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WNT4 AND WNT6 SECRETED GROWTH AND DIFFERENTIATION FACTORS AND NEURAL CREST IN THE CONTROL OF KIDNEY DEVELOPMENT

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Abstract

Secreted signalling molecules are important for the regulation of developmental cell responses. In the developing kidney, signalling occurs between epithelial ureteric bud and metanephric mesenchyme and in between their derivatives.

Wnt6 gene activity was localized to the ureteric bud and newly formed branches of the ureteric tree during early stages of kidney development. In a classic organ culture system, Wnt6 signalling induced the activation of marker genes for early nephrogenesis. The metanephric mesenchymes isolated from the Wnt4 deficient embryos were also induced, and the *Wnt4* gene became activated in the presence of a Wnt6 signalling source. We propose that Wnt-6 is involved as a metanephric inducer and controls nephrogenesis.

Wnt4 is essential for nephrogenesis in mouse and we indicate an additional role for Wnt4 in the control of periureteric stromal differentiation. A failure in vascular development was also found. *Bmp4* expression in the medullar stroma of the Wnt4-deficient kidneys was absent concomitantly with a loss of expression of the smooth muscle marker, α-SMA. *In vitro* Wnt4 signalling induced *Bmp4* expression and local α-SMA production. Hence, we conclude that lack of Wnt4 signalling leads to a loss of the periureteric smooth muscle cells, and Wnt4 may locally regulate this cell population in normal kidneys via regulation of Bmp4 signalling.

The pluripotent neural crest cells are proposed to play regulatory roles in the early metanephros. Here, the use of transgenic animals allowed visualisation of the lumbo-sacral neural crest (NC) cells in close proximity to the early metanephros. The NC cells, however, disappeared in most part of the kidney by E12.5. The Splotch embryos lack the NCs from the early urogenital region. A developmental defect in the kidneys of Splotch embryos was not observed *in vivo* or *in vitro*. The results suggest that the neural crest is not essential for early embryonic kidney development.

In sum, the work presented indicates an important role for Wnt6 in the induction of kidney tubules *in vitro*, for Wnt4 in the specification of kidney smooth muscle cells and for endothelial development in kidney. The neural crest cells apparently have no active morphogenetic role in early kidney development.

Keywords: induction, kidney organogenesis, mouse, neural crest, stromal development, Wnt signalling

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Abbreviations

Apc	adenomatosis polyposis coli
α-SMA	smooth muscle alpha-actin
Bmp	bone morphogenetic protein
Bmpr (Alk)	bone morphogenetic protein receptor
Dkk	dickkopf homolog
Dvl	dishevelled
E	embryonic day
EGF	epidermal growth factor
Eya1	eyes absent 1 homolog
Emx	empty spiracles homolog
Fgf	fibroblast growth factor
Fgfr	fibroblast growth factor receptor
Fox	forkhead box
Fzd	frizzled homolog
Gdnf	glial cell line derived neurotrophic factor
Gfra	glial cell line derived neurotrophic factor family receptor alpha
Gsk	glycogen synthase kinase
GTP	guanidine triphosphate
Hox	homeo box
Jun	Jun oncogene
Kdr (Flk1)	kinase insert domain protein receptor
Lef	lymphoid enhancer binding factor
Lhx (Lim1)	LIM homeobox protein
Lrp	low density lipoprotein receptor-related protein
Myc	myelocytomatosis oncogene
NC	neural crest
Osr	odd-skipped related
Pax	paired box gene
PCP	planar cell polarity
PCR	polymerase chain reaction
Rar	retinoic acid receptor
Ret	ret proto-oncogene
Rho	ras homolog gene family
Robo	roundabout homolog
Ror	receptor tyrosine kinase-like orphan receptor

Ryk	receptor-like tyrosine kinase
Sall	sal-like
sFrp	secreted Frizzled receptor related protein
Shh	sonic hedgehog
Six	sine oculis-related homeobox gene
Slit	slit homolog
Sox	SRY-box containing gene
SPC	spinal chord
Tcf	transcription factor (T-cell specific)
Tek (Tie2)	endothelial-specific receptor tyrosine kinase
Tgf	transforming growth factor
Vegf	vascular endothelial growth factor
Wnt	wingless related MMTV integration site
Wt1	Wilms tumor homolog

List of original articles

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

I Itäranta P, Lin Y, Peräsaari J, Roel G, Destree O & Vainio S (2002) Wnt6 is expressed in the ureter bud and induces kidney tubule development in vitro. Genesis 32:259-68.

II Itäranta P, Chi L, Seppänen T, Niku M, Tuukkanen J, Peltoketo H &Vainio S (2006) Wnt4 signalling is involved in the control of smooth muscle cell fate via Bmp4 in the medullary stroma of the developing kidney. Dev Biol 293:473-83.

III Itäranta P, Viiri K, Kaartinen V &Vainio S (2007) Fate mapping of lumbo-sacral neural crest cells and their function in kidney organogenesis. Manuscript.

Contents

1 Introduction

The mouse metanephric kidney has been an important model to study the molecular background of inductive epithelial-mesenchymal signalling that control the embryonic tissue interactions during mammalian organogenesis. This conclusion is supported by the fact that in no other mammalian species is such detailed previous information of the mechanisms of kidney development (Saxén 1987, Saxén 2001) or genetic methods to address the molecular controlling mechanisms is available. In addition, the early stages of kidney development in mouse are reminiscent of that in human and advances in genetic targeting techniques have generated important knowledge from inherited human kidney diseases and conditions.

The Wnts compose one of the groups of important secreted signalling molecules that operate in various inductive tissue interactions (Cadigan & Nusse 1997, Miller 2002, Perantoni 2003). Accurate genetic control of Wnt signalling is also required after birth e.g. to prevent cancerous growth. The role of Wnts and their signalling mechanisms in mouse and in embryonic kidneys is, however, still relatively poorly understood and under ongoing research.

In mammals, the epithelial nephric duct and mesenchymal metanephric blastema - both derived from the intermediate mesoderm of the embryo - initiate the definitive kidney organogenesis via reciprocal signalling interactions. The neural crest cells emigrate from a dorsal location in the embryo and contribute - in addition to sensory and autonomous innervation - many different cell types to several distant tissues as pointed by avian studies (Le Douarin 1999). In mouse, it has been known for a long time that the neural crest cells are present also in the early metanephros and hence might contribute early to the developing kidney by unknown mechanism(s) (Cullen-McEwen *et al.* 2005). Recent genetic evidence implies that the neural crest might supply a significant proportion of cells to the kidney (Engleka *et al.* 2005). Thus, the putative role of neural crest cells in the developing kidney is of interest and requires investigation.

The first part of this study addresses the presence of Wnt6 in the tissues of the developing mouse embryonic kidney and the signalling role of Wnt6 during kidney organogenesis. The second work investigates the role of another Wnt family member, Wnt4, which is essential for nephrogenesis (Stark *et al.* 1994), and aims to elucidate the possible functions of Wnt4 in the developing kidney in mouse. The third part, in turn, aims to clarify the proposed roles that the

migratory and pluripotent neural crest cells might have during kidney organogenesis.

2 Review of the literature

2.1 Inductive signalling

Signalling interactions between developing tissues create the ordered form and function of an organ. The process is called morphogenesis (Gilbert 2006). The signalling between tissues of an organ during the morphogenetic tissue interactions is sequential and reciprocal. Morphogenetic signalling leads to the stereotypical positioning and arrangement of cells in a developing embryo or in an organ, which is referred to as pattern formation, and this process is called cellular patterning.

Signalling that induces differentiation or otherwise influences cellular behaviour as part of morphogenetic regulation is said to be inductive. Inductive signalling is classified as either instructive or permissive. Instructive signalling from one tissue specifically changes the developmental fate of the recipient tissue. In permissive signalling, the recipient tissue has the potential to express a specific trait, but it requires a suitable surrounding in order to realise that developmental potential. Primary embryonic induction refers to the induction of the embryonic axis and neural development, while all other signalling is called secondary embryonic signalling.

2.1.1 Inductive signalling in embryogenesis

Molecular studies of inductive signalling have revealed that it is usually mediated by intercellular protein morphogens, belonging to secreted growth and signalling factor families such as those of the TGF-β super family and the Wnt, Hedgehog, Fgf and Egf families. The signalling can also be mediated by lipid- or peptide hormones. The concerted action of several signalling morphogens leads to cascades and as a result, to the feedback control of individual signalling pathways. Such feedback control can be positive and negative between interacting tissues and can often be considered as signalling loops (Freeman 2000). The range and amount of the signalling molecules, for example, have been found important for the response in inductive signalling (Eaton 2006).

Findings from molecular studies have also demonstrated that Wnts and modifiers of Wnt signalling, such as antagonists, play central roles in early embryogenesis (Mukhopadhyay *et al.* 2001, Robb & Tam 2004). For example,

Wnt signalling is required for the establishment of the neural crest cell population in *Xenopus* and in chicken, and Wnts play a role in the regulation of premigratory neural crest cells in mouse as well (Knecht & Bronner-Fraser 2002, LaBonne 2002).

2.1.2 Inductive signalling during organogenesis

During the period preceding organ formation, the tissue primordia that give rise to organs are under the influence of inductive signalling from other tissues and can be considered as morphogenetic fields (Freeman 2000, Gilbert 2006). During organogenesis the inductive signalling interactions occur mainly between cells and tissues fated to assemble the organ. Such inductive interactions between epithelial and mesenchymal tissues give rise to several organs including the embryonic kidney and the lung (Saxén 1987, Lin *et al.* 2001, Shu *et al.* 2005).

2.2 Kidney organogenesis

The permanent kidneys in mammals arise from specific metanephric tissue in the embryo, but the mammals also have three embryonic kidneys when the mechanism of kidney development is approached from the evolutionary perspective. The kidneys originate from the intermediate mesoderm and develop through three successive stages (Saxén 1987, Gilbert 2006).

2.2.1 Kidney organogenesis in vertebrates

In the early embryo, the intermediate mesoderm is located between the paraxial mesoderm that forms the somites and the lateral plate mesoderm delineating the body cavity. At the most anterior end of the intermediate mesoderm, the cells are induced to undergo mesenchyme-to-epithelium transformation and to form the paired pronephric ducts that arise at embryonic day 8 (at E8) in mouse and at day 22 in human gestation (Figure 1; Kuure *et al.* 2000). Studies in *Xenopus* and chick suggest that the pronephric duct arises in response to secondary inducing signals from the anterior somites (Seufert *et al.* 1999, Mauch *et al.* 2000). The pronephric duct subsequently elongates caudally by cell proliferation along the surface of the intermediate mesoderm. When the intermediate mesoderm thickens, it forms the nephrogenic cords that locate along the dorsal embryonic body cavity. Besides the pronephric duct, the pronephric tubules are induced in the adjacent mesenchyme

and they may form before the duct (Kuure *et al.* 2000, Mitchell *et al.* 2007). The pronephric duct and the nephrogenic cord grow towards the mesonephric and finally the metanephric regions. The duct is then called the nephric duct or Wolffian duct. In fish and amphibian larval stages the pronephros functions as a secretory organ, but in mammals and birds it represents a transient structure that regresses and disappears while the caudal parts of the nephrogenic cord are still at the early stages (Kuure *et al.* 2000).

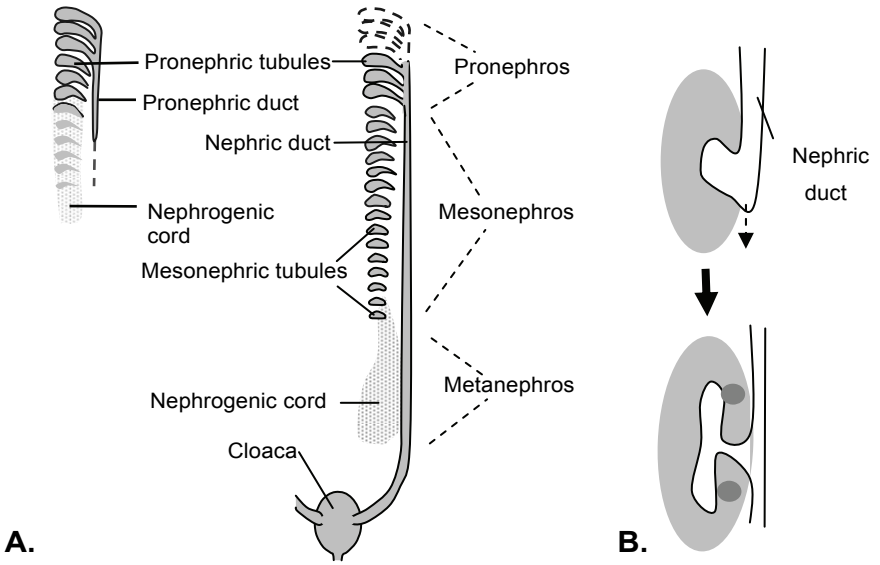

Fig. 1. General representation of the three embryonic kidneys formed in vertebrates. At first, the anterior region of the nephrogenic cord (the rest is not shown) gives rise to the pronephric tubules and pronephric duct. The pronephros extends caudally and more tubules are induced at mesonephric and metanephric regions (not shown) from which the latter gives rise to permanent kidneys in mammals (Sainio *et al.* 1997; Modified from Saxén, 1987). B. In mouse metanephros, the ureteric bud is induced at around E10 before the rest of the nephric duct proceeds (broken arrow) towards the cloaca and urogenital sinus. At E11.5 the ureter has branched into a T-shaped structure and induces pretubular cell aggregates (dark dots) as the first sign of metanephric tubule formation.

At the mesonephric region (Kuure *et al.* 2000, Pietilä & Vainio 2005) the nephric duct induces transient mesonephric tubules that mostly regress except in males. Some of the mesonephric tubules together with the Wolffian ducts contribute to male sex duct development (Sainio *et al.* 1997, Chi *et al.* 2006). In females, the Wolffian ducts regress due to the lack of androgens while another

pair of ducts, the Müllerian ducts, contribute to female sex duct development (Vainio *et al.* 1999a, Heikkilä *et al.* 2005). When the Wolffian duct reaches the metanephric region, it initiates together with the metanephric mesoderm the morphogenetic process that leads to the formation of permanent kidneys in mammals. The metanephric mesenchyme is also referred to as metanephric blastema, indicating that the tissue is composed of as yet undifferentiated cells. Hence, the development of permanent kidneys begins via inductive interactions between tissues of intermediate mesoderm origin, in mouse at E10 (I; Saxén 1987).

2.2.2 Morphogenesis of the mouse kidney

The ureteric bud is induced from the Wolffian duct by signals produced by the metanephric blastema that consists of a group of morphologically similar mesenchymal cells (Saxén 1987, Al-Awqati & Oliver 2002). At first, a diverticulum forms at the end of the duct that then elongates towards, and invades, the blastema. The ureteric bud branches at first into a T-shaped epithelial duct and during the development it arborizes to form an elaborate branched epithelial tree that later forms the collecting ducts and the kidney pelvis as a result of morphogenic interactions with mesenchymal cells and their derivatives (Saxén 1987, Bard 2002, Lin *et al.* 2003, Costantini 2006). The extrarenal part of the ureter elongates to give rise to the permanent ureter that connects the kidney to the bladder (Yu *et al.* 2002).

The mesenchymal cells condense around the ureteric bud and form so-called "cap-cells" (Sariola 2002) which acquire the competence to form epithelial pretubular cell aggregates (Saxén 1987). The rest of the metanephric mesenchyme remains loose and contains the stromal progenitor cells. The tips of the ureter elongate, branch sequentially, and the condensed mesenchyme can be seen around each tip. Each time when preparing for branching, the ureter induces mesenchymal pretubular aggregates on both sides of the branching point initiating the development of presumptive nephrons. Thus, progress occurs towards an increasing amount of developing nephron precursors (Figure 2). The induction of pretubular cells in turn can be seen as the condensation of mesenchymal cells that undergo a mesenchyme-to-epithelium transformation to renal vesicles. The vesicles elongate, form tubular comma-shaped and S-shaped bodies, and fuse to the nascent branches of the ureteric tree. The stromal mesenchyme in turn is

important for the morphogenesis of the ureter and has a regulatory role in the process of tubule induction.

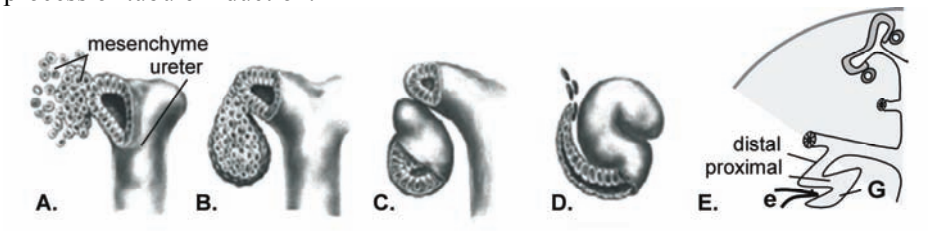

Fig. 2. Early stages of nephron development. A–B. Nephrogenic mesenchyme condenses to form a pretubular aggregate. The aggregated cells transform from mesenchyme into epithelium. These cells form a vesicle that proceeds via comma-shaped body (C) and S-shaped body (D) stages. (A–D: modified from Saxén, 1987). E. The S-shaped body initiates glomerular (G) development together with endothelium (e) and fuses to the branched ureteric tree (only part is shown) while the branching cortical tips still generate new renal vesicles (upper corner).

The endothelial cells migrate at an early stage to one of the clefts of the S-shaped bodies to initiate glomerulogenesis together with the proximal part of the elongated S-shaped tubular epithelium (Loughna *et al.* 1997, Loughna *et al.* 1998). The latter part contributes to the Bowman's capsule and to the cells that specialise to podocytes. In mature glomeruli the podocytes cover the glomerular microvessels and contribute to the filtration of the primary urine (Wartiovaara *et al.* 2004). Mesangial cells, which are specialized smooth muscle cells, are recruited and accompany the capillary tufts from the nearby stromal mesenchyme.

The renal pelvis in mouse begins to form around E14.5. The pelvic development is associated with a bundle-like arrangement of collecting ducts in the papilla that extends and drains to the pelvis (Yu *et al.* 2002). The maturation of nephrons in the mouse lasts for two weeks beyond birth, while the kidneys have reached functional competence by birth.

2.2.3 The migratory cells contributing to the developing kidney

The endothelial cells organise vascular development in the embryo via vasculogenetic and angiogenetic processes (Daniel & Abrahamson 2000, Oettgen 2001). The mesodermal endothelial cells arise as a precursor cell type, angioblasts, that in vasculogenesis differentiate *in situ* from the tissue to form vessels, whereas the angiogenesis refers to a process where pre-existing endothelial cells from an external source organise the vessels into a tissue. The capability of exogenous endothelial vessels to form in embryonic kidneys was

demonstrated by transplantation experiments in the early 80's (Sariola *et al.* 1983, Saxén & Sariola 1987). For example, the endothelial cells from quail chorioallantoic membrane were found to form vessels and glomeruli in the transplanted mouse metanephros, hence suggesting that kidney vasculature could originate via an angiogenetic process. More recent studies imply that a vasculogenetic process could occur in developing kidneys. Most notably, cells expressing markers of endothelial cell lineage have been found in early metanephric mesenchyme in support of a vasculogenetic process. It was also found that the endothelial cells do not target the glomeruli in cultured embryonic kidneys, perhaps due to unfavourable conditions *in vitro* that prevent differentiation of blastemal cells to a specific type of endothelial cells (for reviews see Robert & Abrahamson 2001, Woolf & Yuan 2001).

Exogenous signalling regulates the endothelial cells during the complicated processes of vascular development. Most important of these is the vascular endothelial growth factor (Vegf), a ligand for the Kdr receptor on the endothelial cell surface. Vegf is required for endothelial cell proliferation and survival, and loss of even one *Vegf* allele is deleterious for embryonic vascular development (Carmeliet *et al.* 1996, Ferrara *et al.* 1996). Vegf is produced in the early metanephric mesenchyme, indicating that the metanephros is permissive to vascular development by either one of the mechanisms, and it is also produced later by the induced mesenchyme. Also the podocytes around the glomerular capillary loops produce large amounts of Vegf. Other signalling molecules such as angiopoietins assist Vegf in endothelial cell control. Angiopoietin1 and -2, which target a common Tek receptor presented by endothelial cells, are paracrine factors in the kidney and later regulate (Kolatsi-Joannou *et al.* 2001) the formation and integrity of vessels. Other paracrine and juxtacrine signalling interactions may modulate the migration, proliferation, and the preference of cellular binding partners of endothelial cells during the complicated vascularisation (Risau & Flamme 1995, Risau 1997, Goodwin & D'Amore 2002, Jain 2003).

The neural crest cells are another type of migratory cells that have been implicated in the early stages of kidney development (Karavanov *et al.* 1995, Ito 2003, Cullen-McEwen *et al.* 2005). Interestingly, these cells have been observed to differentiate into neurons and make connections into the induced tubules. However, little is known of their actual role in the developing kidney. The origin of neural crest-derived tissues are best characterised in birds, owing much to the cell lineage mapping research in chick-quail chimeras and the usage of other methods such as vital dyes and antibodies (Le Douarin 1999, Le Douarin &

Dupin 2003, Le Douarin *et al.* 2004). Due to technical problems, such detailed information is not available for neural crest migration in mouse. In addition, the neural crest cells may differentiate to other cell types in target tissues, which on the other hand, present interesting possibilities when considering the cells populating the early kidney region.

2.2.4 The experimental research of mouse kidney organogenesis

The microdissection method and transfilter technique by Clifford Grobstein (Saxén 1999) enables separation of the metanephric mesenchyme and the ureteric epithelium from each other for tissue culture (Saxén 1987). The method made it possible to experimentally approach the tissue interactions in the developing metanephros (Saxén & Sariola 1987, Saxén & Sariola 1987). The pioneering work and the detailed description of the so far known tissue interactions in kidney and of its development in culture has been presented by Lauri Saxén (1987) in "The kidney organogenesis". The monograph has enabled scientists of the present day to address specific questions in a well characterised model system. The two fundamentally important morphogenetic processes in kidney development, the branching morphogenesis of the ureter and metanephric tubule induction, can be addressed by this method. It also enables, for example, analyses of the functional properties of developmentally important metanephric factors.

2.3 Molecular mechanisms in kidney organogenesis

The current research of inductive signalling in metanephros aims to understand the inductive nature of signalling molecules and how these molecules and their functions are regulated during development (Bard 2002, Costantini 2006). By using advanced cDNA microarray technique, the activity of a few thousand genes has been detected in the embryonic kidney (Bard 1999, Challen *et al.* 2005). This technique has been used, for example, to study gene expression profiles in kidneys of normal mice and transgenic animals, which carry controlled mutations in functionally important genes. This technique has also been applied in studies using isolated and cultured kidneys or kidney-derived cell lines (Valerius *et al.* 2002). In addition, studies using *in situ* hybridisation techniques have specifically assigned gene activities in tissues of developing kidney. Hence, we may say that a few thousand genes are known to have some role in developing kidneys.

Transgenic mice have been useful when considering the role of specific genes in the developing kidney. Some transgenic embryos die before a possible role of the gene in the kidney development could be assessed, including many genes that are central to vascular development (Daniel & Abrahamson 2000). In addition, the transgenic animals usually indicate functions in a process where the gene at first becomes critically important and the possible later roles remain elusive. Alternative strategies (Shu *et al.* 2005, van Amerongen & Berns 2006) are available for the targeted disruption of genes in the ureteric epithelium or metanephric mesenchyme (Yu *et al.* 2002, Chi *et al.* 2004, Zhao *et al.* 2004, Carroll *et al.* 2005, Grieshammer *et al.* 2005, Perantoni *et al.* 2005, Costantini & Shakya 2006).

2.3.1 Specification of the intermediate mesoderm and metanephros

The formation of the pronephros is required for the later metanephric development (Sainio *et al.* 1997, Kuure *et al.* 2000, Mitchell *et al.* 2007). Using transgenic animals a few genes have been identified important for the complex patterning of the early intermediate mesoderm, in particular in the region of the pronephros. These genes encode transcription factors Lhx1 (earlier known as Lim1), Osr1 and Pax2 (Shawlot & Behringer 1995, Bouchard *et al.* 2002, Wang *et al.* 2005). The neighbouring tissues may promote the patterning of the pronephric field (Obara-Ishihara *et al.* 1999, Mauch *et al.* 2000).

For the specification of the intermediate mesoderm, it is important that it becomes separated from the neighbouring paraxial presomitic mesoderm and lateral mesoderm. Specification may occur during or soon after the cells forming the embryonic mesoderm migrate through the primitive streak (James & Schultheiss 2005, James *et al.* 2006, Miura *et al.* 2006). A model to explain the patterning of the intermediate mesoderm in *Xenopus* was recently proposed by James and Schulheiss (2005). Dose-dependent Bmp signalling apparently has a major role in the mediolateral control of the mesodermal fatemap in vertebrates. It was proposed that a high level of Bmp signalling determines the lateral mesoderm, and intermediate mesoderm is specified by lower levels of Bmp-induced signalling. In the mesoderm closest to the neural plate, the absence of Bmp signalling would allow the expression of genes specifying the paraxial presomitic fate and these genes, in turn, would repress the genes that are important for the intermediate mesoderm development. Similar Bmp-dependent regulation of mesodermal specification may also function in mouse embryos

(James *et al.* 2006). The signalling molecules that activate genes important for the intermediate mesoderm in mouse remain elusive, however. Interestingly, in cell culture conditions it was recently shown *in vitro* that retinoic acid and activin possess a similar signalling capacity when applied to embryonic stem cells (Kim & Dressler 2005).

In respect to intermediate mesoderm patterning, Eya1 (a homologue of *Drosophila* eyes absent) is considered as a major factor specifying the metanephric blastema within the intermediate mesoderm (Xu *et al.* 1999, Sajithlal *et al.* 2005, Dressler 2006). In humans, the *EYA1* gene associates to an autosomal dominant disorder called Branchio-Oto-Renal (BOR) syndrome that displays also renal anomalies (Sajithlal *et al.* 2005). As for the genetic patterning of metanephric blastema, Eya1 has been proposed to act as a primary regulator of a complex network of transcription factors such as Pax2, Eya1, Six1, Six2, Sall1, Foxc1, Wt1 and Hox11 (Brodbeck & Englert 2004, Dressler 2006). A conserved genetic pathway in the metanephric mesenchyme that involves the Pax-Eya-Six genes is thought to lead to the full expression of the Glial cell line derived neurotrophic factor (Gdnf). Gdnf, in turn, is considered to be the major intercellular signalling molecule that induces ureteric bud formation and during subsequent ureteric development serves as the guiding molecule to the tips of the ureteric tree (Sariola & Saarma 1999).

Wilms tumor homolog (Wt1) is a zinc-finger transcription factor. It is activated at E9.0 in mouse and is expressed in the intermediate mesenchyme and later in the pronephric duct (Kreidberg *et al.* 1993, James *et al.* 2006). Studies suggest that factor Wt1 is required for the ability of nephrogenic mesenchyme to respond to tubulogenic signalling. In humans, mutations in the *WT1* gene are associated with uncontrolled growth of differentiated epithelia in several inherited conditions, and for this reason the gene has been of keen interest to researchers (reviewed recently by Hohenstein & Hastie 2006). While the transgenic mice have not generated similar tumourigenic growth in kidneys, such growth was reported in the mouse kidney organ culture (Davies *et al.* 2004). By applying the recently developed knock-down technique, based on so-called small interfering RNA molecules (siRNA) that are used to inhibit a gene of interest at the mRNA level, Davies *et al.* (2004) were able to determine that in the developing metanephric nephron, Wt1 is controlled by Pax2 and is essential for expression of the gene encoding Wnt4, a nephrogenic factor. Loss of Wt1 resulted in proliferation in developing nephrogenic epithelia.

2.3.2 Signalling molecules during kidney organogenesis

The growth and branching of the ureter is controlled by a complex exchange of morphogenetic information that associates with the patterning of induced nephrogenic mesenchyme (Vainio & Lin 2002, Costantini 2006, Costantini & Shakya 2006). The Gdnf, among other factors, promotes ureteric growth as demonstrated by Sainio *et al.* (1997) and Gdnf signalling is essential for ureteric bud formation. *Gdnf* is expressed in the early metanephric blastema and its receptors, Ret tyrosine kinase receptor (Schuchardt *et al.* 1994, de Graaff *et al.* 2001, Chi *et al.* 2004) and glycophophoinositol-linked coreceptor Gdnf receptor alpha 1 (Cacalano *et al.* 1998), are present in the nephric duct epithelium. The position of the ureteric bud is regulated by attractive Gdnf/Ret signalling in the ureteric epithelium and by factors restricting this signalling. Transcription factor FoxC1 (Kume *et al.* 1998, Kume *et al.* 2000) and Slit2 and its receptor Robo2 (Grieshammer *et al.* 2004) limit the anterior Gdnf expression in the metanephros, and the budding is also inhibited by mesenchymal Bmp4 production (Miyazaki *et al.* 2000). Instead, Bmp antagonist Gremlin1 is produced at the budding site and thus enables the bud to form (Michos *et al.* 2004, Costantini & Shakya 2006). The homeodomain transcription factor Emx2 has been found essential for the ureteric branching. In mice deficient for Emx2, the ureteric buds elongate to the metanephric blastema but do not branch, leading to the agenic kidney phenotype (Miyamoto *et al.* 1997).

Notch signalling is mediated by the binding of Notch receptor molecules to Jagged transmembrane molecules. This is followed by the enzymatic cleavage of Notch by γ-secretase and translocation of the cleaved receptor to the nucleus where it can activate gene expression (Itoh *et al.* 2004, Piscione *et al.* 2004). A Notch receptor Jagged1 is first present in the entire nephric duct, ureteric bud, and periductal mesenchyme and later in metanephros, in the tip area, colocalising with Gdnf1 and Ret/Gfra1 receptors (Kuure *et al.* 2005). When the expression of Jagged1 was genetically targeted to the entire ureteric epithelium, the Ret/Gfra1 receptors were also expressed in the entire ureteric epithelium, suggesting that Jagged1 is involved in the regulation of ureteric growth by controlling Gdnf signalling (McCright *et al.* 2001, Kuure *et al.* 2005). Jagged1 is also present in the induced metanephric mesenchyme and S-shaped bodies, and it is also expressed by endothelial cells in glomerular capillary tufts. Inhibition of Notch signalling *in vitro* results in the lack of the proximal epithelial part of the segmented nephron (Wang *et al.* 2003). A mutation affecting the signalling efficiency of the mouse

Notch2 receptor has lead to several forms of glomerular defects (McCright *et al.* 2001). The findings suggest that Notch signalling is an important juxtacrine signalling factor in several steps of glomerular differentiation.

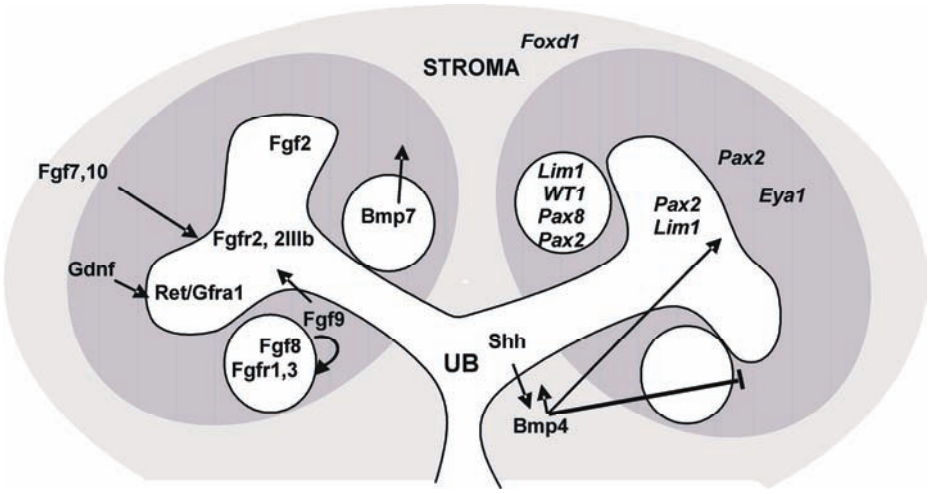

Fig. 3. A presentation of some secreted signalling molecules, receptors and transcription factors that are important in the developing kidney (Perantoni, 2003; Simic and Vukicevic, 2005; Bates, 2007; Cullen-McEwen *et al.* 2005). For abbreviations see pages 7 and 8. UB, ureter bud. Mesenchyme, dark colour. Light circles, induced mesenchyme.

Secreted fibroblast growth factors, Fgfs (Powers *et al.* 2000, Bates 2007), and bone morphogenetic proteins, Bmps (ten Dijke *et al.* 2003, Simic & Vukicevic 2005), are other families of signalling molecules that are important in the developing embryo and also involved in kidney development (Figure 3). The Fgfs stimulate the growth of the ureter and promote nephrogenesis while Bmp signalling has been found to promote branching and elongation of the branched ureteric epithelia (Vainio & Lin 2002, Costantini 2006). The Fgfs signal via homo- or heterodimeric tyrosine kinase receptors presented by target cells and formed by Fgfr1, -2 or -3, that may present multiple alternative splice isoforms, or Fgfr4 (Bates 2007). Fgfr1 and Fgfr2 (Zhao *et al.* 2004) are important in the mouse, acting together in the patterning of the kidney with Fgfs such as Fgf2, -7, -8 and -10, at least (Celli *et al.* 1998, Bates 2007). Depletion of either Fgf7 or Fgf10 activity in mouse has resulted in smaller kidneys and fewer ureteric buds (Qiao *et al.* 1999, Sekine *et al.* 1999, Ohuchi *et al.* 2000). *Fgf8* is expressed at E11.5 in the uninduced nephrogenic mesenchyme (Carroll *et al.* 2005, Perantoni

et al. 2005) and it may cooperate with Wnt signalling in nephron induction (Perantoni *et al.* 2005). *Fgf8* expression is thereafter specifically maintained in nephrons, where Fgf8 signalling is likely to be redundantly transmitted by Fgf receptors. Conditional inactivation of *Fgf8* resulted in the lack of mature nephrons, confirming the role of Fgfs in the control of nephrogenesis (Grieshammer *et al.* 2005, Perantoni *et al.* 2005).

Several extracellular matrix molecules, including the collagen, cadherin, integrin and proteglycan families, play important roles in the regulation of cell-to-cell interactions as well as presenting a substratum of important cell extracellular matrix signalling during kidney development (Horster *et al.* 1999, Gilbert 2006). Integrin signalling is also important for ureteric growth. Functional disruption of the mesenchymal Integrin-α8 leads to a delay in ureteric growth and loss of mesenchyme induction (Müller *et al.* 1997). The proteoglycans are important for the presentation of growth factors, especially Fgfs, and for branching morphogenesis in the kidney. A complete halt in the ureteric branching, like in Emx2-deficient mice, resulted when the gene encoding an enzyme important for heparan sulphate proteoglycans, heparan suphate 2 sulphotransferase, was genetically depleted (Bullock *et al.* 1998). Glypican3 is one of the proteoglycans in kidney and presented by the ureter (Grisaru *et al.* 2001). Syndecan is produced at sites of induced mesenchyme, suggesting a regulatory role in early nephrogenesis (Vainio *et al.* 1992). The targeting of ureteric Glypican3 resulted in the overgrowth of collecting ducts (Cano-Gauci *et al.* 1999).

The metanephric mesenchyme requires signalling for survival, since in tissue culture an isolated mesenchyme undergoes apoptosis that can be rescued by tubulogenesis activating signalling (Saxén 1987, Saxén & Lehtonen 1987). The Wnt signalling molecules play a central role in tubule induction while, Fgf8 (Perantoni *et al.* 2005) and Bmp7 (Dudley *et al.* 1999, Godin *et al.* 1999, Vainio & Lin 2002) may play a cooperative role in line with their proposed roles as survival factors. Disruption of Bmp7 function has resulted in none, or very small and disorganized kidneys (Dudley *et al.* 1995). From the Bmps, Bmp4 is also critical for renal development and the targeting of Bmp4 revealed a haploinsufficient phenotype of several organs. The kidneys were hypoplastic and after birth, they developed cysts. Homozygous embryos died before kidney organogenesis (Dunn *et al.* 1997).

The stroma is an important component in the regulation of kidney development as was first demonstrated by the knockout mice deficient for transcription factor Foxd1 (Hatini *et al.* 1996, Cullen-McEwen *et al.* 2005,

Levinson *et al.* 2005). Homozygous Foxd1 mutant mice are born with hypoplastic disorganised kidneys with few nephrons, while regulation of Bmp4 signalling was disturbed in the cortex with sychronous defect in cortical cellularity. From the stromal growth factors, Fgf7 has been implicated in the regulation of the amount of nephrons. The involvement of stroma in the control of ureteric growth and nephrogenesis was also demonstrated by targeting retinoic acid receptors (Rars) alpha and -beta (Mendelsohn *et al.* 1999) that are expressed in the stroma together with retinaldehyde dehydrogenase 2, which produces retinoic acid (Cullen-McEwen *et al.* 2005). The double-mutant mice had small kidneys, reduced nephron number, impaired collecting duct development and no nephrogenic zone at birth, while mice deficient for either one of the Rars alone were without a kidney phenotype (Mendelsohn *et al.* 1999). In the ureter, the expression of Gdnf receptor Ret was also missing in the double-mutant mice.

The integrity of the extracellular matrix is important for the development of kidney function, as mutations in human Nephrin, present in the glomerular filtration barrier (Wartiovaara *et al.* 2004), were found to associate with the congenital nephrosis most common in the Finnish population (Kestila *et al.* 1998). In support of this, targeting of the mouse homologue of Nephrin1 leads to proteinuria and early death after birth (Putaala *et al.* 2001). From the secreted growth factors, targeting platelet-derived growth factor beta signalling has resulted in loss of glomerular mesangial cells (Hellstrom *et al.* 2001).

2.4 The Wnt family of inductive signalling molecules

The Wnt family consists of secreted signalling molecules (McMahon 1992, Dale 1998, Mikels & Nusse 2006) that play roles in early embryonic development and organogenesis. For example, Wnt3 is expressed in the early mouse embryo and is required for primitive streak formation (Robb & Tam 2004). Wnt signalling is widely used in animal embryos in the regulation of cellular processes such as proliferation, cell movement, cell differentiation, cell polarity, and in the decision between alternative cell fates. Wnt signalling has also been intimately connected to certain types of congenital disorders and to cancer in adults, underpinning the significance of the control of Wnt signalling in a variety of tissues and developmental phases (Cadigan & Nusse 1997, Hoppler & Kavanagh 2007).

In mouse and in human, 19 genes encoding Wnts have been identified (Miller 2002). They may have unique signalling functions (van Amerongen & Berns 2006), but the Wnts may also cooperate (see for example: Mulroy *et al.* 2002,

Guo *et al.* 2004). It is also well known that the Wnts can functionally replace each other *in vitro* (Moon *et al.* 1993, Du *et al.* 1995, Shimizu *et al.* 1997, Kispert *et al.* 1998, Tajbakhsh *et al.* 1998).

The Wnts require assistance for secretion, and the glycosylation of Wnts can be important for the targeting of Wnt secretion (Hausmann *et al.* 2007). The Wnts have also been found to be lipid modified, which may restrict the Wnt signalling range (Willert *et al.* 2003, Schulte *et al.* 2005, Eaton 2006). Once the Wnts are secreted, they may associate to the cell surface or extracellular matrix molecules. The proteoglycans have been found important for Wnt signalling (Eaton 2006). The Wnts may also target the extracellular domains of transmembrane receptors in the recipient cells. Most importantly, the sevenpass receptors belonging to Frizzled family (Bhanot *et al.* 1996, Perrimon 1996, Wang *et al.* 1996, Kikuchi *et al.* 2007) or alternative receptors such as tyrosine kinase receptors Ryk or Ror2 (Halford *et al.* 2000, Nomi *et al.* 2001). Indeed, the molecular research has resulted in the identification of a range of important regulatory mechanisms and molecular interactions of Wnts with their targets (Blitzer & Nusse 2006). These, however, are currently elusive when it comes to the developing kidney.

In responding tissues, the Wnts can activate several intracellular pathways. For example, Wnt5a and Wnt11 are often implicated in the so called "noncanonical Wnt signalling" but recently it was shown that purified Wnt5a protein, while being able to antagonise the canonical Wnt signalling when bound to mouse Ror2 receptor tyrosine kinase, was able to trigger the canonical Wnt signalling in the presence of Fzd4 and Lrp5 receptors (Mikels & Nusse 2006). Thus, the decision of Wnt signalling pathway(s) in responding cells depends on the receptor context. Furthermore, ligands that are structurally unrelated to Wnts can interact with Wnt target receptors and activate Wnt signalling pathways (for a more comprehensive view see Kikuchi *et al.* 2007). From these, R-spondin1 and -3 were recently found also in mouse kidneys at stages E12.5, E15.5 and E17.5 (Nam *et al.* 2007).

2.4.1 The canonical Wnt signalling pathway

At present, it is perhaps reasonable to divide the signalling pathways – rather than the ligands (early findings reviewed in Kühl *et al.* 2000) – into canonical Wnt signal transduction and noncanonical Wnt signalling. The canonical Wnt signalling pathway is relatively well established, owing much to the pioneering discoveries especially in *Drosophila* (Siegfried *et al.* 1994, Bhanot *et al.* 1996)

and to molecular research of intracellular mediators of the pathway. The canonical Wnt signalling pathway was also first identified in the context of Wnt signalling and with a few exceptions, Wnts are considered the main regulators of this pathway and homologous factors mediate the signalling in various species (Strutt 2003, van Amerongen & Berns 2006, Kikuchi *et al.* 2007).

Signalling via the canonical Wnt pathway (Papkoff *et al.* 1996, Hoppler & Kavanagh 2007), results in the stabilization of the cytoplasmic β-catenin and in an increase of its level by a mechanism that prevents the β-catenin from becoming phosphorylated for degradation. The stabilised β-catenin can translocate into the cell nucleus (Molenaar *et al.* 1996, Behrens *et al.* 1998), where it may interact with members of the Tcf/Lef transcription factor family and promote the transcription of Tcf/Lef target genes - often referred to as Wnt target genes. Many of these genes have been identified and encode proteins such as transcription factors Myc (He *et al.* 1998) and Jun (Mann *et al.* 1999) and cyclin D1 (Shtutman *et al.* 1999, Tetsu & McCormick 1999) that are important regulators in proliferating cells, and thus important during development.

β-catenin is a ubiquitous cytoplasmic protein that associates with plasma membrane complexes and is continuously produced in cells (Hoppler & Kavanagh 2007). At the same time, β-catenin is down-regulated in the cells by phosphorylation, which marks β-catenin for degradation (Figure 4). A complex consisting of β-catenin, Axin (Yamamoto *et al.* 1999, Jho *et al.* 2002, Liu *et al.* 2006) and adenomatous polyposis coli (Apc) proteins is necessary for β-catenin phosphorylation by the activity of the glycogen synthase kinase-3 beta (Gsk-3b; Rubinfeld *et al.* 1996).

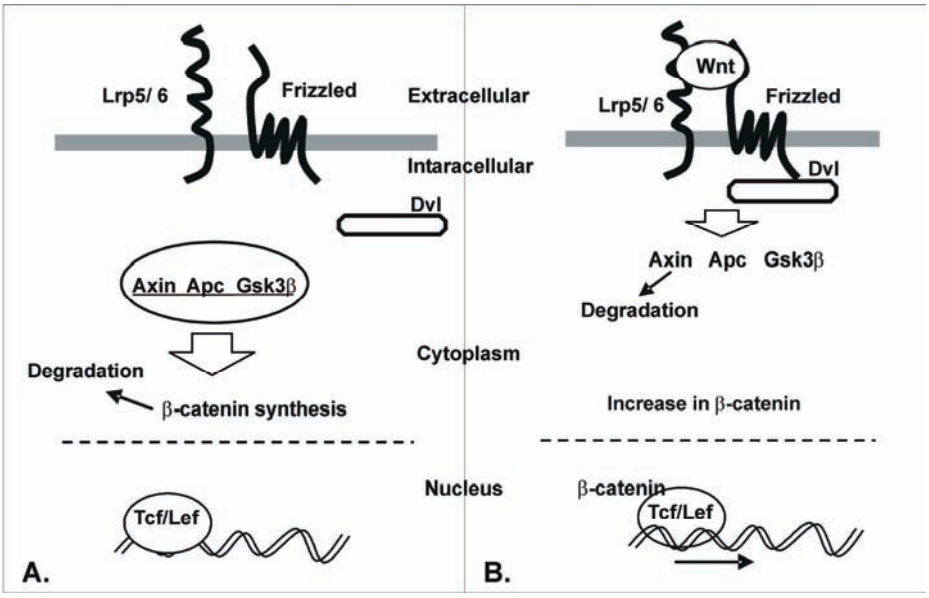

Fig. 4. The canonical Wnt signalling. A. In the absence of Wnt ligand, a phosphorylation complex labels β-catenin for degradation. B. Wnt ligand binding to Frizzled and Lrp5 or Lrp6 results in β-catenin stabilisation, nuclear localisation and activation of gene transcription via interaction of β-catenin and Tcf/Lef.

Binding of the Wnt ligands to the Fzd and Lrp-5/Lrp-6 receptor results in removal of axin from the phosphorylation complex and its degradation. The mechanism behind this process, however, is thus far unclear. Dishevelled (Dvl; Habas *et al.* 2001, Yu *et al.* 2007) proteins that interact with the cytoplasmic tail of the Fzd receptor (Habas *et al.* 2001) are critically required for the process that is also likely to involve the docking of the phosphorylation complex by binding of axin with the phosphorylated cytoplasmic tail of the Lrp coreceptor (Tamai *et al.* 2004).

2.4.2 The noncanonical Wnt signalling pathways

Noncanonical Wnt signalling does not involve the Lrp receptors and is independent of regulation of the intracellular β-catenin level (Miller *et al.* 1999, Kuhl *et al.* 2000, Strutt 2003, Seifert & Mlodzik 2007, Wang & Nathans 2007). In vertebrates, several intracellular pathways can be activated by the binding of Wnts to Fzd receptors (Figure 5).

The Wnt/Ca^{2+} signalling pathway has been found to induce an increase of cytoplasmic Ca^{2+} that triggers the interaction of calcium-sensitive enzymes such as Calcium/calmodulin-dependent Kinase II and protein kinase C with their targets (Miller *et al.* 1999, Kuhl *et al.* 2000). The role of Wnt signalling in intracellular Ca^{2+} regulation during mammalian embryogenesis or organogenesis is, however, an open question (Gwak *et al.* 2006 and references therein). The planar cell polarity (PCP) signalling mediated by Frizzled leads to activation of the Jun-N-terminal kinase (JNK) and small GTP binding Rho family kinases (Malliri & Collard 2003, Strutt 2003) that may also control cytoskeletal arrangements.

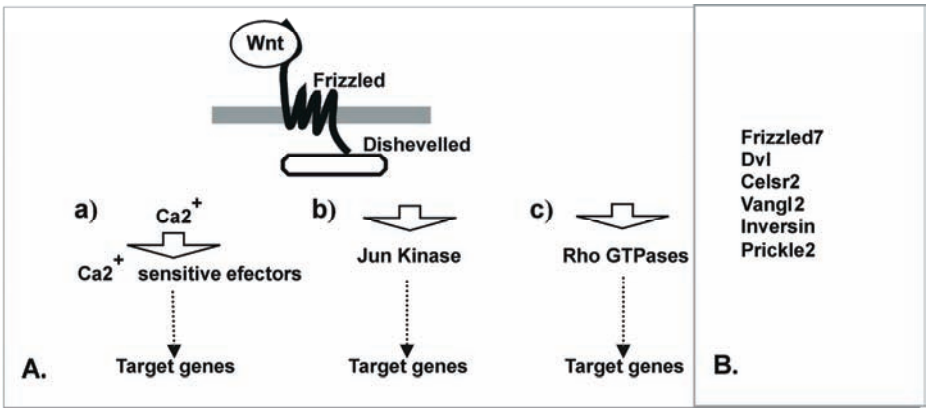

Fig. 5. The noncanonical Wnt signalling pathways in vertebrates. A. In the absence of Lrp coreceptor, Wnt ligand binding may result in a) an increase in cytoplasmic $Ca2^{+}$ ions or b) as an increased Jun kinase activity, or c) as the activity of the of RhoGTPases (Amerongen & Berns 2006;Wang & Nathans 2007; Seifert & Mlodzik 2007). B. A list of examples of mouse homologues of the Drosophila core PCP factors, that have been implied in mouse planar polarity regulation. Inversin can antagonise the canonical Wnt signalling by binding to Dvl, while Prickle-like2 (Prickle2) has been proposed to be involved in Planar polarity signalling in central nervous system (Tissir & Goffinet, 2006; Deans *et al.* 2007).

In *Drosophila*, PCP signalling operates in the control of the polarised behaviour of cells, which manifests as regular structural arrangements of hair and bristles and the eye (Strutt 2003). A group of molecules referred to as the core PCP factors (Figure 5) are involved in all of these processes including the frizzled and the Dvl that are also required for canonical Wnt signal transduction.

The regulation of planar cell polarity in vertebrates (Djiane *et al.* 2000) has been found to encompass homologues of the same group of proteins that belong to core PCP factors in the Drosophila (listed in recent reviews by Seifert &

Mlodzik 2007, Wang & Nathans 2007). In mouse deficient for the Fzd6 receptor, the patterning of hair in the fur is ameliorated, implying that Fzd6 alone is a key mediator of PCP signalling in the skin. Genetic evidence from targeted mice suggest that of the Frizzleds, Fzd3 and Fzd6 play a redundant role in the control of neural tube closure and in orienting the sensory cells in the inner ear. The embryonic tissue culture studies found that Wnt7a is likely to be involved in signals that orient the cochlear hair cell movements (Mayr *et al.* 1997) and demonstrated that JNK signalling is activated in hippocampal neurons by Wnt7b *in vitro* (Rosso *et al.* 2005). This indicates that PCP regulation in mouse can involve signalling of secreted Wnts as an important component (Strutt 2003).

The best known secreted antagonists of Wnt signalling are the secreted Frizzled receptor related proteins (sFrps) and Dickkopfs (Dkks). sFrps are homologous to the exracellular domain of Fzd receptors and can bind directly to Wnts (Leyns *et al.* 1997, Wang *et al.* 1997), thus competing with Fzd receptors for the Wnt ligand. The Dkks specifically antagonise the canonical Wnt signal transduction by sequestering the Lrp receptors from the cell surface (Glinka *et al.* 1998, Mukhopadhyay *et al.* 2001, Mao *et al.* 2002).

2.4.3 Wnt signalling in the metanephric kidney

The Wnt signalling molecules in the branching ureteric epithelium of the embryonic kidney include Wnt6, Wnt7b, Wnt9b and Wnt11, while previous studies have identified Wnt2b and Wnt4 as Wnt members responsible for Wnt signalling from mesenchymal cells (Figure 6; Vainio *et al.* 1999a, Perantoni 2003, Qian *et al.* 2003, Carroll *et al.* 2005). Also several Frizzled receptors are expressed in embryonic kidneys together with Fzd7 (Vainio *et al.* 1999a) that perhaps plays a redundant role with other Fzd receptors (van Amerongen & Berns 2006).

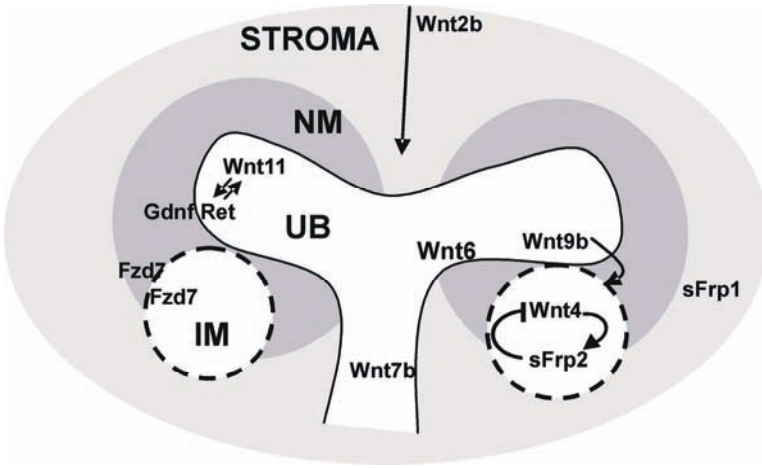

Fig. 6. Wnt signalling molecules and some Wnt interactions (after Majumdar *et al.* 2003; Carroll *et al.* 2005; Yoshino *et al.* 2001; Vainio *et al.* 1999) in the developing kidney. Some positive (arrows) and negative regulatory relationships are indicated. NM: nephrogenic mesenchyme; IM: induced mesenchyme. See text for details.

From the ureteric Wnts, the roles of Wnt6 (see I and **6.1**) and Wnt7b are currently elusive (Kispert *et al.* 1998, Carroll *et al.* 2005). Neither these two nor Wnt11, which shows a distictive expression in the ureteric tips, appear to be critical for kidney development (van Amerongen & Berns 2006). However, Wnt11 was earlier connected to the regulation of Gdnf/Ret signalling *in vitro* (Pepicelli *et al.* 1997) and more recently *in vivo* (Majumdar *et al.* 2003, Carroll *et al.* 2005). The lack of Wnt11 in mouse affected early ureteric branching morphogenesis and led to smaller but functionally normal kidneys. Wnt9b was recently found to be expressed in the nephric duct and in the ureter, and it was shown to be sufficient and required for tubulogenic induction of mesenchyme both in meso- and metanephros (Carroll *et al.* 2005). In embryos lacking functional Wnt9b, metanephric Pax8, Fgf8 and Wnt4 were lost, indicating that the mesenchyme was not induced to differentiate. Genes that associate to uninduced nephrogenic mesenchyme were found to be expressed. Wnt4 is required for the mesenchyme-to-epithelium conversion (Stark *et al.* 1994), while for example Fgf8 is also required for nephrogenesis (Grieshammer *et al.* 2005, Perantoni *et al.* 2005). In addition to the lack of the mesonephric derivatives, the Wnt9b-deficient mouse had remnant kidneys that completely lacked nephrons (Carroll *et al.* 2005). The induction and tubulogenesis could be rescued in the Wnt9b-deficient embryos by genetically targeting *Wnt1* expression to the nephric duct and its

derivatives. This demonstrated the importance of the ureteric Wnt signalling component in the induction of mesenchyme.

A mutation in the Lrp6 receptor results in reduced and cystic kidneys with elongated hydroureter (Pinson *et al.* 2000), implicating a role of canonical Wnt signalling in the kidney. Lin *et al.* (2001a) demonstrated that ureteric branching morphogenesis could be reconstituted by incubating the isolated E11.5 stage ureteric bud in LiCl solution or with Wnt2b expressing cells. The ureteric branching failed without such treatment or if cells expressing ureteric Wnt11 or Wnt6 were used as an inducer. This suggests that the ureteric growth is controlled via canonical Wnt signalling and by Wnt2b that is expressed in the periphery of the early metanephros. Other evidence suggests that the canonical Wnt signalling is involved in the induction of nephrogenic mesenchyme (reviewed in: Perantoni 2003, Kuure *et al.* 2007). The ureteric Wnt9b and mesenchymal Wnt4 are the most obvious candidates for being responsible for the canonical Wnt signalling in the induced mesenchyme; however it should be noted that the details of their signalling mechanisms in metanephros remain elusive (Carroll *et al.* 2005).

When it comes to the Wnts and noncanonical Wnt signalling in the developing kidney, it should be noted that in mouse it is still poorly understood. Noncanonical Wnt signalling, however, has been implied in the kidney (Perantoni 2003) where, for example, it could function in the maturation of the ductal epithelia at the time when the glomerular filtration begins (Simons *et al.* 2005, Fischer *et al.* 2006). Even though studies have important implications for human congenital conditions, such as nephronophthisis type II that leads to death before two years of age and polycystic kidney disease, affirmative evidence of noncanonical Wnt activity in the kidney epithelia was not shown (Wang & Nathans 2007).

Some of the secreted antagonists of Wnt signalling are also present in the metanephros, and of these sFrp2 is induced in the pretubular cells in response to Wnt4 signalling (Lescher *et al.* 1998), while another sFrp, sFrp1, is produced by the stromal component of the kidney. sFrp2 can bind Wnt4 and is thought to restrict Wnt4 signalling, but the role of these factors in the developing kidney as well as other possible modifiers in the regulation of Wnt signalling awaits further elucidation.

3 Outline of the research

The kidney organogenesis begins and proceeds with reciprocal inductive interactions of mesenchymal and epithelial tissues. During the developmental process, the mesenchyme forms the nephrons that are early integrated by endothelial cells, contributing to the vascular development of the kidney (Sariola *et al.* 1983, Robert & Abrahamson 2001). Another migratory cell population, the pluripotent neural crest cells, initially produced by the dorsal margin of the developing neural tube, may contribute cells to the developing kidney (Ito 2003, Cullen-McEwen *et al.* 2005, Anderson *et al.* 2006), not only the neurons innervating the kidney.

As a model of developing kidney, mouse embryos have a strong research background (Saxén 1987, Saxén & Lehtonen 1987, Saxén & Sariola 1987) and the molecular biology of kidney organogenesis is at present in the keen interest of developmental biologists. However, the complex and precisely arranged structure of the kidney is not yet fully characterised. Therefore, further investigation of molecular mechanisms regulating the developmental program of kidney organogenesis is needed.

Certain members of the Wnt family of regulatory factors are known to possess important functions in kidney development (Vainio *et al.* 1999b, Perantoni 2003, Carroll *et al.* 2005), but Wnt signalling in the developing mammalian kidney is not yet well established. Besides the research of molecular aspects of kidney organogenesis, also the origin of kidney cells requires more clarification. For instance the migratory neural crest cells may have important roles in the developing kidney.

Therefore, the main aims of this study were:

1. to investigate the expression of the epithelial *Wnt6* gene and its role in the kidney morphogenesis,
2. to investigate the function of mesenchymal Wnt4,
3. to investigate the migration of neural crest cells to the early developing kidney and characterise their role in kidney development.

4 Materials and methods

A more detailed description of the materials and methods can be found from the original articles I-III.

4.1 Mouse lines and cells (I-III)

The CD-1, *Wnt4* (Stark *et al.* 1994, Kispert *et al.* 1998), 206 (Echelard *et al.* 1994), *Splotch*^{2H} (Epstein *et al.* 1991), *Wnt-1-Cre* (Danielian *et al.* 1998, Chai *et al.* 2000), *R26R* (Soriano 1999) and *Tie1/LacZ* (Korhonen *et al.* 1995, Loughna *et al.* 1997) mouse lines and untransfected NIH3t3 fibroblast cells or NIH3t3 cell lines expressing Wnt-4, Wnt-6 or Wnt-11 were used for the experiments.

Embryos homozygous for a defective allele of the *Wnt4* gene were obtained by mating heterozygous parents. Splotch embryos homozygous for the defective *Splotch*^{2H} allele of the *Pax3* gene were obtained by mating heterozygous parents from the *Splotch*^{2H} mouse line. The embryos from such crossings were genotyped by PCR.

Cells expressing Wnt4 were generously provided by Dr. Andreas Kispert and the cell lines expressing Wnt6 or Wnt11 were generated using a retroviral procedure as described in I.

4.2 Isolation of organs and tissues from embryos (I-III)

The organs and tissues were dissected from embryos of different stages in ice-cold Dulbecco's PBS. The vaginal plug was considered as evidence of mating (E0.5 of gestation). For organ culture experiments, the metanephric kidney was isolated from embryos at E11.5 or later at E13.5 (II) depending on the purpose of the experiment. Pieces of the spinal cord to be used in organ culture were isolated from embryos at E11.5. For other experiments, the embryos at E10.5 – E18.5 were used for isolation of the kidneys with surrounding tissues depending on the process in kidney development that was of interest.

4.3 Organ culture methods and cultured cell lines

4.3.1 Normal organ culture (I-III)

The various organ cultures and the experiments with different inducers in combination with isolated metanephric mesenchyme were essentially done as described in Saxén (1987) and Saxén & Lehtonen (1987). Most often the metanephroi of embryos were isolated at E11.5 for culturing, but in some experiments (II) the use of the E13.5 metanephric kidneys was more relevant in regards to the question addressed. In the latter, the embryonic kidneys were isolated and split in two halves to expose the kidney interior to exogenous inducers.

The embryonic organs were grown on pieces of Nucleopore filter supported by stainless steel grids. The medium under the grids was changed every 2 days and consisted of DMEM with glutamax I supplemented with 10% (v/v) of fetal bovine serum, 100 units/ml penicillin, and 100 µl/ml streptomycin. The cultures were incubated in 5%CO_2/95% air at 37^{o}C for varying lengths of time depending on the purpose of the experiment. The same culture conditions were used in tissue recombination experiments where the isolated metanephric mesenchymes were combined with different inducers.

In some organ culture experiments, the effect of growth factors, such as the recombinant human Bmp-4 (II; Vainio *et al.* 1993) or neurotrophic factor-3 (III; Karavanov *et al.* 1995), was tested. The proteins were supplemented in the culture medium.

4.3.2 Embryonic tissue recombination (I-III)

For experimental induction of the metanephric mesenchymes, the ureter buds were separated form the mesenchyme after incubation of the metanephric kidneys in 3% pancreatin/trypsin (GibcoBRL) in Tyrode's solution. The mesenchymes were then combined with fragments of isolated ureters that were preincubated in 10mM LiCl as described in I and in Lin *et al.* (2001), or with different cell lines. In other experiments SPCs that were genetically identical (I,II) or different (II) were combined with the isolated mesenchymes.

Following the culture on pieces of Nucleopore filter, the embryonic rudiments were fixed to visually examine the result and to prepare them for further use in different applications such as histological examination (I), whole-

mount *in situ* hybridisation (I,II), LacZ staining (II,III), and immunostaining assays (II,III).

4.3.3 Construction and use of cells expressing Wnts in tissue recombination (I,II)

Stable cell lines expressing Wnts were generated by retroviral techniques (Miller & Rosman 1989, Pear *et al.* 1993) as described in I. Shortly, a retroviral expression vector pLNCX with cDNA encoding Wnt6 or Wnt11 was constructed and transfected into a packaging cell line, Bosc 23, using Fugene reagent (Boehringer-Mannheim). The viral supernatant of the packaging cells was harvested 2-3 days later and transferred to NIH3T3 cells that were subcultured for 3 weeks in the presence of 1mg /ml neomycin. The remaining cells survived the selection with the antibiotic due to the expression of a vector-supplied resistance gene and were successfully infected.

These cells (I) and the cell line expressing Wnt4 (II; Kispert *et al.* 1998) were then used to assay tubule induction in tissue recombinants as described in Kispert *et al.* (1998). In such experiments, trypsinized cells were seeded on pieces of Nucleopore filter ($5x10^5$ cells / 0.2ml of medium on each) one day before tissue recombination. On the next day, the filters were transferred onto stainless steel grids and overlayed with freshly isolated mesenchymes.

Also the Wnt4 expressing cells could also be used (II) by following the same procedure or as in some experiments, these cells were removed with an injection needle from a subconfluent culture instead of resuspension after the enzyme treatment. The cells were then placed in contact with one edge of an isolated metanephric mesenchyme as a cluster, or transferred as a draft underneath an E13.5 kidney split in half.

The untransfected NIH3T3 cells served as controls and were treated according to the procedure applied in various experiments with the Wnt-expressing cells (Figure 7).

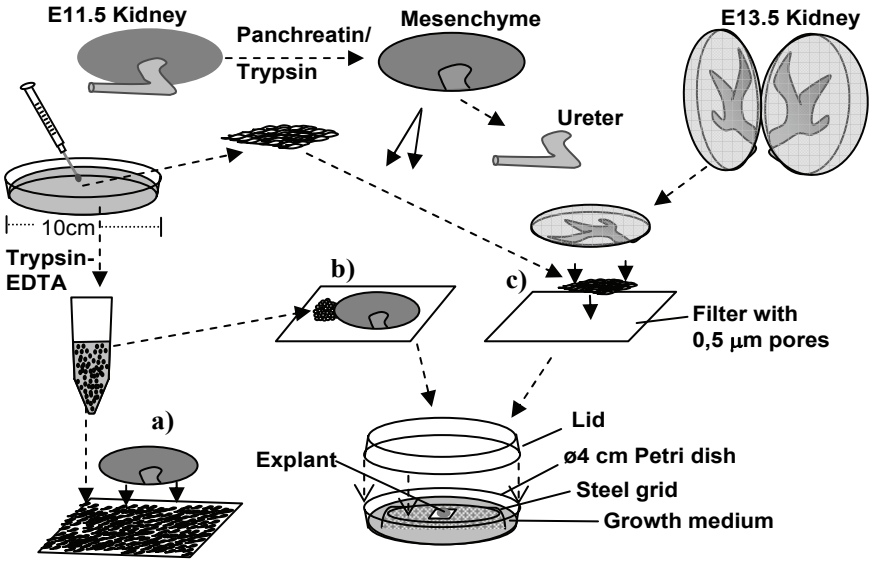

Fig. 7. Different experimental arrangements used in the present kidney induction studies. (Upper left) The ureter was separated from E11.5 kidneys after a short period of enzyme treatment and the mesenchyme was used for induction experiments with cells (Saxén, 1987). The cells were seeded on nucleopore filters one day before combining with isolated mesenchymes (a) or used as a cluster for a local inductive effect (b). (Upper right) The E13.5 kidney was split in two halves in order to expose the inner medulla for inductive cells that were dissected and removed from a cell culture dish as a draft and (c) combined.

4.4 Preparation of embryonic tissues for histology (I,III) and expression analysis (I-III)

The embryonic tissues for histology were fixed with Bouin's solution (I) or in 4% paraformaldehyde (PFA) in PBS (II, III). The latter was the fixative of choice for tissues when targeting the mRNA products of genes using *in situ* hybridisation techniques. The tissues were fixed overnight at 4^{o}C and then dehydrated. The embryonic tissues for analysis using the whole-mount *in situ* hybridisation were dehydrated in a methanol series and stored at -20^{o}C in 100% methanol until needed. For histological analysis and for the *in situ* hybridisation or immunoassaying on sections, the tissues were dehydrated in an ethanol series and stored in 75% ethanol at -20^{o}C until embedded in paraffin and sectioned (5 μm in thickness).

Haematoxylin and eosin were used for staining histological sectons.

4.5 Probes and *in situ* hybridisation methods (I-III)

The cDNA for the analysis of *sFRP2* expression was obtained as an expressed sequence tag cDNA (Gbacc w08345, Genome Systems, USA), verified by sequencing and then used as a template to prepare a probe. For the expression analysis of other genes, cDNAs were obtained as gifts from several laboratories. The other analysed genes were: *Pax2* (Dressler *et al.* 1990), *Pax8* (Plachov *et al.* 1990), *Wnt4* and *Wnt6* (Gavin *et al.* 1990), *Wnt7b* (Kispert *et al.* 1996), *Wnt11* (Kispert *et al.* 1998), *E-cadherin* and *c-ret* (Stark *et al.* 1994, Kispert *et al.* 1996), *Tie2* (Loughna *et al.* 1998), *Notch2* (McCright *et al.* 2001), *Shh* (Chiang *et al.* 1996, Yu *et al.* 2002), *Patched1* (Yu *et al.* 2002), *Bmp4* (Miyazaki *et al.* 2000), *Rarß* (Benson *et al.* 1995), *Foxd1* (Dolle *et al.* 1990) and *Hoxa10* (Hatini *et al.* 1996).

The analysis of gene expression using whole-mount *in situ* hybridisation was performed according to Kispert *et al.* (1996) and Zhang *et al.* (2001). The probes were labelled with digoxygenin UTP and were used for *in situ* hybridisation (Vainio *et al.* 1999a). The same probes were also used for nonradioactive expression analysis on sections (Zhang *et al.* 2001) and in II. Preparation of ^{35}S-UTP labelled probes and radioactive *in situ* hybridisation on sections were performed according to Wilkinson & Nieto (1993).

4.6 PCR and RT-PCR methods (II,III)

Amplification of DNA fragments by PCR was used to screen for the wild-type and defective alleles of the *Wnt-4* gene (II; Stark *et al.* 1994). PCR was also used to screen for wild-type and defective *Pax-3* genes in *Splotch^{2H}* mice and for screening the transmission of the *LacZ* transgene by the 206 parents (III).

PCR was also used for screening the presence of transcripts of genes (III) known to be expressed by neural crest-derived cells (Anderson *et al.* 2006), and then the DNA template for the PCR reaction was obtained by converting isolated total mRNA to DNA by reverse transcription.

In brief, total RNA was isolated from pieces of embryonic tissue using the RNeasy plus mini kit (Qiagen) and treated with DNAse I enzyme (Promega) to ensure the absence of contaminating genomic DNA. After treatment to inactivate the DNAse I enzyme, the RNA was reverse transcribed with sequence specific primers using the RevertAid First Strand Synthesis Kit (Fermentas). The

synthesised DNA could then be used as a template for PCR amplification by a thermo stable enzyme.

4.7 Antibodies and immunoassaying methods (II,III)

The antibodies for immunodetection were from commercial sources. The antibody targeting Pecam-1 (Platelet/endothelial cell adhesion molecule 1; CD31) was from BD Biosciences (USA), and the one targeting α-SMA (alpha Smooth muscle actin) from Sigma Aldrich (USA). The Troma-I antibody directed against cytokeratin Endo A (Keratin 8) was from the Developmental Studies Hybridoma Bank (USA), and the antibody against the Pax2 transcription factor was from Nordic BioSite (Sweden). The secondary antibodies used were: Horseradish Peroxidase (HRP)-conjugated rabbit anti-mouse from DAKO (Denmark) and fluorescent antibody conjugates, Alexa fluor 647 goat anti-rat IgG and Alexa fluor 488 goat anti-mouse IgG were obtained form Invitrogen (USA). The liquid DAB Substrate Kit (Zymed), containing 3,3'-Diaminobenzidine tetrahydrocloride as the substrate for HRP, was used for colour development.

For whole-mount immunostaining, embryonic kidneys and cultured tissue explants were fixed overnight at 4ºC in a solution with DMSO and fetal bovine serum (1:4 v/v) and then processed for staining with substrates (Lin *et al.* 2001b, Chi *et al.* 2004). The immunohistochemistry was performed using paraffin sections that were dewaxed and rehydrated for the staining purpose.

4.8 Analysis of apoptosis and cell proliferation (II)

Proliferating cells were detected using a cell proliferation kit (Amersham) based on the incorporation of Bromo-deoxy-Uridine (BrdU) into DNA in cells that duplicate their genomes for cell division, and probing such DNA. A solution containing BrdU was intraperitoneally injected into pregnant mice at a dose of 50mg/kg body weight. The assay was performed following the manufacturer's instructions and it included treatment with diaminobenzidine (DAB) substrate for the colour reaction.

The apoptotic cells in the kidneys were analysed using an *in situ* cell death detection kit (Boehringer-Mannheim) that contained reagents for the terminal deoxinucleotidyl transferase-enzyme mediated labelling of fragmented DNA of apoptotic cells. Colour development was based on DAB treatment.

The results were photographed and analysed as described in II.

4.9 Detection of β-galactosidase in embryonic tissues (II,III)

The β-galactosidase enzyme, produced by the endothelial cells in tissues of *Tie1/LacZ* embryos (II) or by cells of neural crest origin (III) in embryos from 206 mice as well as in those obtained from *Wnt-1-Cre* and *R26R* intercrosses, were visulized by X-gal staining according to Kispert *et al.* (1996). For details see III. After staining, the embryos, urogenital regions of embryos or explants from organ and tissue culture experiments were examined under a stereomicroscope. For a more detailed histological analysis, the embryonic tissues were paraffin embedded for sectioning.

4.10 Photography and image analysis (I-III)

Processed samples were photographed with Leica DC100 digital camera attached to an Olympus SZH10 research stereo microscope. Fluorescence was detected with Olympus DP50 camera attached to Leica MZFLIII stereomicroscope. The digital images were composed and analysed using computer programs such as Adobe Photoshop 5.0 and Scion Image software.

4.11 Axis induction in *Xenopus* (I)

Murine Wnt-6 cDNA was cloned in the pT7TS-2 plasmid vector (Zorn & Krieg 1997) for ectopic expression in *Xenopus* embryos by injection into the ventral blastomeres. The embryos were later scored for the presence of a second axis.

5 Results

5.1 The source of tubule inducing signalling resides in the ureteric tips (I)

At first, we compared the ability of the dissected uretric tip region and the ability of the rest of the ureter, the stalk region, to induce the tubulogenic program in isolated metanephric mesenchymes from early embryonic kidneys and found that this property was restricted to the ureteric tip region. Nine from a total of 14 dissected ureteric tips were successful in the tubule induction and maintaining the combined mesenchyme in the organ culture. All five cultures where the stalk region was combined with the mesenchyme resulted in the failure in the induction and in a loss of the mesenchymal tissue during the culture period of 72h. The result suggested that the likely source of tubule-inducing signalling resides in, and is restricted to, the tip area of the kidney ureter.

5.2 Expression of *Wnt6* in the normal embryonic kidney (I)

In the study addressing the possible function of Wnt6 in the kidney, we first found that *Wnt6* was expressed in the whole epithelium of the ureteric bud, invading the metanephric blastema at E10.5 in the mouse. It was also seen in the Wolffian duct from which the ureteric bud had diverted. During the subsequent serial branching and elongation of the ureter, the expression of *Wnt6* was found to be maintained in the newly formed branches excluding their extreme tips. Downregulation of *Wnt6* expression could also be seen in the stalk region of the ureter and in parts of the ureteric tree that had already branched at least once. Thus, *Wnt6* expression associated to the vicinity of the extreme tips of the growing ureter; however, the expression was not seen beyond the stage of E14.5 kidneys.

5.3 Expression of *Wnt6* in the *Wnt4* mutant kidney (I)

Similar analysis of kidneys of embryos homozygous for a targeted mutation in the *Wnt4* showed that the defect in the mesenchymal *Wnt4* affected the regulation of ureteric *Wnt6*. *Wnt6* expression was upregulated and was not restricted to newly formed branches of the ureter as was found in normal, wild-type kidneys.

5.4 Wnt6 signalling can induce an ectopic axis in *Xenopus* and tubule formation in mouse metanephric mesenchyme (I)

For functional studies we prepared a NIH3t3 fibroblast cell line expressing Wnt6 and another cell line for the expression of Wnt11 that is also expressed in the mouse ureter (Kispert *et al.* 1996). The cells were subsequently used in organ culture to test the ability of Wnt producing cell lines and the parental NIH3t3 cells, each in turn, to induce the mesenchymal tubulogenic program *in vitro*. The isolated mesenchyme was maintained and became induced for tubule development only by the cells expressing Wnt6. It occured in a fashion similar to such cultures in which the mesenchyme was combined with a piece of embryonic spinal cord. The mesenchymes cultured in contact with Wnt11-producing cells or with the parental NIH3t3 cells all failed to become induced and degenerated during the culture period.

The induction of the early tubulogenic program in isolated metanephric mesenchymes combined in organ culture with the cells expressing Wnt6 could be confirmed by the histological analysis of the induced tubules and by the results from the expression analysis. The latter included experiments where the metanephric mesenchymes were at first cultured together with Wnt6-expressing cells and then subjected to analysis for mRNA products of genes that were known to become active in the induced tubules of the metanephric kidneys of mice. The expression of genes including *Pax2*, *Pax8*, *sFRP2*, *E-cadherin*, and *Wnt4* could be demonstrated. The expression was found to be restricted to induced mesenchyme and was absent from the cells of the mesenchyme surrounding the clusters of induced mesenchyme. The expressional activity also increased in parallel with the time that the mesenchymes were cultured and in a manner suggesting progress in the differentiation of the induced mesenchyme.

We also demonstrated that in *Xenopus* embryos, mouse Wnt6 belongs to the group of Wnts that are capable of inducing the secondary axis as such a structure was observed in embryos that were experimentally manipulated for ectopic Wnt6 expression. The result demonstrated that while Wnt6 signalling and Wnt4 signalling have a similar effect in regards to tubule induction (Kispert *et al.* 1998) in mouse metanephric kidney, in other inductive systems they can act differently.

5.5 The loss of Wnt4 function is associated with vascular defect (II)

The Wnt4 defective kidneys offered a unique experimental opportunity to address *in vivo* the role of nephrogenesis in different developmental processes in the kidney. In the kidneys of *Wnt4*$^{-/-}$ mutant embryos, tubulogenesis, and thereby nephrogenesis, is lost and the size of the kidneys at birth is rudimentary compared to normally developing kidneys (Stark *et al.* 1994). We therefore investigated the development of mutant kidneys by comparing the apoptosis and cell proliferation of *Wnt4*$^{-/-}$ mutant kidneys to those that we could observe in the wild-type kidneys. The studies resulted in the conclusion that there was no contrasting difference in the amount of apoptotic cells, nor in the amount of proliferating cells observed in *Wnt4*$^{-/-}$ mutant or wild-type kidneys.

The examination of vascular development by visualisation of blood vessel forming endothelial cells revealed that the endothelial cells were able to migrate in the early presumptive kidney mesenchyme (at E11.5) and thereafter populated the developing *Wnt4*$^{-/-}$ mutant kidneys. The manner, however, was quite reminiscent of that observed in the case of wild-type kidneys. For example, the endothelial cells coalesced and formed tubular structures at sites were the major vessels formed in normal kidneys. The network of endothelial cells was, however, less complex already at E13.5 in comparison to that seen in the wild-type kidneys, and as the development proceeded, it became more evident that the vascular development of *Wnt4*$^{-/-}$ mutant kidneys was severely disturbed. While the perinatal kidney of normal embryos at E18.5 contained well defined tubules of nascent blood vessels, such tubules were absent from *Wnt4*$^{-/-}$ mutant kidneys at the same stage. Although the endothelial cells were found in *Wnt4*$^{-/-}$ mutant kidneys, they formed an arbitrary pattern of clustered cells instead of vessels with a clear tubular appearance. This finding suggested a defect in vascular development in *Wnt4*$^{-/-}$ mutant kidneys.

5.6 The loss of Wnt4 function leads to the loss of stromal smooth muscle cell differentiation (II)

To address the presence and distribution of differentiating smooth muscle cells (Yu *et al.* 2002), we immunoassayed and compared the presence of alpha smooth muscle actin in the kidneys of *Wnt4*$^{-/-}$ mutant embryos with those of wild-type embryos. The production of α-SMA appeared first in cells in the vicinity of major vessels in wild-type kidneys at E14.5, but was not found in the *Wnt4*-deficient

kidneys. This difference between kidneys of wild-type and *Wnt4*$^{-/-}$ mutant embryos was more pronounced at later stages as the amount of perivascular α-SMA increased in the wild-type kidneys and was then found also in the periureteric stromal cells of the kidney. However, differentiating α-SMA positive cells were absent from the *Wnt4*-deficient kidney proper still at E16.5. α-SMA-expressing cells appeared thereafter in the region closer to the developing cortex at E18.5, but the inner medulla was devoid of staining. This loss of α-SMA positive cells in the periureteric stroma coincided and colocalised with the respective site of *Wnt4* gene activity in the periureteric stroma of normal kidneys, suggesting a link between Wnt4 signalling and the differentiation of periureteric stromal cells.

5.7 Loss of Wnt4 function in the kidney leads to the loss of Bmp4 activity (II)

It was known that Bmp4 can act as a differentiation factor in wild-type kidney, promoting α-SMA-expression (Raatikainen-Ahokas *et al.* 2000, Yu *et al.* 2002, Miyazaki *et al.* 2003). *Bmp4* expression, in turn, is known to be promoted by the secreted Shh that also enhances the expression of *Patched1* (Yu *et al.* 2002). Therefore, the activity of the *Shh*, *Patched1* and *Bmp4* genes were next addressed in *Wnt4*-deficient kidneys by expression analysis.

Bmp4 expression was lost from *Wnt4*-deficient kidneys at stage E14.5, but the expression of *Shh* and *Patched1* were retained, the former being produced by the ureteric epithelium and the latter in the adjoining mesenchyme. This suggests that the epithelial-mesenchymal interaction was to some degree similar to normal in the medulla of *Wnt4*-deficient kidneys. However, the likely activator of α-SMA, Bmp4, was not present at stage E18.5, when the amount of smooth muscle cells is normally increasing in the stroma. Colocalizing abnormal activities of *Bmp4, Shh* and *Patched1* were later, at E18.5, present in the peripheral capsular region of the *Wnt4* deficient kidneys, while in the medulla the activity was lost. On the contrary, in normal embryonic kidneys the expression of *Bmp4, Shh* and *Patched1* focused on medullar tissues at the same stage.

5.8 Ectopic Wnt4 signalling can induce *Bmp4* expression in embryonic *Wnt4*-deficient kidneys *in vitro* (II)

The loss of α-SMA positive cells coincided and colocalised with the loss of *Bmp4* expression in the embryonic medulla of *Wnt4*-deficient kidneys, while both were present in wild-type kidneys. *Wnt4* expression had been reported (Stark *et al.* 1994) in the same tissue at the same stage of the developing kidney. Thus, the loss of α-SMA positive cells and the loss of *Bmp4* expression coincided and colocalised in embryonic kidney tissue defective for functional Wnt4 signalling. We therefore tested *in vitro* if Wnt4 signalling is connected to the regulation of *Bmp4* activity. This was done in the experiments where kidneys from *Wnt4*-deficient embryos were placed in contact with cells expressing Wnt4 underneath the explant or with control cells. After an overnight period in culture, the expression of *Bmp4* was probed in kidney explants by using the whole mount *in situ* hybridisation method. *Bmp4* expression was found to be absent when the *Wnt4*-deficient kidneys were cultured alone or cultured in contact with control cells. When *Wnt4*-deficient kidney explants had been in contact with Wnt4-expressing cells, *Bmp4* expression was present in the stromal mesenchymal cells. The finding suggests that inductive Wnt4 signalling can regulate *Bmp4* expression in the stromal mesenchyme.

5.9 Wnt4 signalling can induce differentiation of the smooth muscle cells in *Wnt4*-deficient embryonic kidneys *in vitro* (II)

E13.5 kidneys were prepared and placed in culture in order to address the appearance of smooth muscle cells, after a period of time in culture, by immunoassaying the α-SMA. The main finding was that when an E13.5 *Wnt4*-deficient kidney explant was cultured in contact with Wnt4-expressing cells placed on one side, staining concentrated to cells at the contact site. In contrast, such staining was absent when control cells were placed in contact with a *Wnt4*-deficient kidney rudiment.

In both experimental settings the rest of the kidney explants were weakly stained as were the explants cultured alone and stained after only a short period in culture (two hours). When E13.5 *Wnt4*-deficient kidney explants were cultured alone in the presence of recombinant human Bmp4 protein, the staining revealed positive cells in all areas of the explant. This result demonstrated that kidney cells deficient for *Wnt4* function are competent for α-SMA production in the presence

of activating signals. The amount of α-SMA positive cells increased in normal kidneys without a need for stimulating factors.

5.10 Wnt4 signalling can induce sprouting of the metanephric endothelial cells *in vitro* (II)

The effect of Wnt4 signalling (Wright *et al.* 1999, Cheng *et al.* 2003) on the metanephric endothelial cells was also addressed by analysing the distribution of endothelial cells in normal isolated E13.5 kidney explants that were cultured in contact with Wnt4-expressing cells or without such contact. The endothelial cells in E13.5 kidney explants cultured alone were distributed all over the tissue and formed a pattern of semi-regular network arranged around the developing tubules. The pattern was consistent irrespective of the technique that was used for visualising the endothelial cells (the β-galactosidase assay of endothelial cells in kidneys when the embryos were positive for *Tie1/LacZ* reporter gene and the immunoassay of kidney explants using an antibody targeting Pecam-1). Other embryonic kidney explants were placed in contact with Wnt4 expressing cells underneath the explant. Visualisation of endothelial cells in such kidney explants revealed that many endothelial cells had migrated outside the explant at the contact site and formed a network of cells on top of Wnt4-expressing cells. When two kidneys were placed in contact with a graft of Wnt4-expressing cells, the endothelial cells formed a contiguous network between the explants. The endothelial cells did not migrate as extensively if a control cell line was used, and the sprouting in some cases (4/9) did not result in a contiguous network. Thus, the Wnt4-expressing cells were more efficient inducers of metanephric endothelial sprouting *in vitro*. The result suggests that Wnt signalling can be involved in the regulation of endothelial cells of the developing kidney.

5.11 Neural crest cells transiently surround the metanephros in *Wnt1* reporter mouse embryos (III)

Previous studies had shown that neural crest cells can be marked with β-galactosidase expression either permanently by using crosses of *Wnt1-Cre* (Danielian *et al.* 1998, Chai *et al.* 2000) and *R26R* mouse lines (Soriano 1999) or transiently by using the 206 mouse line (Echelard *et al.* 1994). Both approaches take advantage of the use of the gene regulatory elements of *Wnt1* to activate β-galactosidase enzyme production that is expressed in the embryonic neural tissue

that produces the neural crest cells. In our study these mouse lines were used to investigate the migratory neural crest cells, and a Splotch mouse line in which a migration defect in neural crest cells was documented by others.

The distribution of neural crest cells was first studied in tissues and tissue sections of the embryos where the neural crests cells and their derivatives were permanently marked by β-galactosidase enzyme expression and could be visualised by X-gal staining. The study revealed that neural crest cells had migrated to close proximity of the metanephros at E10.5 and partially mantled the metanephric kidneys at E11.5, but such cells could not be found after E12.5. Investigations at stages E14.5 and E16.5 revealed cellular extensions towards the kidneys from neural crest cells that localized medially to the kidneys; however, staining indicating migration of undifferentiated neural crest cells into the kidney stroma was not found. The stromal cells and the epithelia of the embryonic kidneys remained unstained. Based on this investigation it seemed unlikely that neural crest would contribute to non-neural cell lineages in the kidney.

5.12 Neural crest cells are not essential for the early kidney organogenesis (III)

The neural crest cells in the 206 mouse line do not express β-galactosidase outside the neural crest region and the visualisation of these migratory cells was based on the β-galactosidase protein that the cells carried within them. Such cells outside the neural crest could be visualised by X-gal staining at stages E10.5, E11.5 (Karavanov *et al.* 1995) and E12.5 in the urogenital region of the 206 embryos. However, similar staining did not occur, indicating the absence of neural crest cells, in the urogenital region of the 206 embryos that were also carrying a homozygous mutation resulting in the Splotch phenotype (Epstein *et al.* 1991). Expression analysis based on RT-PCR resulted in the finding that the *Phox2b* and *Sox10* genes, early markers of neural crest cells, were not expressed in the urogenital tissues that were isolated at stages E11.5 and E12.5 from Sploch embryos. Expression of these genes was detected, however, in the urogenital tissues of the phenotypically normal littermates.

The analysis of Splotch embryos suggested that the migration of neural crest cells to the early metanephros did not occur in Splotch mutants and the mouse line would be suitable for addressing the possible role of neural crest cells that mantle the kidney at E11.5. The histological analysis of kidneys from Splotch embryos at stage E14.5 showed a similar arrangement of epithelial and

mesenchymal tissue components as could be observed in the normal kidneys. No detectable change was observed in the distribution of stromal cells in the kidneys of Sploch embryos at stage E14.5 based on the expression analysis of stromal marker genes. Expression analysis of *RARß*, *Foxd1* and *Hoxa10* was performed with the *in situ* hybridisation method. The results suggested that the neural crest cell defect in Splotch embryos had no observable *in vivo* effect in the developing kidneys.

The possible influence on kidney development resulting from the lack of neural crest cells was addressed *in vitro* using embryonic organ and tissue cultures. No change was observed in the growth of Sploch kidneys. The metanephric mesenchyme isolated from Splotch embryos was also competent for tubulogenesis based on cultures where the embryonic SPC was used as an inducer.

5.13 Neural crest cells can migrate to isolated kidney mesenchyme *in vitro* (III)

The migratory behaviour of neural crest cells to the kidney was addressed in tissue culture experiments, where isolated embryonic SPC tissue in combination either with metanephric kidney or metanephric mesenchyme was cultured for 72 hours or longer. In these experiments X-gal staining was used to visualise the β-galactosidase-positive neural crest cells in tissues of embryos collected from 206 mothers, while the wild-type embryos from CD-1 mothers were negative for such staining. The analysis revealed that β-galactosidase-positive neural crest cells could not be demonstrated in metanephric kidneys after a 72 hour period in culture regardless of the origin of embryos. However, the embryonic SPC tissue and surrounding mesenchyme were positive for neural crest cells still after a 96 hour period in culture, when the donors of the isolated explant were from 206 mothers.

When wild-type metanephric mesenchymes were cultured in contact with SPC from 206 embryos for 96 hours, an extensive amount of β-galactosidase-positive cells could be observed intercalated with the induced mesenchyme. The finding was in contrast to cultures where metanephric kidneys were cultured in contact with SPC from 206 embryos, as only the cells outside the metanephric kidney were stained after 96 hours in culture. The experiment suggested that neural crest cells could not migrate to isolated metanephric kidneys *in vitro*.

In sum, the neural crest cells could migrate extensively to isolated metanephric mesenchymes but not *in vitro* into isolated metanephric kidneys.

6 Discussion

6.1 Wnt6 signalling plays a redundant role in the induction of metanephric tubules

The induction of mesenchymal cells to tubulogenesis in the metanephros involves two consecutive events: the conversion of cells in metanephric mesenchyme competent to the inducing signal and the induction triggering the tubulogenesis. In the latter process the activation of Wnt4 has been found important. The early experiments by Grobstein and Saxén led to the conclusion that secreted signalling molecules from the ureter are required for the induction, and later *in vitro* experiments pointed to the role for Wnt family of signalling molecules (I; Kuure *et al.* 2007). Several other molecules were also studied as candidate mediators in the inductive signalling, for example the Leukocyte inhibitory factor and those belonging to the interleukin family (Horster *et al.* 1999, Sariola 2002).

We found that Wnt6 was expressed in the early ureteric bud that was known to present inducing activity and thereafter in the branching ureteric tree, localising in a manner that suggested the involvement of Wnt6 in tubule induction. Also, the cells expressing Wnt6 could induce tubulogenesis and Wnt4 expression in the isolated metanephric mesenchyme *in vitro*. In addition, we found that the mouse Wnt6 was able to induce an ectopic axis in *Xenopus* like Wnt1 and Wnt7a, which were shown by Kispert *et al.* (1998) to induce tubulogenesis in isolated metanephric mesenchymes from mouse embryos. The Wnt6 expressing cell line has been successfully used to address the regulatory function of Wnt6 signalling in tooth development (Sarkar & Sharpe 1999, Kettunen *et al.* 2000, Laurikkala *et al.* 2001) and somitogenesis (Schmidt *et al.* 2004, Otto *et al.* 2006), where Wnt6 may maintain and regulate the Pax3 and Pax7 transcription factors in the muscle precursors. Wnt6 has also been found in the gastric epithelium of developing stomach (Nyeng *et al.* 2007).

Some of our evidence, such as the relatively weak expression in normal embryonic kidneys and the fact that *Wnt6* was not expressed in the entire ureteric tip epithelium, was against a critical role for Wnt6 as a ureteric signalling molecule in mesenchymal tubule induction. In addition, the expression could not be demonstrated after stage E14.5, while the inductive signalling thereafter continuously produced new nephrons in the renal cortical zone. More recently Wnt9b, first reported in adult kidney and in the developing glomeruli of embryos

at stage E16.5 (Qian *et al.* 2003), was shown by Carroll *et al.* (2005) to be expressed also in the early nephric duct and ureter. By using genetic targeting strategies, Carroll *et al.* (2005) demonstrated that Wnt9b is a critical factor in the nephric duct and its derivatives, sufficient for the epithelial conversion of the mesenchymal component. In addition, the Wnt6 knockout mice are viable (van Amerongen & Berns 2006) suggesting that the kidney development is not critically affected. Hence, as a factor with a tubule inducing property, Wnt6 is likely to play a less critical supportive role like some other signalling molecules that are expressed in the ureter, while the other possible signalling roles of Wnt6 remain elusive.

Lin *et al.* (2001) studied the function of Wnt6 signalling in the branching of ureter *in vitro* and reported that Wnt6 producing cells were inefficient in promoting ureteric bud branching morphogenesis. In consideration of other possible signalling roles of Wnt6 in urogenital development, it is also possible that Wnt6 function integrates into the regulation of nephric duct and/or ureteric bud epithelial cells while Wnt6 is also expressed in the genital ridges of both sexes at first and later in developing male gonads (Cory *et al.* 2007).

6.2 Wnt4 plays a role in the periureteric stroma and positively regulates smooth muscle cell differentiation via Bmp4 activity

Wnt4 is shown to be involved in the development of several organs such as was found in female sex determination (Vainio *et al.* 1999a, Heikkilä *et al.* 2005), and Wnt4 plays important roles in the developing pituitary (Treier *et al.* 1998) and adrenal glands (Heikkilä *et al.* 2002). In the developing thymus Wnt4 regulates the cellularity together with Wnt1 (Balciunaite *et al.* 2002, Mulroy *et al.* 2002). Wnt4 has been implicated in neuronal development in the CNS (Lyuksyutova *et al.* 2003). Wnt4 is also needed for mammary epithelium branching during pregnancy (Brisken *et al.* 2000). Thus, Wnt4 signalling plays multiple roles in developing tissues. In the embryonic kidney Wnt4 was found to be essential for the epithelial conversion of induced mesenchyme (Stark *et al.* 1994, Kispert *et al.* 1998).

We propose also a later role for Wnt4 signalling in the kidney medulla as a regulator of *Bmp4* expression and, via Bmp4, stromal smooth muscle differentiation. We support this argument with the following findings: 1) *Bmp4* gene activity was lost in Wnt4 mutant kidneys at the site were the *Wnt4* deficient allele was active; 2) the stromal smooth muscle differentiation marker, α-SMA,

was not found at this site; 3) Wnt4-expressing cells were able to stimulate the *Bmp4* expression in the Wnt4-deficient kidney *in vitro* and 4) Wnt4-expressing cells were able to induce a local differentiation of smooth muscle cells in the Wnt4-deficient kidney *in vitro*. Previous reports suggest that Bmp4 is a local signalling cue for smooth muscle cell migration and differentiation in cultured metanephric kidneys (Raatikainen-Ahokas *et al.* 2000, Miyazaki *et al.* 2003).

Thus, the loss of Wnt4 signalling, *Bmp4* expression, and smooth muscle differentiation in the stroma of Wnt4-deficient kidneys colocalise, and the experimental analysis placed Wnt4 signalling upstream of *Bmp4* and smooth muscle differentiation. The result implies that Wnt4 signalling in the periureteric stroma expressing *Wnt4* in normal kidneys can stimulate *Bmp4* expression. Yu *et al.* (2002) reported that Sonic hedgehog (Shh) produced by the ureteric epithelium can upregulate Bmp4 expression in the metanephros. However, the Bmp4 signalling was sufficient for metanephric development in embryos with a conditionally inactivated ureteric *Shh* gene (Yu *et al.* 2002). The canonical Wnt signalling activity in the developing lung has been recently reported in the regulation of Bmp4 activity by Shu *et al.* (2005) and concomitantly leads to downregulation of the smooth muscle differentiation marker, αSMA. At present, however, the signalling mechanism by which Wnt4 can induce *Bmp4* expression is unclear.

6.3 Lack of Wnt4 leads to a defect in vascular development

The complex vascular network in the kidney develops as a result of secreted signals, receptors, and interactions of cells with their surrounding (Sariola *et al.* 1983, Puri *et al.* 1995, Loughna *et al.* 1997, Loughna *et al.* 1998). We found a defect in vascular maturation in the *Wnt4*-deficient kidneys as the vascular pericytes that were marked by αSMA staining in normal kidneys were missing from the *Wnt4*$^{-/-}$ mutant ones. When considering if the Wnt-4 signalling would be directly responsible for the loss of vascular smooth muscle cells, it should be noted that the expression of *Wnt-4* has not been found in endothelial cells nor is it confined to the immediate vicinity of nascent vessels in the kidney. The notion that the mammalian Wnts are likely to signal within a short distance supports this proposal. Hence, we suggest that the loss of vascular smooth muscle cells observed in Wnt4 deficient kidneys results from secondary reasons rather than being a direct effect of Wnt4 signalling.

6.4 Neural crest cells are not essential for stromal development in the early metanephros

In the past, evidence has been provided implying neural crest involvement as a putative source of cells in the early metanephros. Quite recently, when the Cre recombinase coding region was targeted to the *Pax3* locus in mouse, replacing the first exon of the gene (Engleka *et al.* 2005), it was discovered that a significant proportion of mesenchymal derivatives in kidneys including the developing nephrons were labelled with LacZ expression. The result thus suggests that in some point in their history these cells were expressing *Pax3*. The result also implies that the kidney cells could originate from a source outside of the intermediate mesoderm such as the neural crest, while the mouse line has also been used for the selective inactivation of genes in the metanephric mesenchyme. Hence, the role of neural crest cells in the developing kidney has remained unclear. One of the main reasons is perhaps that the neural crest cells proliferate and differentiate while migrating, making the reliable labelling of neural crest cell derivatives difficult. The autonomous nervous system that innervates, on the other hand, the kidney is of neural crest origin (Sariola *et al.* 1988). The innervation has been found to regulate glomerular morphogenesis in later development; while dissecting the nerves innervating the kidney, has affected the filtration capacity but not the function of the kidneys.

We used the Wnt1Cre mice to target the early lumbosacral neural crest cell precursors with a stable production of β-galactosidase enzyme. The study of the developing metanephric region of these mice revealed that the neural crest cells were encapsulating the early metanephros, but did not migrate into the metanephric mesenchyme in order to contribute cells to the metanephric kidney. We also found that neural crest cells were not migrating in recombinant culture assay *in vitro* to metanephric kidney explants. Thus, it seems unlikely that the non-neuronal cells in the kidneys would originate from the neural crest. For example, White and Andersson (1999) have grafted rat neural crest cells from the lumbosacral region to chicken embryos and found that the grafted cells were able to contribute e.g. to the lumbar plexus, but the contribution of the cells to chicken kidneys was not reported, supporting our view. Also, we found that the early metanephros may produce attractive molecules to neural crest cells; however, the cells were not capable of entering into the intact isolated kidney, which is in line with the observations from LacZ reporter mice.

Signalling activity that restricts the amount of nephrons in the developing kidney has been associated to the stromal component. Thus, signalling that would affect stromal development in the metanephros might result in the aberrant development of the kidney. We addressed this possibility in Splotch mice in which the neural crest migration in the trunk is deficient. Hence, the metanephros is not targeted by neural crest cells. When comparing the kidneys lacking neural crest with normal kidneys, we did not find structural or functional differences, thus providing evidence that the neural crest is not essential for the regulation of early development. Most of the Sploch embryos die around stages E13.5-E14.5 due to failure in heart development. Hence, addressing later kidney development using Splotch mice is not possible.

7 Conclusions

While the ureteric bud grows and branches, *Wnt6* expression is maintained in the newly formed branches of the ureter, and the expression of Wnt6 is present in the ureteric epithelia towards the tips. However, Wnt6 expression in metanephros after stage E14.5 was undetectable by the *in situ* hybridisation method. We also demonstrate that the tubule-inducing property is located on the tip of the branching ureter, and *in vitro* Wnt6 signalling can induce tubulogenesis in isolated metanephric mesenchyme. Thus, Wnt6 is involved in tubule induction. The data suggests that the Wnt6 could play, at the early stages of development, a role as a secreted signal from the ureteric epithelium that regulates tubulogenesis. The mice deficient for Wnt6, however, are known to be viable, thus providing evidence that Wnt6 signalling is not critical in the developing kidney and in addition, it was recently found that *Wnt9b* expression overlaps with *Wnt6* expression in the Wolffian duct and also partially in the ureteric bud, where *Wnt9b* expression was found at the extreme tips of the branching ureteric tree. Wnt9b was also demonstrated to play a critical role in providing the tubule-inducing capacity for the branching ureteric bud epithelia. Hence, we conclude that Wnt6 is likely to play, at most, a redundant role in the induction of mesenchymal tubulogenesis in developing metanephros while, on the other hand, it may possess distinct functional properties that might be elucidated in such mice where both Wnt6 and Wnt9b are functionally inactive. We also demonstrate that Wnt6 is able to induce an ectopic axis in *Xenopus* embryos and hence could serve as one of the several Wnts that can be used in studies concerning the axis inducing mechanism.

The Wnt6 mutant mice may further elucidate the Wnt6 signalling function *in vivo*. In addition, a double mouse mutant defective in Wnt6/Wnt9b signalling may provide important knowledge of Wnt signalling in the nephric duct. *In vitro*, Wnt6 signalling function could be addressed for example by using the knock-down approach by Davies *et al.* (2004).

Wnt4 is a critical factor in the developing metanephros, regulating the mesenchyme-to-epithelium conversion of mesenchymal cells induced by the ureter. Here we provide evidence that connects stromal Wnt4 signalling to stromal smooth muscle differentiation in the developing kidneys. We show that Wnt4 expression in the periureteric stromal component of normal kidneys can be demonstrated in mouse at the embryonic stage when the differentiating smooth muscle cells can be visualised in this tissue. The fact that the gene activity of a

disrupted *Wnt4* allele could be localised also to the perureteric cells of *Wnt4*-deficient kidneys at this stage implies that at least to some degree, the molecular regulation of this tissue component is reminiscent of normal kidneys. In contrast to this notion, we show that Bmp4 expression in the medullar stroma of Wnt4 deficient kidneys is absent, thus suggesting a requirement of functional Wnt4 signalling in the regulation of the periureteric stromal tissue. The absent Wnt4 signalling in the medullar stroma of Wnt4-deficient kidneys is likely to affect the interactions between the stromal cells and the nascent collecting duct epithelia which, on the other hand, is poorly understood at later embryonic stages. Our expression data also suggest that Sonic hedgehog signalling, which may regulate the level of Bmp4 in kidneys, cannot alone activate *Bmp4* expression as Sonic hedgehog signalling was found in the absence of *Bmp4* expression in Wnt4-deficient kidneys. We show that *in vitro* Wnt4 signalling can directly or indirectly activate the *Bmp4* expression, while recombinant human Bmp4 can induce smooth muscle differentiation marker production in the Wnt4-deficient kidneys. In sum, we conclude that Wnt4 signalling may activate *Bmp4* expression in the periureteric smooth muscle cells and via regulation of the Bmp4 signalling contribute to cell differentiation.

We also found that the interaction between vascular endothelial cells and pericytes is disturbed in Wnt4 deficient kidneys likely due to reasons secondary to the Wnt4 signalling. The smooth muscle marker protein produced in nascent pericytes was detected around the vessels in the normal embryonic kidneys at stage E14.5 and later, but could not be found in the Wnt4-deficient kidneys. The molecular interactions behind the defect, however, remain elusive. *In vitro* we also show that the nascent blood vessel endothelial cells in the embryonic metanephros can respond to Wnt signalling by increasing in amount and networking, implying that Wnt signalling could regulate the endothelial cells in the developing kidney.

In addition we addressed a question that concerns the putative contribution of the migratory neural crest cells in the developing kidney and, by using transgenic mice, mapped the neural crest cells associating to the early developing metanephros. We also show that in Splotch embryos with a defect in neural crest migration and, therefore, in the absence of neural crest cells in the metanephric field, the early metanephros is not morphogenetically different from controls while the neural crest derivatives later integrate to the normal metanephric kidney by making cellular processes. The isolated metanephric kidneys from Splotch embryos were not different from controls in their tubule-forming capacity and

ureteric branching morphogenesis either, suggesting that neural crest cells do not provide signalling that is necessary for the morphogenetic regulation of the early metanephros. The neural crest cells are not essential for early stromal development and they do not provide essential signalling to the early metanephros.

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