Furthermore, kidney organogenesis is also stimulated by derivatives of vitamin A (Vilar *et al.* 1996). Hence there may be a regulatory link between Wnt signalling and retinoic acid in the kidney as well. The pattern of Wnt2b expression and data showing that this expression can be regulated by retinoic acid in cultured human cells (Wakeman *et al.* 1998) suggest that Wnt2b may be one of the functional signals during early kidney development. Unlike Wnt4, it does not induce tubulogenesis, but it does support ureteric bud epithelial development, and it promotes kidney reconstitution, while Wnt4 does not. Wnt2b is expressed some distance away from the ureteric bud epithelium, however, so that it may diffuse and move closer to the ureteric bud, or else its signalling may be redundant and mimic the activity of the other Wnts present in mesenchymal cells. Wnt4 is localized to the kidney mesenchyme, but ureteric branching is initially unaffected in Wnt4 knockout embryos (Stark *et al.* 1994) and Wnt4 and Wnt2b signalling cause two distinct phenomena (present data). Thus Wnt2b and Wnt4 are unlikely to be redundant, and specific Wnts such as Wnt4 regulate mesenchymal differentiation and tubule induction (Kispert *et al.* 1998), while another member of the family, Wnt2b, acts reciprocally to regulate ureteric branching. Wnt6, Wnt11 and Wnt7b are expressed in the ureteric bud (Kispert *et al.* 1996, 1998) and could thus be involved in mediating the effect of *Wnt2b* on the ureteric bud or the phenocopy activities of *Wnt2b* signalling. *Wnt6* (Itäranta *et al.* 2001) and *Wnt11* are expressed at the branching ureteric tip, but cells expressing *Wnt6* are

not capable of promoting branching of the isolated ureter, which excludes its role in the process. *Wnt11* gene expression is initiated in reconstituted explants of ureteric bud and kidney mesenchyme, but is less intense than in control samples, which suggests that *Wnt11* is not involved in mediating *Wnt2b* signalling to the ureteric epithelium. *Wnt7b* signalling can induce tubulogenesis *in vitro* (Kispert *et al.* 1998), but it is not expressed at the ureteric tips. Hence *Wnt7b* is also unlikely to phenocopy the activity of *Wnt2b*.

Lithium reproduces the effect of cells expressing *Wnt2b*, suggesting that *Wnt* signalling acts directly on the ureteric bud to regulate its branching, and interestingly, both epithelial and mesenchymal responses can be induced by it (Davies *et al.* 1995, present data), suggesting that both tubule induction and maintenance of the bud's inductive activity and branching are mediated by activation of the β-*catenin* signalling pathway rather than alternative signal transduction pathways. The opposing roles of *Wnt2b* and *Wnt4* suggest that the signal transduction cascade upstream of *GSK-3*β, which is capable of responding to *Wnt4* and *Wnt2b*, may not be shared by the ureteric bud epithelium and kidney mesenchyme. Interestingly, *Wnt2b* can induce ectopic limb development in the chick via sequential activation of *FGF10* and *FGF8*, which can also induce ectopic limb development (Kawakami *et al.* 2001). *FGF10* is expressed in the developing lung and kidney as well (Min *et al.* 1998, Sekine *et al.* 1999, Clark *et al.* 2001) and can promote branching of the lung epithelium (Park *et al.* 1998). Hence *Wnt2b* function may be conserved and operate in the lung and kidney to regulate epithelial branching via control over *FGF* signalling. *FGF7* and *GDNF* (Sainio *et al.* 1997, for a review, see Sariola and Sainio, 1997) are additional mesenchymal signals that have been shown to regulate ureteric development. Receptors for *GDNF*, *C-ret* and *FGF7* are present in the ureteric epithelial tips, further supporting a role for these in reciprocal tissue interactions (Qiao *et al.* 1999). *Wnt2b* expression in the kidney is most intense at E11.5 and E12.5 and diminishes after that, which suggests that *Wnt2b* may be functional in promoting the initiation of epithelial branching at the early stages of kidney development, when *GDNF* has also been shown to be active.

Taken together, the expression pattern of the *Wnt2b* gene in mouse embryonic tissues suggests a role in epithelial-mesenchymal interactions. *Wnt2b* signalling is capable of inducing ureteric bud growth and branching, and indirectly also tubulogenesis, via the epithelium. Lithium reproduces the effects of *Wnt2b* signalling, suggesting that signalling to the ureteric bud is direct. *Wnt2b* may thus act as a mesenchymal signal mediating reciprocal signalling from the mesenchyme to the epithelium in order to coordinate early kidney organogenesis. Finally, the data currently available indicate that *Wnt* signalling in the kidney is specific to either the ureteric bud or the kidney mesenchyme, depending on the *Wnt* family member that is active.

6.3 *Type XVIII* collagen regulates modes of epithelial morphogenesis in kidney and lung

The mechanisms by which the rather limited number of embryonic signalling molecules form networks and regulate the spatial organisation of cells during morphogenesis remain poorly understood (Hogan, 1999). To address these questions, two embryonic model systems were used, the lung and the kidney.

Type XVIII collagen is expressed throughout the epithelial bud at the initiation of lung and kidney organogenesis, but becomes localized to the epithelial tips in the lung during the early stages of epithelial branching, while its expression in the kidney is confined to the epithelial stalk region and is lost from the nearly formed ureter tips, thus displaying the reverse pattern to that in the lung. In recombinants between ureter bud and lung mesenchyme, the *type XVIII* collagen expression pattern in the ureter bud shifts from the kidney to the lung type, with an associated shift in *shh* expression and induction of *SP-C* expression. Thus the lung mesenchyme acts as an instructive inducer of the ureteric bud to respecify and reverse these marker gene patterns in recombinants. *Wnt11*, which is a marker of the ureteric tip, is weakly expressed in the recombinant, suggesting an association between *type XVIII* collagen and the *Wnt* signalling pathway. *Proteoglycan* (*PG*) and *metrix* metalloproteinases (*MMPs*) may involve in this process. *Type XVIII* collagen contains a *Fz* domain that antagonizes *Wnt* signalling and plays the opposite role during ureteric bud branching morphogenesis. The down-regulation of *type XVIII* collagen would help the tip *Wnts* diffuse into the adjacent mesenchyme and contribute to the induction of tubules (Stark *et al.* 1994, Kispert *et al.* 1998, Vainio *et al.* 1999). Up-regulation of *Wnt11* and *Wnt6* at the tips may cause down-regulation of *Shh*, since there is evidence that *Wnts* can specifically down-regulate *Shh* and *Ptc* (Sarkar *et al.* 2000).

We also demonstrated here that *type XVIII* collagen is functional during lung branching morphogenesis. Loss of *type XVIII* collagen from the branching tips by antibody blocking causes a dramatic reduction in *Wnt2* gene expression, but *FGF10* and *Shh* do not show a similar decrease. It has been reported that a *Shh* mutant causes *Wnt2* down-regulation but not that of *BMP4* or *Wnt7b*. This indicates that *type XVIII* collagen may cooperate with *Shh* in the epithelium and interact with mesenchymal *Wnts* such as *Wnt2* and Wnt2b, eventually promote the characteristic morphogenesis of the epithelial type during lung organogenesis. This hypothesis is based on the fact that *type XVIII* collagen may function as a *PG* (*proteoglycan*) (Halfter *et al.* 1998) and *PG* are necessary for *Wnt* signalling (Lin& perrimon, 1999). The high level of expression of *Wnt2/2b* in the lung mesenchyme is very close to the tips, where *type XVIII* collagen is expressed, and *Wnt7b* is expressed in the epithelium, which would also support interactions between *type XVIII* collagen and *Wnts* and their roles in lung branching morphogenesis. The mesenchymal inductive signals mediated by *Shh* or *BMP4* in the epithelium may induce *type XVIII* collagen at the branching tips and repress its expression in the stalk. At the same time, feedback inhibitors, e.g. *TGF*-β, *Shh* and *BMP4*, may send signals to the mesenchymal cells to regulate the secretion of mesenchymal growth factors (for a review, see Kaplan, 2000). The notion that *Shh* secreted by the distal tip epithelium may regulate the expression of *BMP4* and/or *Wnt2* in the adjacent mesenchyme is also supported by a previous report (Bellusci *et al.* 1996). The localization of *type XVIII* collagen expression

in the branching tips and its loss from the stalk may be important, as this may ensure that important signals such as the *Wnts* are located at the right site and exert their appropriate function. The culturing of lungs in the presence of a *type XVIII* collagen antibody leads to a reduction in epithelial branching. The expression of *Wnt2*, which remains expressed in heterotypic recombinants, is dramatically reduced in lungs treated with the antibody, while the expression of *Shh* and *FGF10* is not so clearly reduced. Therefore, specific mesenchymal factor(s), e.g. *Wnt2*, may function independently of or in combination with other growth factors, interacting with epithelial factors, e.g. *Shh* and *type XVIII* collagen, and repatterning the branching morphogenesis of the ureteric bud towards the early lung type.

The fact that the *type XVIII* collagen antibody reduced the number of epithelial tips in lung explants and almost completely blocked epithelial morphogenesis in heterotypic recombinants suggests an important role for *type XVIII* collagen in the chain of molecular events associated with the reprogramming of ureteric bud development. The marked decrease in *Wnt2* expression in antibody-blocked explants supports a role for *type XVIII* collagen in epithelial development as well. The respecification of *type XVIII* collagen at the tip in UB/LM, which may have been mediated by *Shh* and *BMP4*, could be involved in binding *Wnts* to the branching epithelial tips and concentrating them there in order to promote typical morphogenesis. To summarize, we have demonstrated an intra-organ patterning process that involves *type XVIII* collagen, an extracellular matrix protein containing endostatin and frizzled domains. The repatterning of *type XVIII* collagen expression was accompanied by ectopic expression of lung *SP-C* and the morphogen *Shh*, and this was associated with changes in the early epigenesis of the ureteric bud towards the early lung type.

Our data support a model according to which differential forms may be regulated by patterning cues exchanged during morphogenetic inductive tissue interactions, and these localized signalling activities of growth factors involve extracellular matrix molecules such as *type XVIII* collagen, *laminin*, *integrin*, *PG* and *MMPs*.

7 Conclusions and future perspectives

We have analysed here the expression of three *spry* genes during kidney organogenesis. The expression profiles suggest roles for these *spry* genes in the epithelial-mesenchymal interactions that govern organogenesis. In order to obtain a better understanding of the function of *spry* in kidney development, we generated a $Pax2^{hSpry2}$ transgenic mouse model. Overexpression of *hspry* in the ureteric bud resulted in small, ectopic and cystic kidneys, while *Hspry2* expression in the kidney led to reduced cell proliferation and enhanced apoptosis. *Spry2* overexpression in the $Pax2^{hSpry2}$ kidneys led to reduced expression of *FGF2* in the ureteric epithelium and mesenchyme, accompanied by reduced expression *FGF7*, *FGF8* and *FGFR1* in the mesenchyme but up-regulated *FGFR3* expression. Furthermore, transgenic expression of *hspry2* led to changes in the *GNDF* signal, which may be associated with down-regulation of *Wnt11*, *BMP4* and *Pax2* in the ureteric bud. The phenotype was more severe in *in vitro* organ culture, where the addition of *FGF7* and *GDNF* protein was able to restore the branching of the ureteric bud in the $Pax2^{hSpry2}$ kidneys towards the situation in the controls. Interestingly, transgenic expression of *hsrpy2* led to an ectopic ureteric bud and administration of *FGFs* and *GDNF* to the induction of supernumerary budding from the *Wolffian* duct in the $Pax2^{hSpry2}$ kidneys, which was not seen in the wild types. Taken together, our data reveal that *spry2* apparently plays a critical role in signalling by *FGFs* and in that way may coordinate the expression of several signalling pathways in the epithelium and mesenchyme during kidney development. The *spry* transgenic mouse line provides a model for further study of the relationship between *spry* and the signalling pathway of the *FGFs* and other signalling pathways such as *Pax2*, *GDNF-Cret* and *BMP4*. The $Pax2^{hSpry2}$ transgenic mice also developed a phenotype in the reproductive tract and ear which will be analysed in detail.

In addition to the *spry* gene, the roles of *type XVIII* collagen and *Wnt2b* during organogenesis were investigated. We demonstrated that *type XVIII* collagen was differentially expressed and that the molecule could play a role in shaping the specific form of the organs during morphogenesis. Our data point to an intra-organ patterning process revealed by the expression of *type XVIII* collagen that may be regulated by the

position of inductive factors and matrix molecules. *Wnt2b* may act as an early mesenchymal signal controlling the morphogenesis of epithelial tissue, and the *Wnt* pathway may regulate ureteric branching directly.

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