Kidney Induction:
Control by Notch, Wnt and GDNF/Ret signalling

Satu Kuure

Biochemistry and Developmental Biology
Institute of Biomedicine
Faculty of Medicine
University of Helsinki
HBGS

ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Faculty of Medicine,
University of Helsinki, in Auditorium 3, Biomedicum Helsinki,
Haartmaninkatu 8, on June 8th 2007 at 12 o’clock

Helsinki 2007
The developmental biology of my life became true in my sons Otto and Oula.
TABLE OF CONTENTS

ABBREVIATIONS 6
ORIGINAL PUBLICATIONS 7
ABSTRACT 8
1. REVIEW OF THE LITERATURE 10
   1.1 Introduction to kidney development 10
      1.1.1 Pro- and mesonephros 10
      1.1.2 Metanephros 11
   1.2 Classical induction studies 13
   1.3 Molecular control of renal differentiation by transcription factors 15
      1.3.1 *Pax2* and *Pax8* are required for nephric lineage determination 15
      1.3.2 *Wt1* is essential for mesenchymal survival and nephrogenic competence 17
      1.3.3 Wolffian duct formation depends on *Gata3* 18
      1.3.4 *Eya* and *Six* interact in early specification of metanephric mesenchyme 19
      1.3.5 *Lim1* functions at several stages of renal differentiation 21
      1.3.6 *Sall1* defines nephron progenitors within metanephric mesenchyme 22
      1.3.7 *Emx2* may regulate the expression of genes encoding ureteric bud derived signals 22
      1.3.8 *Hox* genes control ureteric budding via *Six2* and *Gdnf* 23
      1.3.9 *Foxc1/Foxc2* suppress *Gdnf* expression 24
   1.4 GDNF-activated Ret signalling 24
      1.4.1 Role of GDNF signalling in kidney morphogenesis 27
   1.5 Notch signalling 29
      1.5.1 Notch signalling in nephron formation 32
   1.6 Wnt signalling 34
      1.6.1 Wnt signalling in nephron induction 37
      1.6.2 Wnt signalling in branching morphogenesis 38
2 AIMS OF THE STUDY 39
3 MATERIALS AND METHODS 40
4 RESULTS AND DISCUSSION 42
   4.1 Overexpression of *Jag1* in Wolffian duct derivatives activates
Notch signalling and causes renal aplasia or hypodysplasia (I) 42
4.2 Changes in the GDNF/Ret signalling disturb the ureteric budding and branching in Jag1 transgenic mice (I) 44
4.3 Suppression of Bmp4 by BAMBI may explain spontaneous extra ureteric budding in Jag1 transgenic kidneys (unpublished) 46
4.4 Canonical Wnt activity and β-catenin localisation suggest dual function for Wnt signalling in renal development (II, III) 47
4.5 Activation of canonical Wnt pathway through stabilisation of β-catenin arrests ureteric branching and induces nephron formation (II, III) 48
4.6 In vivo manipulation of β-catenin specifically in the ureteric epithelium results in similar ureteric branching defect as its in vitro stabilisation 50
4.7 Cellular mechanisms in epithelium specific β-catenin mutant mice 51
4.8 Ureteric epithelium specific β-catenin manipulation disturbs Ret-signalling and causes loss of ureteric tip identity 52
4.9 β-catenin stabilisation triggers nephrogenesis in isolated mesenchymes 56
4.10 Genetic stabilisation of β-catenin in mouse mesenchymes induces nephrogenesis 57
4.11 Establishment of nephrogenic competence does not require the ureteric epithelium 57
5 CONCLUDING REMARKS 59
6 ACKNOWLEDGEMENTS 61
7 REFERENCES 63
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGM</td>
<td>aorta-gonad-mesonephros</td>
</tr>
<tr>
<td>A-P</td>
<td>anterior-posterior</td>
</tr>
<tr>
<td>AS</td>
<td>antisense</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BIO</td>
<td>6-bromoindirubin-3′-oxime</td>
</tr>
<tr>
<td>BAT</td>
<td>β-catenin-activated transgene</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>DBA</td>
<td>Dolicos biflorus lectin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF1, Suppressor of Hairless, Lag-1</td>
</tr>
<tr>
<td>dpc</td>
<td>days post coitum</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ENU</td>
<td>ethynitrosourea</td>
</tr>
<tr>
<td>gal</td>
<td>galactosidase</td>
</tr>
<tr>
<td>GFL</td>
<td>GDNF family ligand</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidyl inositol</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparin sulphate proteoglycans</td>
</tr>
<tr>
<td>IM</td>
<td>intermediate mesoderm</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun terminal kinase</td>
</tr>
<tr>
<td>LRP</td>
<td>low density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>LT</td>
<td>Lotus tetragonolobus lectin</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madine Darby Canine Kidney</td>
</tr>
<tr>
<td>MEN</td>
<td>multiple endocrine neoplasia</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>PA</td>
<td>polyA</td>
</tr>
<tr>
<td>PCP</td>
<td>planar cell polarity</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RUB1</td>
<td>rat ureteric bud cell line</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>spc</td>
<td>spinal cord</td>
</tr>
<tr>
<td>TK</td>
<td>tyrosine kinase</td>
</tr>
<tr>
<td>YAC</td>
<td>yeast artificial chromosome</td>
</tr>
<tr>
<td>WT</td>
<td>Wilms’ tumor</td>
</tr>
</tbody>
</table>
ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by their Roman numerals (I-III) in the text. In addition, some unpublished results will be presented.


* These authors contributed equally to this work.
§ These laboratories contributed equally to this work
ABSTRACT

The permanent mammalian kidney (metanephros) develops as a result of complex reciprocal tissue interactions between a ureteric epithelium and the renal mesenchyme. The overall goal of the research in this thesis was to gain data that will eventually help in elucidating the formation of congenital renal malformations. The experiments in my thesis aimed to reveal the mechanisms by which Notch, Wnt and GDNF/Ret signalling pathways regulate the development of functional kidney.

The function of Notch pathway was studied by a transgenic mouse model, where a Notch ligand, Jag1, is overexpressed in the presumptive and definitive ureteric epithelium. In line with the results from other organs, it was shown that renal development is sensitive to the dosage of Notch activity. Overactivation of Notch signalling disturbs kidney development and alters the expression of Gdnf and Ret/GFRα1. This indicates that Notch signalling interplays with GDNF/Ret in the regulation of the primary ureteric budding and its subsequent branching. The data also suggested that strict spatio-temporal regulation of these two pathways is required for determination of ureteric tip-identity, which appeared to be crucial for the branching.

The function of Wnt signalling in the ureteric morphogenesis was studied by in vivo and in vitro methods to show that a canonical pathway is required for ureteric branching. Stabilisation of β-catenin in the cultured kidneys or specifically in the ureteric epithelium results in renal aplasia/hypodysplasia. These defects originate from severe blockage of ureteric branching due to the disrupted Ret signalling. Consequently, ureteric tip specific markers are lost and stalk identity is expanded throughout the whole epithelium. Strikingly, also deletion of β-catenin from ureteric epithelium results in a similar phenotype but through a different mechanism. In mice with epithelial specific β-catenin deletion Ret expression is lost and the phenotype mimics that of Ret-deficient kidneys. Thus, the data demonstrates that the Wnt/β-catenin pathway plays an essential role in the patterning and branching of the ureteric epithelium.

A novel in vitro method was generated and utilised in nephron induction studies to reveal the mechanisms through which nephrogenesis is triggered. Transient GSK3 inhibition in isolated renal mesenchymes efficiently induced nephron formation through the stabilisation of β-catenin. Also genetic stabilisation of β-catenin specifically in the renal mesenchyme results in spontaneous nephrogenesis. Activation of the canonical Wnt/β-catenin pathway also induced nephrogenesis in two mouse models where the mesenchyme had never been in the contact with the epithelium demonstrating that the
nephrogenic competence is established without the influence of the Wolffian duct or ureteric bud. The results show that activation of the canonical Wnt pathway is sufficient to initiate nephrogenesis, and suggest that this pathway mediates the nephron induction in murine kidney mesenchymes.

Taken together, this thesis demonstrates Notch and Wnt signalling pathways as novel regulators of ureteric branching morphogenesis, and that activation of the canonical Wnt pathway is sufficient for nephron induction. The studies also indicate that the Notch and Wnt pathways cross-talk with GDNF/Ret signalling in the patterning of ureteric epithelium.
1. REVIEW OF THE LITERATURE

1.1 Introduction to kidney development

Embryonic kidney has been for decades a useful model to study induction as well as cellular and molecular mechanisms of organogenesis. Like most organs in the body, the kidney develops as a result of inductive interactions between the epithelial and mesenchymal tissues. Here the term kidney induction covers all inductive events that control the formation of ureteric bud, its subsequent branching and differentiation of nephrons. The morphological steps of kidney development have been well characterised, but only after extensive mouse genetics, we begun to understand the signalling pathways and complex molecular networks regulating the formation of the functional organ. In this literature review, I will first describe the morphological steps of the kidney development, then go through the transcription factors critical for kidney induction, and finally review what is known about the role of GDNF, Notch and Wnt signalling in this process.

1.1.1 Pro- and mesonephros

In mammals and birds functional kidney develops in three spatially and temporally distinct stages: pronephros, mesonephros and metanephros (Fig. 1). Only the metanephros differentiates into the permanent kidney in mammals. The two others are transient structures during the embryogenesis of higher vertebrates but they contribute to the development of gonads and adrenal glands. All three kidneys are derived from the intermediate mesoderm (IM) that first forms a nephric ridge in the dorsal wall of the body cavity. After this specification, the formation of the Wolffian (nephric) duct begins by caudal migration of mesenchymal cells. These migrating mesenchymal cells differentiate into the epithelial tubule and which finally reaches the cloacae.

**Pronephros** is a functional organ in amphibians and fish during the larval stages (for a review, see Saxén, 1987). It is noteworthy that similar inductive
interactions, signalling molecules and pathways seem to control the development of all three kidney types demonstrating a conserved control also at the genetic level (Carroll and Vize, 1996; Heller and Brandli, 1997; Heller and Brandli, 1999). Therefore, pro- and mesonephros have proved to serve as simplified models to study nephron development.

**Mammalian mesonephros** develops from mesodermal region also called AGM (aorta-gonad-mesonephros). It is not known what are the factors involved in specification of the AGM and how the area is further subdivided into distinct regions. The AGM participates in the development of aorta and gonads, but it is also the source of haematopoietic stem cells (Medvinsky and Dzierzak, 1996; Durand and Dzierzak, 2005). Cells derived from this region will eventually migrate to e.g. liver and bone marrow where they contribute to definitive haematopoiesis.

Much of the knowledge of how mesonephros forms and functions is based on the studies in amphibians and chicken. The mesonephros develops when the Wolffian duct reaches the prospective mesonephric mesenchyme and induces adjacent mesenchymal cells to condense. Thereafter the condensates will form mesonephric tubules leading to the formation of nephrons (Saxén, 1987). In principle, both morphologically and genetically, the process of nephrogenesis occurs in a very similar manner as seen later in metanephros, which will be described in more detail in section 1.1.2. However, the mesonephric tubules are segmented in a row next to the Wolffian duct, whereas in the metanephric kidney the tubules are organized in more complex three-dimensional orientation.

### 1.1.2 Metanephros

Main information of the cellular basis and tissue interactions driving the kidney development comes from the studies of Grobstein and Saxén (Grobstein, 1956a; Grobstein, 1956b; Saxén and Lehtonen, 1978; Saxén and Lehtonen, 1987). The development of the metanephros, which is the permanent and functional kidney in higher vertebrates, begins in mouse at E10.5-11 (Saxén, 1987) and in humans at around E30. The differentiation of metanephric kidney depends on a series of reciprocal inductive interactions between two tissues: the metanephric mesenchyme and the Wolffian duct-derived ureteric bud. Currently many of the signals mediating these interactions are known but we still understand poorly how different regulators are integrated with each other.
Kidney development begins with the signals from the metanephric mesenchyme that induce the emergence of a single ureteric bud from the Wolffian duct, and its invasion into the metanephric mesenchyme (Fig. 2A-B). Next in response to mesenchymal signals, the ureteric bud branches for the first time to form T-bud. At this stage, the two ureteric bud branches transmit signals that induce loose metanephric mesenchymal cells first to condense, and then to form pretubular aggregates at the armpits of the T-bud (Fig. 2C). The pretubular aggregates continue their epithelialisation and differentiate via comma- and S-shape bodies into the various cell types of functional nephrons (Saxén, 1987). Simultaneously to the induction of the first nephrons, the ureteric bud continues its branching (Fig. 2D), which is crucial for the growth and further differentiation of the kidney. The branching pattern not only determines the shape and size of the organ (al-Awqati and Goldberg, 1998) but also influences on nephron number. The same scheme of branching and induction is repeated time after time to produce approximately 30,000 nephrons in mouse and one million in human (al-Awqati and Goldberg, 1998).

Ureteric branching follows always certain patterns: the tip branches preferably in a bifurcation manner but occasionally during the second round of branching, also trifurcated pattern is observed. Very rarely new branches are formed from the stalk of the ureteric bud (Lin et al., 2001; Watanabe and Costantini, 2004; Costantini, 2006). The cap condensate, which is a specific cell population within the metanephric mesenchyme surrounding ureteric bud tips (Fig. 2), expresses genes that regulate the ureteric branching (Sariola, 2002). In addition to the reciprocal signalling between the ureteric epithelium and mesenchymal cap condensate, also the stroma of the developing kidney contributes to control of ureteric branching (Mendelsohn et al., 1999; Batourina et al., 2001; Batourina et al., 2002).

Figure 2. Metanephros development. (A) Mesenchyme induces thickening of Wolffian duct (WD), (B) which elongates to form a ureteric bud (UB). (C) The first branching of the UB induces pretubular aggregate formation. (D) UB continues its branching and induction of nephrons.
During renal differentiation also vessel formation and innervation must occur for the proper function of the organ (Sariola et al., 1988). Recently it was shown that vascular endothelial growth factor (Vegf) signalling in the renal angioblasts is required for initiation of kidney differentiation (Gao et al., 2005). In addition, factors produced by neurons may affect kidney morphogenesis (Saxén, 1987; Sariola et al., 1988).

1.2 Classical induction studies

Much of the knowledge of the cellular basis of tissue interactions involved in the kidney development comes from the early tissue culture studies of Grobstein and Saxén (Grobstein, 1956a; Grobstein, 1956b; Saxén, 1970; Saxén and Lehtonen, 1978; Saxén and Lehtonen, 1987). In such organ culture system the metanephric mesenchyme is separated from the ureteric epithelium and cultured on the filter with heterologous inducer tissues such as spinal cord (spc) (Fig. 7). The early experiments suggested that direct cell-cell contacts between the mesenchyme and the inducer are crucial in triggering the nephron differentiation (Lehtonen, 1976; Saxén et al., 1976). The signals produced in vitro by the spc are though to be the same, or very similar, to those provided by the ureteric bud in vivo. Although spc induction mimics in vivo nephrogenesis fairly well, it results in only a fraction of nephrons that are normally formed in intact kidneys suggesting a lack of proliferation of undifferentiated mesenchymal cells. This, together with the fact that the whole mesenchyme is turned into nephrons by spc, suggest that ureteric bud epithelium secrets additional signals that potentially maintain and stimulate proliferation of nephron progenitors. It may be also

Figure 7. Experimental set-up for in vitro mesenchymal induction studies After enzymatic treatment, ureteric bud is manually dissociated from the metanephric mesenchyme of E11 kidney. The mesenchyme is then placed on top of porous filter, which allows cell contacts with the inducer tissue, the spinal cord, placed on the other side of the filter (Saxen, 1987).
possible that in the intact kidney, positional information within the kidney mesenchyme prevents its differentiation into nephrons, and thereby potentially promotes the stromal fate.

It has been speculated that Wnt proteins (see chapter 1.6) expressed in the spc trigger the nephron differentiation (Kispert et al., 1998; Cho and Dressler, 2002). Wnt-expressing cells in the contact with mesenchyme can induce nephron differentiation (Kispert et al., 1998) but so far, no reports have been published showing that purified Wnt proteins could trigger nephrogenesis. Similarly, intracellular activation of Wnt signalling in isolated renal mesenchymes fails to induce nephrons even though the pretubular aggregates are formed (Davies and Garrod, 1995). Inability of Wnt proteins to induce nephron formation may result from the requirement of cell-cell contacts in the induction process, or of the glycoprotein chemistry, which makes production of functional soluble Wnt protein very challenging. The latter possibility is unlikely, since purified Wnt proteins have been successfully used in other systems (Schulte et al., 2005; Naito et al., 2006; Oloumi et al., 2006). Also the first possibility is improbable, since nephron induction with soluble factors in isolated rat mesenchymes has been demonstrated with rat pituitary extract (Perantoni et al., 1995) and also with certain fractions of conditioned media from rat ureteric bud (RUB1) cell line (Karavanova et al., 1996). Leukaemia inhibitory factor (LIF), which was then identified as an inductive protein from RUB1 cells, and LIF in combination with basic FGF (FGF2) and TGFβ1/TGFβ2 is able to trigger tubulogenesis in isolated rat mesenchymes (Barasch et al., 1999; Plisov et al., 2001). The molecules of LIF and TGF pathways are expressed in the developing mouse kidney (Pelton et al., 1990; Pelton et al., 1991; Nichols et al., 1996), but the genetic evidence does not support their role in induction of nephrons (Stewart et al., 1992; Escary et al., 1993; Nichols et al., 1996; Ware et al., 1995) since the kidneys in the mice lacking either the LIF receptor gp130 or TGFβ2 are normal albeit small (Sanford et al., 1997; Barasch et al., 1999). Thus, the inductive potential of above mentioned proteins appears to be quite limited as their function in mouse kidney is neither sufficient (Cho and Dressler, 2002) nor essential for nephrogenesis.

The experiments described above suggest that either the inductive signals for nephrogenesis are different in closely related rodent species, or that additional signals are needed to trigger tubulogenesis in mice. However, based on these findings the molecular basis of nephron induction remains vague.
1.3 Molecular control of renal differentiation by transcription factors

Transcription factors are proteins, which regulate the expression of other genes. They, often in a complex with other proteins, recognize and bind short DNA sequences in the promoters or enhancers. Depending on the mode of action, transcription factors can either activate or repress the expression of downstream genes.

Generation of targeted (Soriano, 1995) and gene trap (Chen and Soriano, 2003) mutations in genes expressed in developing embryo have greatly facilitated our understanding of the molecular mechanisms, which regulate embryogenesis. For example, much of the knowledge in the genetic control of renal differentiation has been obtained by deleting the function of a gene expressed in the developing kidney. However, the conventional knockout mice are sometimes non-informative. Certain genes are indispensable in early embryogenesis, for example during the gastrulation or formation of the vasculature system and their deletion causes embryonic lethality prior to the initiation of kidney organogenesis. The function of such genes can be studied by conditional deletion strategy (Hadjantonakis et al., 1999; Nagy, 2000), where the gene is deleted in an organ- or tissue-specific manner with the help of tissue specific promoters and Cre-lox technique (Soriano, 1999). Redundancy may also mask the importance of certain genes, which will only be revealed when redundant genes are co-deleted from the genome.

In the following chapters I will introduce the transcription factors, which have the established role in the early specification and differentiation of kidney. Gene functions will be discussed in chronological order up to the T-bud stage. Due to the obvious complexity and obscurity in the regulation, signalling pathways controlling the transcription factors are only briefly mentioned if relevant for the pathways studied in this thesis.

1.3.1 Pax2 and Pax8 are required for nephric lineage determination

Pax proteins belong to a family of paired box-containing transcription factors, which are involved in the developmental control of several organs e.g. kidney, ear and eye (Lang et al., 2007). In humans, mutations in PAX2 cause a rare renal-coloboma syndrome characterised by optic nerve colobomas and renal abnormalities (Eccles et al., 2003). In the mouse kidney, the expression of Pax2 and -8 is initiated already in the IM and maintained throughout the development of the pro- and mesonephros (Bouchard et al., 2000). In metanephros, Pax2 is expressed in the ureteric bud and in the induced
mesenchyme. Pax8 co-localizes with Pax2 prior to the metanephric induction, but when the ureteric branching start, Pax8 expression gets restricted exclusively to the mesenchyme, while Pax2 is still detected in the ureteric epithelium (Plachov et al., 1990).

Deletion of Pax2 in mice results in a complete lack of kidneys, ureter and genital tract. The metanephric mesenchyme is morphologically detectable in the Pax2-null embryos, but no ureteric buds are formed showing the indispensable function for Pax2 in the initiation of ureteric budding (Torres et al., 1995). The Pax2-deficient kidney mesenchyme fails to express a key regulatory molecule of ureteric budding, Glial cell line-Derived Neurotrophic Factor (GDNF). Pax2 binds to Gdnf promoter and can activate its expression in a metanephric mesenchyme-derived cell line (Brophy et al., 2001) suggesting that Pax2 may directly regulate Gdnf expression.

The genetic inactivation of Pax2 indicates that it is required already for the differentiation of the IM. In the absence of Pax2, the gonads form but they lack the structures that are derived from the IM, such as Müllerian ducts in females and Wolffian duct-derived vas deferens and epididymis in male mouse embryos. The development of mesonephric tubules is also disrupted in Pax2-/ mice (Torres et al., 1995) and, consistent with that, the metanephric mesenchyme fails to respond to induction cues also when stimulated by heterologous inducers (Brophy et al., 2001). The expression of Pax2 is still detected during the glomerulogenesis suggesting that the protein functions during the later nephrogenesis. This is further supported by congenital nephrotic syndrome –like defects in Pax2-overexpressing mice (Dressler et al., 1993).

The mice deficient for Pax8 die at about 3 weeks of age due to thyroid defects, but kidney development appears normal (Mansouri et al., 1998). Pax8 can functionally replace Pax2 in zebrafish otic placode induction demonstrating redundancy between these factors (Hans et al., 2004; Mackereth et al., 2005). The mice deficient for both Pax2 and -8 show the additive requirement of the genes in the specification of the nephric lineage in the IM. The mesenchyme-to-epithelium transition normally required for Wolffian duct formation does not take place in Pax2/8 mutants, which also fail to initiate the expression of Lim1 and Ret (Bouchard et al., 2002). Recently it was shown that Pax2 and Pax8 regulate ureteric branching also after primary budding as the number of ureteric tips and nephrons is significantly reduced in compound heterozygote mice (Narlis et al., 2007).
1.3.2 Wt1 is essential for mesenchymal survival and nephrogenic competence

Wilms’ tumour gene 1 (Wt1) encodes a zinc-finger tumour suppressor, which transcriptionally controls target genes but also participates in RNA processing (Hohenstein & Hastie, 2006). Paediatric cancer of the kidney, the Wilms’ tumours (WT), affects 1:10 000 individuals and accounts approximately 8% of all malignancies in children. WT is thought to rise due to a metanephric mesenchyme differentiation failure (Chen and Soriano, 2003). WTI germ line mutations are found in 10% of WT cases and splice alteration are detected in nearly 90% of sporadic tumours (Gubler and Jeanpierre, 2003). WTI mutations are also associated with several syndromes characterised with genital and renal malformations such as WAGR (WT, Aniridia, Genital abnormalities, and mental Retardation), Denys-Drash and Frasier (Gubler and Jeanpierre, 2003).

Wt1 is a critical regulator of normal renal development also in mouse. Similarly to Pax2 and -8, Wt1 is detected already in IM and differentiating mesonephros. It is expressed in the metanephric mesenchyme even before ureteric bud formation, which after the expression becomes up-regulated in the induced mesenchyme. Later, Wt1 is highly expressed also in glomerular podocytes (Pelletier et al., 1991; Armstrong et al., 1993). The rich expression pattern suggests functions at three stages of renal development: the determination of the kidney area, the differentiation of nephrons and the maturation of glomeruli.

Wt1-deficient mice confirmed its necessity for the inception of kidney development; in mutant mice the cells that normally form kidneys die by apoptosis (Kreidberg et al., 1993; Davies et al., 2004). Mutant mesenchyme cannot be induced to form nephric tubules (Kreidberg et al., 1993) suggesting that Wt1 is cell-autonomously required for nephron differentiation. Wt1 reciprocally controls the ureteric budding and branching, because no ureteric bud is formed in Wt1-deficient mice, which die between E13 and E15 due to defects in heart, lung and mesothelium development. Heart defects can be completely rescued with the expression of a human WTI YAC construct in mutant mice but the urogenital development is only partially restored (Moore et al., 1999) indicating that Wt1 function is necessary also after the initiation of kidney organogenesis. This is supported by siRNA-based repression of Wt1, which prevents nephron differentiation and causes abnormal proliferation and these both possibly mimic the aspects of Wilms’ tumour biology (Davies et al., 2004). It has been suggested that Wt1 may regulate apoptosis in the developing kidney (Kuure et al., 2000), but the increased cell death in
Wt1-/- kidneys may also reflect its requirement for mesenchymal survival. Due to the lack of mesenchymal induction, Wt1 thereby indirectly contributes to the control of normal proliferation and apoptosis.

Several Wt1 target genes have been identified by *in vitro* overexpression and reporter assay methods in the cultured cells. Genetic studies have proven that at least *amphiregulin* (Wagner et al., 2003), *Sprouty* (Gross et al., 2003), *nestin* (Wagner et al., 2006), *nephrin* (Wagner et al., 2004), *TrkB* (Wagner et al., 2005) and *POU4f2* (Wagner et al., 2003) are regulated by Wt1 also *in vivo*. These targets are activated rather than suppressed by Wt1 suggesting a dual function for the protein in transcriptional regulation.

1.3.3 Wolffian duct formation depends on Gata3

The zinc-finger transcription factor *Gata3* is expressed in the Wolffian duct epithelium from the time of its emergence in IM and the expression is maintained also later in the ureteric epithelium (Labastie et al., 1995). Mutations in human *GATA3* gene cause hypoparathyroidism, sensorineural deafness and renal anomaly (HDR) syndrome indicating that the protein is essential for development of the parathyroid glands, auditory nerves and kidneys (Van Esch et al., 2000). Deletion of mouse *Gata3* has revealed its’ indispensable function in the development of noradrenalin neurons of the sympathetic nervous system (Lim et al., 2000), T-cell lineage determination (Ting et al., 1996) and expansion of T-cell progenitors (Hendriks et al., 1999). The role of Gata3 in renal development was shown when embryonic lethal phenotype of *Gata3*-deficient mice was partially rescued with drug treatment and the surviving mice exhibited complete lack of kidneys (Lim et al., 2000). The renal aplasia is caused by the defects in Wolffian duct formation and migration. *Ret* and *Wnt11* expressions are lost in *Gata3*-deficient epithelium suggesting that Gata3 may regulate *Ret* expression in the developing Wolffian duct, but also later in the tips of ureteric buds. Lack of *Ret* and *Wnt11* is unlikely the cause of the phenotype in *Gata3*-deficient mice, but suggests that Gata3 might play a role also in the regulation of ureteric branching morphogenesis, which was recently confirmed (Hasegawa et al., 2007). Microarray and loss of function studies in mice double-deficient for *Pax2/8* indicate regulatory relationship between the *Pax* genes and *Gata3* so that Pax2 together with Pax8 may directly regulate *Gata3* expression during the Wolffian duct formation (Grote et al., 2006).
Deletion of mouse *odd-skipped related 1* (*Odd1*), a zinc finger transcription factor related to *Drosophila* pair rule gene *odd skipped*, results in defective Wolffian duct formation resembling that of the *Gata3* mutants (Wang et al., 2005). However, the mechanism for the similar phenotypes is probably different in *Gata3*- and *Odd1*-deficient mice. *Gata3* is cell-autonomously needed for the Wolffian duct development. On the other hand, *Odd1* is expressed in IM cells and thereafter exclusively in the mesenchymal compartment of developing kidney suggesting that mesenchyme reciprocally regulates Wolffian duct development. Interestingly, *Odd1* expression gets down-regulated when the mesenchyme differentiates into pretubular aggregates (So and Danielian, 1999; James et al., 2006). In chick, *Odd1* up-regulates *Pax2* and *Lim1* expression but its continuous expression in mesonephric precursors prevents their differentiation into nephrons (James et al., 2006). These findings suggest that *Odd1* is transiently needed to initiate nephrogenesis.

1.3.4 *Eya and Six interact in early specification of metanephric mesenchyme*

Six, Eya and Dach form a complex of highly conserved proteins, which synergistically participate in the control of organ development by regulating transcription of target genes. Only the Six proteins have a DNA-binding ability while Eya and Dach function as co-factors. Interestingly, the Eya proteins exhibit a phosphatase domain, which upon activation switches repressor function of Six-Dach to a transcriptional activator (Li et al., 2003). Mutations causing human branchio-oto-renal (BOR) syndrome characterised by loss of hearing and kidneys were first identified in the *EYA1* gene (Abdelhak et al., 1997) but later also in *SIX1* (Ruf et al., 2004). The mice heterozygous for *Six1* allele show full penetrance of deafness and partial penetrance of severe renal abnormalities (Xu et al., 2003; Zheng et al., 2003).

*Dach1* is expressed in the undifferentiated mouse mesenchyme as well as in early nephron segments (Ayres et al., 2001), but no renal abnormalities have been reported from either *Dach1* or -2-deficient mice (Davis et al., 1999; Davis et al., 2006). Three *Six* genes are expressed in the developing mouse kidney; *Six1* and -4 are first detected in the uninduced and then in induced kidney mesenchyme but get later restricted to the subset of cells in the collecting duct epithelium (Li et al., 2003; Xu et al., 2003; Kobayashi et al., 2007). *Six2* is exclusively expressed in the induced metanephric mesenchyme (Brodbeck et al., 2004; Self et al., 2006), where it co-localises with *Six1* and *Eya1* (Xu et al., 1999). *Eya2* is also detected in the metanephric mesenchyme (Kuure et al.,...
unpublished observation), but its function in renal differentiation remains to be elucidated.

Both Eya1- and Six1-deficient mice lack kidneys due to a failure of the ureteric budding and branching (Xu et al., 1999; Li et al., 2003; Xu et al., 2003). In Eya1-deficient mice, Pax2 expression is normal but Gdnf and Six1 are not detected in the mesenchyme. The expression of Pax2, Sall1 and Six2 is reduced in Six1-deficient kidneys, but quite surprisingly Gdnf remains normal. However, Six4 deletion in the Six1-deficient background (Kobayashi et al., 2007) deteriorates the renal phenotype and abolishes Gdnf and Pax2 expression from the double-mutant kidneys. Previously it was also shown that Six1 function is essential for normal mesonephric development (Kobayashi et al., 2007). Therefore it is possible that Six1 is required for the metanephric induction and differentiation. Thus, the lack of ureteric bud in Six1-deficient kidneys could rather be secondary than primary defect originating from the loss of mesenchymal induction. All in all, it is difficult to make conclusions on the function of these genes in regulation of ureteric branching morphogenesis based on above described genetic studies. Maybe some players in the kidney are still missing, since otherwise it is almost impossible to understand the regulatory relationships of Eya1 and Six in the protein complex controlling the down-stream gene expression.

Six2 expression depends on Six1 (Xu et al., 2003) but on the contrary to Six1- or Eay1-deficient phenotypes, Six2 is not necessary for ureteric budding and first round of branching. Instead, deletion of the gene results in an interesting renal phenotype with ectopic nephron formation accompanied by depletion of nephron progenitors (Self et al., 2006). Six2 appears to restrict the nephrogenic fate within the metanephric mesenchyme as the pretubular aggregates and the expression of genes related to epithelialisation process (Wnt4 and sFRP2) are abnormally located in Six2-deficient kidneys. Additionally, lack of Six2 does not affect proliferation of renal cells but rather increases apoptosis within the metanephric mesenchyme supporting the idea that nephron progenitors are exhausted in the kidneys lacking Six2. The reason for the perturbed ureteric branching, which eventually leads to renal hypoplasia in Six2-deficient mice, may derive from defective inductive interactions between the mesenchyme and ureteric epithelium. Another prediction is that when the whole cap condensate, which normally expresses Six2 and is therefore able to initiate Gdnf expression (Brodbeck et al., 2004), differentiates into pretubular aggregates, the condensate cells that are required for guiding the ureteric branching are depleted causing the arrest in ureteric branching. Loss
of Wnt11 at T-bud stage in Six2-deficient kidneys suggests defects in GDNF/Ret signalling although both transcripts are detected in the mutant kidneys.

1.3.5 Lim1 functions at several stages of renal differentiation

The homeobox gene Lim1 is expressed from IM to developing tubules in the metanephric kidney. The expression is detected in ureteric epithelium and in induced mesenchymal aggregates of early kidney as well as later in comma and S-shaped bodies (Fujii et al., 1994; Karavanov et al., 1998). Deletion of Lim1 in mouse leads to death at E10 because of abnormal gastrulation. However, few knockout animals develop further and are born without head, kidneys and gonads (Shawlot and Behringer, 1995). The cause of renal aplasia in Lim1-deficient mice originates from disorganisation in IM, which fails to differentiate into Wolffian duct (Tsang et al., 2000). Tissue-specific deletions and BAC complementation studies show that Lim1 is indeed required at several stages of renal differentiation (Tsang et al., 2000; Kobayashi et al., 2005). The BAC complementation results in a hypomorphic mouse model, which retains only traces of Lim1 expression and shows retarded development of ureteric buds as well as complete lack of nephrons or their precursor structures (Kobayashi et al., 2005). Deletion of the gene from Wolffian duct and its derivatives causes hypoplastic but functional kidneys with abnormalities in distribution of nephron precursors and in distal ureteric development. Ureteric budding is delayed and branching disturbed when Lim1 expression is deleted from the epithelium. Interestingly, Wnt11 remains in the tips of ureteric bud although Ret expression is not up-regulated in the Lim1-deficient ureteric epithelium arguing against previous data, which suggested that Ret-signalling is required for Wnt11 expression (Pepicelli et al., 1997). Lim1 may potentially regulate Ret expression in the ureteric tips but it is clearly also cell-autonomously required for normal ureteric budding and branching.

Metanephric mesenchyme –specific deletion of Lim1 causes arrest of nephrogenesis at pretubular aggregate stage without affecting the expression of Wnt4 (Kobayashi et al., 2005), which is required for further epithelialisation of nephrons (Stark et al., 1994). In the absence of Lim1 the expression of genes known to pattern the future nephron, Dll1 and Brn1 (Pou3f3) is lost. This data suggest that Lim1 specifies the nephron segments possibly by being a permissive (Kobayashi et al., 2005), rather than inductive signal for mesenchyme-to-epithelium transformation.
1.3.6 *Sall1* defines nephron progenitors within metanephric mesenchyme

*Sall* genes encode homologues of the *Drosophila* region-specific homeotic gene *spalt* (sal). In humans, heterozygous mutations in *SALL1* lead to Townes-Brocks syndrome, which is an autosomal dominant disorder characterised by multiple birth defects including renal, ear, anal and limb malformations (Nishinakamura and Osafune, 2006). In mouse, *Sall1* is expressed from E10.5 onwards in mesonephric tubules and distal part of the Wolffian duct, while in the definitive kidney it is detected in the metanephric mesenchyme. Later it is detected also in the comma-shaped bodies of developing nephrons (Nishinakamura et al., 2001). Other family members, *Sall2-4*, are largely co-expressed with *Sall1* in the developing kidney (Nishinakamura et al., 2001; Ott et al., 2001; Warren et al., 2007).

Deletion of *Sall1* in mouse causes failure of ureteric bud elongation and invasion into the metanephric mesenchyme leading either to complete lack of kidneys, unilateral renal aplasia or bilateral hypoplasia (Nishinakamura et al., 2001). *Sall1*-deficient mesenchyme is capable of nephron formation but the number of *Pax2*-expressing mesenchymal cells is diminished suggesting that Sall1 might regulate the size of mesenchymal progenitor cell population. Indeed, isolated Sall1-positive mesenchymal cells can reconstitute nephrons in vitro (Osafune et al., 2006). These cells exhibit some of the progenitor cell characteristics as they can produce colonies after single cell suspension and can give rise different nephron segments in the presence of *Wnt4*-expressing cells.

*Sall2* (Sato et al., 2003) and *Sall3* (Ott et al., 2001) mutant mice do not exhibit kidney defects, and *Sall1/Sall2* double mutants exhibit similar phenotype as deletion of *Sall1* alone (Nishinakamura et al., 2001). *Sall4*-deficient mice mimic the defects of Okihiro syndrome patients, who are characterised by radial ray defects and Duane anomaly and have also anal, renal, cardiac, ear, and foot malformations, as well as hearing loss (Sakaki-Yumoto et al., 2006; Warren et al., 2007). *Sall4/Sall1* heterozygote exhibit an increased incidence of kidney agenesis among other defects (Sakaki-Yumoto et al., 2006) showing that these two factors genetically interact.

1.3.7 *Emx2* may regulate the expression of genes encoding ureteric bud derived signals

*Emx2*, which is a homologous to *Drosophila empty spiracles*, encodes a homeobox transcription factor expressed in the ureter of the developing mouse kidney. Metanephros development in *Emx2* knockout mice is arrested soon after the ureter bud
formation but the expression of Wt1, Gdnf and Ret is not disrupted in the absence of Emx2. This suggests that Emx2 acts downstream of these genes and is not regulating the ureteric budding or branching. In the Emx2 mutant kidneys no tubules are formed and the signal required for the epithelialisation of the mesenchyme, Wnt4, is not up-regulated. Thus, since ureteric budding happens in the absence of Emx2, it is possible that Emx2 regulates ureteric derived signals required for further differentiation of kidneys.

1.3.8 Hox genes control ureteric budding via Six2 and Gdnf

The mammalian homeobox (Hox) complex is divided into four linkage (A-D) groups containing 13 sets of paralogous genes (33 in total), which are functionally highly redundant. Several Hox genes are expressed during early development of the nephrogenic area in mouse and their expression pattern has been described in detail (Patterson and Potter, 2004). For example, Hoxb7 is detected first in mesonephros and later in Wolffian duct, ureter and collecting ducts. The promoter of Hoxb7 gene (Kress et al., 1990; Vogels et al., 1993) has been widely used to express genes in the ureteric epithelium of developing mouse kidney (Srinivas et al., 1999a; Patterson et al., 2001; Shakya et al., 2005a). Hoxa11–c11 and –d11 are also expressed in the IM but unlike other regulators of kidney development in the area, these genes are detected only at the level of hind limb buds, specifically at the site where ureteric bud is formed. This has raised speculations that Hox-genes determine the site where kidneys are formed in the anterior-posterior axis of the developing embryo but genetic experiments do not fully support this. Double mutants of Hoxa11/Hoxd11 have renal defects that are not present in either of the single mutants. Hoxa11/Hoxd11-deficient animals have either no kidneys or show only one hypoplastic kidney with reduced number of glomeruli, which are however well developed. Only one wild type allele of either Hoxa11 or Hoxd11 is sufficient for normal kidney development (Srinivas et al., 1999b; Patterson et al., 2001). Removal of the last Hox11 paralogous member, Hoxc11, results in the complete loss of metanephric kidney induction. However, morphologically visible metanephric mesenchyme expressing Pax2 and Wt1 is formed in the triple mutants (Wellik et al., 2002) suggesting that at least Hox11 paralogue is not required for the positional guidance of kidney formation although a possibility for redundancy between nonparalogous genes remains (Patterson and Potter, 2003). Arrest of renal development to the early state by the loss of Hoxa11/Hoxd11/Hoxc11 was shown to be due to the
changes in the regulation of ureteric budding and branching. *Gdnf* expression, which is reduced already in *Hoxa11/Hoxd11* mutant kidneys, is either absent or greatly reduced in triple mutants, and also *Six2* expression appears to depend on the number of functional *Hox* alleles (Patterson et al., 2001). As *Six2* operates up-stream of GDNF and is able to bind *Gdnf* promoter to activate its expression (Brodbeck et al., 2004), it is likely that the reduced *Gdnf* levels in *Hox11* triple mutants are due to effects on *Six2* rather than directly on *Gdnf*.

### 1.3.9 Foxc1/Foxc2 suppress Gdnf expression

The *Fox* genes encode forkhead/winged helix transcription factors that share very high homology in the DNA binding domain. At least *Foxc1* (previously *Mf1*) and –2 (*Mfh1*) as well as *Foxd1* (Bf2) and –2 (*Mf2*) are expressed in the developing mouse kidney. Of these, *Foxc1/2* and *Foxd2* are detected in the IM and condensing metanephric mesenchyme with almost overlapping patterns (Kume et al., 2000a), while *Foxd1* localizes into the renal stroma (Hatini et al., 1996). *Foxd2* ablation in mouse causes renal hypoplasia and hydroureters at low frequency (Kume et al., 2000b). Relatively mild phenotype most probably reflects redundancy with *Foxc1* and -2 (Kume et al., 2000b). Deletion of *Foxc1* in mouse results in extra mesonephric tubule formation and duplex kidney, or double ureter formation (Kume et al., 2000a). These are likely due to abnormal *Gdnf* expression, which is anteriorly extended in the mutant embryos and induces spontaneous extra ureteric bud formation along the Wolffian duct. It is tempting to speculate that *Foxc1* provides positional information for the site where kidney will develop by regulating *Gdnf* expression via *Eya1*, which is also extended in *Foxc1*-mutant kidneys. More evidence is still required to prove this since it is equally possible that normally metanephric mesenchyme migrates caudally to its definitive position and in the mutant mice the migration is delayed or does not occur. To prove the latter alternative would require fate mapping of IM cells, which has not been carried out yet.

### 1.4 GDNF-activated Ret signalling

GDNF signals through Ret tyrosine kinase (TK) by first binding to membrane anchored glycosylphosphatidyl inositol (GPI) –co-receptor called GDNF-family receptor-α1 (GFRα1), which then brings two molecules of Ret together (Fig. 3). After formation of such a functional receptor complex, tyrosine residues in the TK domains of
Figure 3. Formation of functional GDNF-Ret receptor complex. GDNF brings two molecules of GFRα1 together, which triggers homodimerization of Ret (Sariola & Saarma, 2003).

The receptors are transphosphorylated, and this triggers the activation of context dependent intracellular pathways (reviewed in Sariola and Saarma, 2003; Arighi et al., 2005). The cellular responses to Ret activation include proliferation, cell survival, migration and cell fate determination.

The high-affinity binding of GDNF to Ret is mediated by GFRα1 (Jing et al., 1996; Treanor et al., 1996) suggesting that Ret is unable to bind GDNF on its own (Jing et al., 1996). Gdnf mutants deficient in GFRα1 binding can however activate Ret indicating that at least some Ret molecules can weakly associate with GFRα1 before GDNF binding (Treanor et al., 1996). Additionally, GFRα1 expression in several tissues does not co-localize (Suvanto et al., 1997; Trupp et al., 1997; Ylikoski et al., 1998; Kokaia et al., 1999) with Ret suggesting that GDNF can also signal Ret-independently. This pathway triggers Src-family kinase activation through GFRα1 and phosphorylation of MAP kinase, PLC-γ, CREB as well as induction of Fos in Ret-deficient cell lines and primary neurons (Poteryaev et al., 1999; Trupp et al., 1999).

**GDNF** is a neurotrophic factor, which belongs to GDNF family ligands (GFLs). The other members are artemin (ARTN), neurturin (NRTN) and persephin (PSPN). GFLs associate to the transforming growth factor-β (TGF-β) superfamily and typically for many secreted factors, are initially produced as precursors, preproGFLs. Upon secretion, the signal sequence is cleaved and the activation of the pro-form probably occurs by proteolytic cleavage (Arighi et al., 2005). GFLs function as homodimers and seem to bind side chains of extracellular-matrix (ECM) heparin sulfate proteoglycans (HSPG) (Hamilton et al., 2001). Without heparan sulphate, GDNF-induced receptor phosphorylation, axonal growth and scattering of epithelial cells do not occur, or occur
only at very high concentrations of GDNF (Barnett et al., 2002), suggesting that HSPGs, such as syndecans and glypicans, locally modulate GDNF signalling.

GDNF was originally purified as a growth factor maintaining embryonic midbrain dopaminergic neurons, which are non-functional in Parkinson's disease (Lin et al., 1993; Tomac et al., 1995). In addition, GDNF maintains and induces differentiation of certain peripheral neurons, including subpopulations of sympathetic, parasympathetic, sensory and enteric neurons. GDNF has important functions also outside the nervous system (Airaksinen et al., 1999; Sariola and Saarma, 2003) since it acts as a morphogen in regulating the differentiation of spermatogonia, and as discussed more in details later, promotes ureteric budding as well as branching during kidney development (de Graaff et al., 2001; Jain et al., 2006).

Ret is a classical oncogene, which encodes a transmembrane receptor of the TK family of proteins. Three isoforms of Ret proteins are generated by alternative 3’ splicing (Tahira et al., 1990; Myers et al., 1995). The long, intermediate, and short Ret isoforms, which differ by 51, 43, and 9 amino acids in the C-terminus, and are referred to as Ret51, Ret43, and Ret9, respectively. The two major isoforms, Ret51 and Ret9, are highly conserved (Carter et al., 2001) but recent evidence indicates that distinct isoforms potentially exert different physiological functions (reviewed in Arighi et al., 2005).

Ret protein (Fig. 3) is composed of three domains; four cadherin-like repeats form an extracellular ligand-binding domain, which is followed by a cysteine-rich region and hydrophobic transmembrane domain. The cytoplasmic part is split into two TK domains by an insertion of 27 amino acids between the kinase active domains. Mutations in these domains of Ret, or others that activate signalling, can cause thyroid cancer or multiple endocrine neoplasia type 2 (MEN2A or MEN2B) disorders. Loss-of-function mutations on the other hand cause colonic aganglionosis and Hirschprung’s disease (reviewed in Arighi et al., 2005). Like several other proto-oncogenes, Ret is active already during the development being expressed primarily on neural crest-derived cells and in developing renal system (Pachnis et al., 1993; Avantaggiato et al., 1994). Accordingly, Ret function is required for differentiation of kidneys and peripheral nervous system but it also regulates spermatogenesis (Durbee et al., 1996b; Schuchardt et al., 1996; Meng et al., 2000; Jain et al., 2004).
1.4.1 Role of GDNF signalling in kidney morphogenesis

The role of GDNF-activated Ret signalling in the development of kidneys is indispensable. Ret remained for long time an orphan receptor. Due to the expression patterns of Gdnf and Ret in the adjacent tissues of several organs (Hellmich et al., 1996; Suvanto et al., 1996), GDNF was a ligand candidate for the Ret well before the actual evidence was shown. In the kidney, Gdnf is expressed in the mesenchyme prior to the invasion of ureter bud, while Ret transcripts are first found throughout the Wolffian duct and later only in the tips of ureteric bud epithelium (Pachnis et al., 1993). Quite soon both biochemical and experimental data confirmed that GDNF binds to Ret and activates its phosphorylation and subsequent down-stream signalling (Durbec et al., 1996a; Trupp et al., 1996; Worby et al., 1996).

Disruption of the GDNF-activated Ret signalling perturbs kidney development already at the early state (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). Gdnf-, Ret- and GFRα1-deficient animals show abnormalities in the ureteric bud formation, which most frequently fails to grow out from the Wolffian duct. Also isoform specific deletions of Ret have been generated, but the results from these experiments are contradictory. The mice lacking Ret9 isoform exhibit the same phenotype as the Ret-null animals, and its expression under Hoxb7-promoter rescues the malformations in Ret-deficient mice (de Graaff et al., 2001). On the other hand, exclusive expression of either of the human isoforms, Ret9 or Ret51, is enough to support the normal development (Jain et al., 2006). The opposite phenotypes may arise from differences in the genetic backgrounds of the mice, or from the different properties of the human Ret51 and chimeric murine Ret51. However, the in vivo experiments together with the results obtained from in vitro studies, where the GDNF-releasing beads induce ureter budding from Wolffian duct (Pepicelli et al., 1997; Sainio et al., 1997), confirm that GDNF-activated Ret signalling is needed for the ureteric bud formation and branching. In approximately 30-50% of Ret-deficient animals a ureteric bud is formed and rudimentary kidney can be observed at birth (Schuchardt et al., 1994; Sanchez et al., 1996; Schuchardt et al., 1996). The same occurs also in Gdnf knockout mice but at somewhat lower frequency (unpublished data). The fact that sometimes ureteric budding and branching take place in mice lacking Gdnf/Ret/GFRα1 suggests that partially redundant signals can induce ureteric bud outgrowth. These signals are unknown, but candidates could include fibroblast growth factor (FGF)-, Notch- and Ephrin-signalling pathways. Also Met signalling may...
participate in the control of ureteric budding since GDNF partially restores branching morphogenesis in Ret-deficient kidney explants and this restoration occurs potentially through the Met pathway (Popsueva et al., 2003).

Additional insight into the function of GDNF/Ret signalling in the kidney was obtained from genetic studies in which ES cells homozygous for a Ret null mutation and simultaneously positive for the Hoxb7/GFP transgene, were injected into wild-type blastocysts to generate chimeric embryos (Shakya et al., 2005b). In the resulting genetically mosaic kidneys, the GFP marker was used to follow the ability of the Ret-deficient cells to participate in Wolffian duct and ureteric bud morphogenesis. Ret-deficient cells contributed extensively to the Wolffian duct and to the trunk of the ureteric bud, but they were specifically excluded from the tips of the ureteric bud. This indicates that Ret is needed in the ureteric bud cells to contribute to the tip domain, and suggests a critical function for Ret signalling in establishing the ureteric bud tip identity. Taken together, this study and the conventional knockout data (see previous chapter, reviewed in Costantini and Shakya, 2006) indicates that tip identity generated by active Ret signalling, which up-regulates Wnt11 (Kispert et al., 1996; Majumdar et al., 2003 and see chapter 1.5.1), is essential for normal ureteric branching morphogenesis.

In vitro experiments with GDNF releasing beads (Pepicelli et al., 1997) demonstrate that ureteric bud cells respond to GDNF by increasing proliferation (Sainio et al., 1997; Michael and Davies, 2004) but suggest also that GDNF might act as a chemo attractant during ureteric budding, similarly to its role in migrating enteric neural crest cells (Young et al., 2001; Natarajan et al., 2002). This is not, however, supported by the studies where Gdnf is ectopically expressed in the Wolffian duct and ureteric epithelium (Shakya et al., 2005a). In such transgenic mice, like in those expressing constitutively active Ret under the same promoter (Srinivas et al., 1999b), normal ureteric budding still occurs. More importantly, also spontaneous extra budding is observed showing that local expression of Gdnf/Ret is not required for ureteric bud formation. These experiments suggest either existence of unidentified Ret signalling inhibitors, or that other, potentially suppressive signals determine the site and orientation of the ureteric bud. Such regulators related to negative control of Gdnf/Ret expression are at least Foxc1, Slit2/Robo2 signalling and Sprouty1 (Fig. 4).
In the absence of these regulators, spontaneous extra ureteric budding occurs due to either expanded Gdnf expression domain or interference with intracellular Ret signalling as in Sprouty1-deficient mice (Chi et al., 2004). Bmp4 and Gremlin act down-stream, or in parallel with Ret signalling to negatively fine tune the ureteric budding site. Bmp4 heterozygote embryos exhibit duplicated ureter (Miyazaki et al., 2000) and in organ culture, Bmp4 inhibits GDNF-induced ureteric bud formation (Brophy et al., 2001). In Gremlin1-deficient embryos, ureteric bud outgrowth is completely blocked, and it was therefore postulated that Gremlin normally blocks the inhibitory effect of Bmp4 on ureteric budding (Michos et al., 2004).

As discussed in Chapter 1.2, several transcription factors positively regulate the expression of GDNF/Ret pathway molecules (Fig. 4). Pax2 and Six2 can activate the expression of Gdnf (Brophy et al., 2001; Brodbeck et al., 2004), while the former also regulates Ret (Clarke et al., 2006). Eya1 (Li et al., 2003) and Hox11 paralogues (Patterson and Potter, 2003), potentially via Six2, are required for Gdnf expression. Sall genes may also contribute to the control of Gdnf expression (Nishinakamura et al., 2001).

Very little is known about the targets of GDNF-activated Ret signalling in the developing kidney. Intracellularly, Ret activation in the ureteric epithelium cells can be mediated at least via PI3K-Akt, Ras-Erk MAP kinase pathways (Fisher et al., 2001; Tang et al., 2002; Watanabe and Costantini, 2004) and phospholipase C (PLC) gamma pathway (Jain et al., 2006). Currently we are only about to start to reveal the target genes, which are activated upon GDNF/Ret signalling.

1.5 Notch signalling

The notching wing phenotype associated with the haploinsufficiency of the Notch locus was one of the first genetic variations observed in Drosophila melanogaster (reviewed in Wright, 1970). Later it led to the cloning and characterization of Notch gene (Artavanis-Tsakonas et al., 1983) and over the years, Notch signalling has been shown to function in an enormous diversity of developmental processes and its dysfunction is implicated in many cancers. Notch signalling has been linked to the cell fate decision, especially in the nervous system, but it also guides patterning and formation of other organs including thymus, haematopoietic system, heart, pancreas, tooth and kidney in both invertebrates and vertebrates.
The Notch receptor and its ligands, Delta and Serrate (known as Jagged in mammals) are both single-pass transmembrane proteins with large extracellular domains that consist primarily of epidermal growth factor (EGF)-like repeats. There are four Notch receptors (Notch1-4), four Delta (Dll1-4) and two Jagged (Jag1-2) ligands in mammals (Table 1). Ligand binding to Notch receptor activates two types of proteolytic cleavages. The first cleavage is catalyzed by ADAM-family metalloproteases and prepares the receptor for the second cleavage, which is mediated by presenilin-\(\gamma\)-secretase complex. This second cleavage releases the Notch intracellular domain (NICD), which then translocates into the nucleus. The translocation events are poorly understood and there are no known cellular parameters that could influence them (Louvi and Artavanis-Tsakonas, 2006). In the nucleus, the NICD fragment interacts with members of the CSL (CBF1, Suppressor of Hairless, Lag-1) family of transcription factors. This interaction converts the CSL proteins from transcriptional repressors into transcriptional activators and thereby stimulates the expression of bHLH repressors, namely Hairy and Enhancer of the Split (\textit{Hey} and \textit{Hes} genes in mammals) (Fig. 5).

<table>
<thead>
<tr>
<th>Component type</th>
<th>\textit{Drosophila melanogaster}</th>
<th>Vertebrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor</td>
<td>Notch</td>
<td>Notch1-4</td>
</tr>
<tr>
<td>Ligand</td>
<td>Delta, Serrate</td>
<td>Delta1-4, Serrate, Jagged1-2</td>
</tr>
<tr>
<td>CSL DNA-binding protein</td>
<td>SuH</td>
<td>CBF1/RBPkJ</td>
</tr>
<tr>
<td>Co-activator</td>
<td>Mastermind</td>
<td>Mastermind1-3</td>
</tr>
<tr>
<td>Co-repressor</td>
<td>Hairless</td>
<td></td>
</tr>
<tr>
<td>(\gamma)-secretase complex</td>
<td>Presenilin, nicastrin,</td>
<td>Presenilin1-2, nicastrin,</td>
</tr>
<tr>
<td></td>
<td>APH1, PEN2</td>
<td>AHP1, PEN2</td>
</tr>
<tr>
<td>Glycosyl transferase</td>
<td>Fringe</td>
<td>Lunatic fringe, radical fringe,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Manic fringe</td>
</tr>
<tr>
<td>Metalloprotease, receptor</td>
<td>Kuzbian, kuzbian-like</td>
<td>ADAM10, TACE/ADAM17</td>
</tr>
<tr>
<td>Cleavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic inhibitor</td>
<td>Numb</td>
<td>Numb, Numb-like</td>
</tr>
<tr>
<td>Positive regulator</td>
<td>Sanpodo</td>
<td></td>
</tr>
<tr>
<td>bHLH repressor, target genes</td>
<td>E(spl)bHLH</td>
<td>HES/ESR/HEY</td>
</tr>
</tbody>
</table>
Figure 5. Notch signalling. Binding of Ser/Dll ligand to Notch receptor results in cleavage of the receptor, which translocates to the nucleus and activates the expression of bHLH type of repressors (Artavanis-Tsakonas et al., 1995).

Notch receptors, and thereby the signalling, are extracellularly modulated by Numb, Numb-like and Fringe (Lunatic, Radical and Manic) proteins (Table 1). CSL-independent signalling has been identified in Drosophila (Rusconi and Corbin, 1998; Zecchini et al., 1999) and potentially also in mammals (Lindsell et al., 1995; Shawber et al., 1996; Eiraku et al., 2005) but the possible other mediator(s) of this pathway are not known. Therefore, it is unclear whether the CSL-independent activity of Notch is mediated by a ligand binding or is an intrinsic property of Notch, or whether it also requires cleavage of the Notch protein (Martinez Arias et al., 2002).

The most familiar outcome of Notch signalling is fate determination of cells within an equivalence domain, in which all cells are originally able to adopt at least two different fates. In such a case, Notch functions via ‘lateral inhibition’, which is a process to limit the number of cells adopting a certain fate. Prominent examples of lateral inhibition include formation of neuroblasts in fruit flies (reviewed in Skeath and Thor, 2003) and development of sensory hair cells in the inner ear of vertebrates (reviewed in Riley and Phillips, 2003); both processes involve high levels of Delta expression. Here, the Delta-expressing cell adopts the primary fate, and by activating Notch signalling in the next cell, inhibits the surrounding cells from taking the same fate. In contrast to the mosaic pattern of cell fates established by lateral inhibition, Notch signalling can also generate cell domains with the same fate by inducing differentiation towards certain lineage, or by inhibiting from acquiring another. This ‘lateral induction’ process
(reviewed in Lewis, 1998) defines proneural domains in the ear and the eye, and is involved in the sharpening of somite boundaries as well as in the formation of the limb bud margin (reviewed in Irvine and Vogt, 1997).

1.5.1 Notch signalling in the nephron formation

Several expression studies show that Notch signalling molecules are expressed throughout the nephrogenesis (Leimeister et al., 2003; Piscione et al., 2004; Sharma et al., 2004; Chen and Al-Awqati, 2005). The expression of Notch pathway molecules in kidney, like in other organs, appears to be very dynamic, which may explain some discrepancies observed between the reported patterns. All four receptors (Notch1–4), the four transmembrane ligands (Delta1, -4, Jag1 and -2) as well as intracellular effectors (the Hey1, -L and -2 and Hes1 and -5 genes) and extracellular modulators (Lfng, Mfng, Rfng) are detected in partially overlapping pattern in the epithelialising nephron structures. In addition, Notch3, -4 and Delta4 are expressed also in the developing vasculature of the kidney. Due to the obvious overlapping patterns, it is difficult to discern ligand-receptor specificity based on their expression. Expression patterns however suggest redundancy at least between the ligands. Although several transgenic mouse models (Ohtsuka et al., 2006; Ong et al., 2006; Souilhol et al., 2006; Vooijs et al., 2007) have been generated to visualize the Notch activation in situ, they do not help in elucidating the activity in the developing kidney. Only Hes1- and Hes5-promoters driving the expression of GFP (Ohtsuka et al., 2006) show activity in early nephron structures, but the detailed analysis is missing. Additionally, it is reasonable to believe that the reporter mice do not fully recapitulate all signalling activity due to the complex nature of the pathway. For example Notch1 function does not correlate with the probability of its activation: the expression pattern of Notch1 specific NICD is much wider (Vooijs et al., 2007) than the spectrum of affected organs in Notch1-deficient animals (Kiernan et al., 2001). This is in line with the findings in kidney, where NICD is detected in the developing nephrons (Cheng et al., 2003; Wang et al., 2003), but tissue-specific deletion of Notch1 does not affect renal differentiation (Cheng et al., 2007).

It has been shown that development is sensitive to the dosage of Notch activity (McCright et al., 2001; Duarte et al., 2004; Krebs et al., 2004). Hypomorphic Notch2 mice survive beyond the initiation of organogenesis (McCright et al., 2001) unlike the null allele animals (Hamada et al., 1999), and demonstrate the importance of Notch
signalling in kidney development. The hypomorphic allele results in reduced Notch2 signalling, which causes renal hypoplasia and disrupts nephron patterning as well as glomerular development. Mesenchymal condensation and pretubular aggregation are normal in these mutant animals but the number of differentiating glomeruli is substantially reduced (McCright et al., 2001) suggesting that functional signalling is required also for the normal progress of ureteric branching. Inhibition of the proteolytic cleavage of Notch disturbs the earlier steps of glomerulogenesis since no comma- or S-shape bodies develop in mice double mutant for presenilin1 and -2 (Wang et al., 2003). Similar although milder effect is observed when presenilins are chemically inhibited in kidney cultures, in which fewer renal epithelial structures are observed, with a severe deficiency in the development of proximal tubules and glomerular podocytes (Cheng et al., 2003). Although the above described experiments suggest involvement of Notch signalling in the guidance of ureteric branching morphogenesis, the specific function there has not been determined. Recently, conditional Pax3Cre-driven deletion of Notch2 from the metanephric mesenchyme (Cheng et al., 2007) confirmed the previous findings (McCright et al., 2001) showing that signalling through Notch2 is required for the differentiation of proximal nephron structures. It would be interesting to know whether the epithelial specific deletion, for example with Hoxb7Cre, results in phenotype, which would reveal a role for Notch signalling in ureteric bud outgrowth or branching.

The phenotype of the compound heterozygote for Jagl and Notch2 indicate that Jagged1 is the ligand for Notch2 during kidney development. No renal phenotypes have been reported from other Notch ligands expressed in the kidney; either due to the embryonic lethality prior to the onset of organogenesis (Hrabe de Angelis et al., 1997) or due to obviously normal renal differentiation (Jiang et al., 1998; Gale et al., 2004; Hellstrom et al., 2007). In human, mutations in JAG1 gene cause an autosomal dominant Alagille syndrome with developmental abnormalities in several organs including the kidney (Li et al., 1997). An extensive survey of mutations of JAG1 in Alagille patients suggests that haploinsufficiency for the JAG1 gene is the primary cause of the syndrome (Spinner et al., 2001). Heterozygous Jagl-deficient mice exhibit only the eye defects characteristic of Alagille patients, whereas Jagl/Notch2 compound heterozygotes show all phenotypes of the syndrome including defects in nephron differentiation and maturation (McCright et al., 2001; McCright et al., 2002). Homozygous Jagl-deficient mice die prior to the initiation of kidney development due to vascular defects (Xue et al., 1999). In addition, two different Jagl mutations have
been created by ENU mutagenesis. Headturner mice contain a missense mutation in the Jag1 (Tsai et al., 2001) and the homozygote embryos exhibit similar phenotypes as Jag1-null animals including growth retardation, vasculature defects and the neural tube defects similar to Notch1 mutant mice (Kiernan et al., 2001). Slalom homozygote mice survive one day later (up to E12.5) and demonstrate similar although slightly milder phenotype (Tsai et al., 2001) as the two other mouse models. In conclusion, none of these genetically modified mice help in elucidating the function of Jag1 in kidney morphogenesis and therefore there is a great need for conditional deletion (Brooker et al., 2006; Kiernan et al., 2006; Weller et al., 2006) of the gene in the kidney.

1.6 Wnt signalling

The mouse mammary proto-oncogene Int-1 was identified as a homologue (Rijsewijk et al., 1987) of Drosophila segment polarity gene Wingless (Nusslein-Volhard and Wieschaus, 1980), which was named after the phenotype caused by its mutations in flies (Sharma and Chopra, 1976). Members of this large family of related glycoproteins have been renamed Wnts, an amalgam of int and wingless (reviewed in Siegfried and Perrimon, 1994). The Wnt signalling plays multiple roles during normal development and aetiology of diseases (reviewed in Wodarz and Nusse, 1998; Schmidt and Patel, 2005; Brade et al., 2006; Clevers, 2006; de Iongh et al., 2006). Wnts are thought to act as a morphogens, so that they are long-range signals whose activity is concentration dependent (Logan and Nusse, 2004), but it is unclear how these gradients are generated and affect the target cells. One possibility is that extracellular HSPGs function in the transport or stabilisation of Wnt proteins. For instance, flies carrying mutations in Dally, which encodes a GPI-anchored HSPG, or in genes encoding enzymes that modify HSPGs, resemble wingless mutants (Lin, 2004). It has also been suggested that Wnt proteins act as a short-range signalling substance, which function in a contact-dependent manner. This mode of action has been shown at least for Wnt7b in the regression of hyaloids vasculature of the eye (Lobov et al., 2005).

In vertebrates, the Wnt ligand family consists of at least 17 members, which bind seven-pass transmembrane Frizzled (Fz) receptors. At least four distinct intracellular pathways can be activated by Wnt binding to its receptors, but ligands themselves do not distinguish the pathway that will be activated. For example the best characterised pathway, the canonical (β-catenin-dependent) pathway is activated by a complex formation between a lipoprotein receptor-related (LRP) co-receptor, Fz and Wnt. This
activates by an unknown mechanism dishevelled (Dsh), which inhibits glycogen synthase kinase-3β (GSK3β). Consequently β-catenin gets stabilised in the cytosol and is subsequently translocated into the nucleus where it interacts with Lef/Tcf transcription factors and regulates their expression among other target genes (Fig. 6) (Clevers, 2006).

Inhibition of GSK3β is a key event in the activation of canonical pathway. According its name, GSK3 was originally identified as enzyme of glycogen metabolism but later its function in canonical Wnt pathway was established. It appears that the GSK3β, which is involved in the control of β-catenin is “insulated” from other signalling pathways (Patel et al., 2004). Partially this may be a consequence of its subcellular localisation and complex formation with adenomatous polyposis coli (APC) and axin. In addition to β-catenin, axin and APC are also GSK3 substrates. Phosphorylation of axin by GSK3 increases its stabilisation and affinity to β-catenin (Jho et al., 1999; Yamamoto et al., 1999; Willert et al., 1999) and therefore it has been suggested that stabilisation of axin rather than β-catenin itself is actually the rate limiting step leading to the activation of canonical pathway (Tolwinski and Wieschaus, 2004).

The signalling events of the non-canonical Wnt pathways are relatively poorly understood. The three non-canonical pathways involve activation of calcium/calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC) in a Wnt/calcium pathway, and direct activation of phospholipase C (PLC) and phosphodiesterase (PDE) by Frizzled-receptors through heterotrimeric GTP-binding proteins. Lastly, the planar cell polarity (PCP) pathway activates the Jun-N-terminal kinase (JNK) and, perhaps, small GTP-binding proteins. Interestingly, calcium has been

Figure 6. Activation of canonical Wnt pathway. (A) In the absence of Wnt activity, β-catenin is targeted to degradation by GSK3 phosphorylation. (B) Wnt binding to Fz-receptor brings it together with LRP5/6, which inactivates GSK by phosphorylation. This allows cytosolic β-catenin stabilization resulting in its translocation to nucleus where it interacts with TCF transcription factors.
implicated as an important second messenger in all of these pathways. This, together with the fact that for example PKC, PLC and JNK also participate in the signal transduction downstream of other signalling pathways, makes dissecting the mechanisms of non-canonical pathway very difficult. However, non-canonical Wnt pathways control several aspects of the vertebrate development including gastrulation movements, cochlear hair cell and heart induction, and neuronal migration (reviewed in Veeman et al., 2003).

The Wnt-Fz interaction appears loose, so that a single Wnt can bind multiple Fz proteins and vice versa. In canonical pathway, binding of Wnt ligand requires Fzs to cooperate with a single-pass transmembrane molecule of the LRP family known as Arrow in Drosophila (Wehrli et al., 2000) and LRP5 and -6 in vertebrates (Pinson et al., 2000; Tamai et al., 2000). Although it has not been formally demonstrated that Wnt molecules form trimeric complexes with LRP5/6 and Fz, surface expression of both receptors is required to activate the canonical pathway. Wnt proteins can bind also to an unusual receptor called Derailed, which belongs to a RYK subfamily type of transmembrane tyrosine kinase receptors. This alone is able to activate the canonical pathway alone in Drosophila (Yoshikawa et al., 2003) and together with Fzs in mammals (Lu et al., 2004). Additionally, at least two non-Wnt-related proteins, Norrin (Xu et al., 2004) and R-spondins (Nam et al., 2006), can activate the Fz/LRP receptors.

Wnt signalling can be inhibited by different ways but the mechanisms are still poorly understood. The secreted Dickkopf (Dkk) proteins directly bind to LRP5/6 (Glinka et al., 1998) and by this way crosslink LRP6 to another class of transmembrane molecules, the Kremens (Mao et al., 2002), thus promoting the internalisation and thereby inactivation of LRP6. Another type of secreted Wnt inhibitor Wise and its family member SOST, also act by binding to LRP (Itasaki et al., 2003; Li et al., 2005; Semenov et al., 2005). Yet other inhibitors of Wnt signalling include soluble Frizzled-Related Proteins (sFRPs), which resemble the ligand-binding domain of the Fz family of Wnt receptors (Hoang et al., 1996), and WIF proteins, which are secreted molecules with similar extracellular portion than Derailed/RYK class of transmembrane Wnt receptors (Hsieh et al., 1999). Depending on context, sFRPs and Wise may also promote signalling by stabilizing the Wnts or by facilitating the secretion or transport of the proteins. It should be taken into the consideration that for example Wise is also known as a Bmp inhibitor called ectodin (Kusu et al., 2003; Laurikkala et al., 2003; Kassai et al., 2005) and thus this extracellular modulator is not apparently receptor specific.
1.6.1 Wnt signalling in nephron induction

Several Wnt ligands are expressed in the developing kidney, but the receptors mediating these signals are not known. Wnt4 is so far the only family member, which is detected in the mesenchymal compartment of the developing kidney being expressed in the condensing mesenchyme and pretubular aggregates (Stark et al., 1994). In the absence of Wnt4 metanephric mesenchyme fails to form pretubular aggregates and nephron differentiation does not occur (Stark et al., 1994; Saulnier et al., 2002) or is greatly reduced (Kobayashi et al., 2005). Wnt4 has been suggested to act as an autoinducer of the mesenchyme-to-epithelial transition during nephron differentiation. Accordingly, Wnt4- but also non-mesenchymal Wnt1-, Wnt3a-, Wnt7a-, Wnt7b- (Kispert et al., 1998) and Wnt6- (Itaranta et al., 2002) expressing cells trigger tubulogenesis in isolated metanephric mesenchymes confirming the inductive potential of Wnts. Currently it is not fully understood what signals prior to Wnts are required in the metanephric mesenchyme for nephrogenic competence.

In mouse, Wnt11, Wnt9b, Wnt7b and Wnt6 genes are expressed in the ureteric bud epithelium in an almost overlapping pattern (Kispert et al., 1996; Itaranta et al., 2002; Carroll et al., 2005), and Wnt9b appears to be an early inductive signal required for triggering the nephron differentiation. Deletion of Wnt9b arrests renal development at the T-bud stage and no mesonephric or metanephric tubules are formed (Carrol et al., 2005). The Wolffian duct development appears normally in Wnt9b-deficient mice. Arrest of ureteric branching is accompanied by reduced Gdnf expression but no changes in epithelial expression of Pax8 or Lim1 are detected. Metanephric mesenchyme differentiation does not proceed beyond the mesenchymal condensation step in Wnt9b-deficient mice since the mesenchyme fails to express Wnt4, Pax8 and Lim1. This shows that Wnt9b acts up-stream of Wnt4, which is itself competent to activate the entire tubulogenic program without Wnt9b. Endogenous Wnt4 is, however, not able to rescue tubulogenesis in Lim1-deficient kidneys (Kobayashi et al., 2005). Normal Lim1 expression is essential for Wnt9b expression (Pedersen et al., 2005) as shown by deletion of Lim1 with Pax2-Cre, which results in reduced and patchy expression of Wnt9b. Even though Wnt signals are crucial for the induction of nephrons it appears that Lim1 acts prior to their function and therefore it would be interesting to know, if Lim1-deficient mesenchyme is capable of undergoing nephrogenesis when induced for example by Wnt-expressing spinal cord.
Transgenic experiments where Wnt9b is substituted with classical canonical ligand, Wnt1, suggest that Wnt9b can activate canonical pathway (Carroll et al., 2005), but whether it does so during nephron induction depends on the receptors that it binds to, and these are currently unknown. In renal cell line (MDCK) also Wnt4 has been shown to signal through canonical pathway (Lyons et al., 2004). Additionally, β-catenin signalling is activated in tubular epithelial and interstitial cells after experimentally induced renal injury (Surendran et al., 2005) but its overactivation in the epithelial cells of kidney causes cyst formation (Saadi-Kheddouchi et al., 2001). All these experiments suggest that canonical pathway may participate in renal differentiation. However, Wnt4-induced colony forming capacity appears to depend on PCP, which regulates renal progenitor cell population size in isolated mesenchymes (Osafune et al., 2006). Based on the current data, the conclusive evidence indicating role for any of the intracellular Wnt pathways is still missing.

1.6.2 Wnt signalling in branching morphogenesis

β-catenin-activated transgene-galactosidase (BAT-gal) reporter mouse line is widely used tool to visualise the sites of active canonical pathway in situ (Maretto et al., 2003). In the kidney it has revealed canonical activity exclusively in the Wolffian duct and ureteric bud epithelium. Similar pattern was seen with GFP-reporter mice (Moriyama et al., 2007) but was undetectable in yet another reporter line (DasGupta and Fuchs, 1999). The lack of renal phenotype in Wnt6 (personal communication with S. Vainio), Wnt7a and –b deficient mice (Parr and McMahon, 1995; Yang and Niswander, 1995; Lobov et al., 2005) imply redundancy between these genes but that remains to be shown. The only genetic evidence for participation of Wnt signalling in the control of ureteric bud morphogenesis comes from Wnt11-deficient mice where ureteric branching is slightly reduced and consequently kidneys are hypoplastic in newborn mice (Majumdar et al., 2003). It has been suggested that Wnt11 functions by maintaining normal expression levels of Gdnf (Majumdar et al., 2003), which in turn triggers Ret-signalling to up-regulate Wnt11 (Kispert et al., 1996; Majumdar et al., 2003) indicating interaction for these pathways in the control of branching morphogenesis. Also Pax2/Pax8 appears to control Wnt11 expression, which is significantly reduced in the compound heterozygote kidneys, while Ret/Gdnf expression remains normal (Narlis et al., 2007). This suggests that Pax2/Pax8 controls Wnt11 expression either parallel or downstream of Ret signalling.
2. AIMS OF THE STUDY

Kidney development has been a subject of intensive research for decades but only recently we have started to understand how different signalling cascades regulate activation or repression of transcription factors, and how various pathways cross-talk with each other in the guidance of renal differentiation. The experiments in the present study have elucidated the function and regulatory relationships of two developmentally important signalling pathways, Notch and Wnt, in regards to GDNF-activated Ret signalling. My specific aims were to:

1. examine the possible role of Notch signalling in early kidney development by overexpressing Jag1 in Wolffian duct and its derivatives
2. study the potential function of the canonical Wnt pathway in ureteric morphogenesis
3. analyse the inductive capability of canonical Wnt pathway in of two rodent species
3. MATERIALS AND METHODS

The methods used are described in details in the “Materials and Methods” section of the respective publications. Experimental procedure using laboratory animals were performed according to ethical guidelines by local authorities. Here, table 2. summarises the in situ hybridization probes, table 3. shows the experimental methods and table 4. indicates the mouse strains used in this thesis.

Table 2. The probes used for in situ hybridization

<table>
<thead>
<tr>
<th>Probe</th>
<th>Reference</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg2</td>
<td>IMAGE-clone 5254031</td>
<td>II</td>
</tr>
<tr>
<td>Bambi</td>
<td>(Grotewold et al., 2001)</td>
<td>I</td>
</tr>
<tr>
<td>Bmp4</td>
<td>(Raatikainen-Ahokas et al., 2000)</td>
<td>I, II</td>
</tr>
<tr>
<td>Eya1 and -2</td>
<td>(Xu et al., 1997)</td>
<td>I, unpubl.</td>
</tr>
<tr>
<td>Erm</td>
<td>(Blak et al., 2007)</td>
<td>II</td>
</tr>
<tr>
<td>Gdnf</td>
<td>a gift from Dr JG Pichel</td>
<td>I, II</td>
</tr>
<tr>
<td>GFRα1</td>
<td>(Suvanto et al., 1997)</td>
<td>I, III</td>
</tr>
<tr>
<td>Hes1</td>
<td>A gift from Dr R. Kagayama</td>
<td>I</td>
</tr>
<tr>
<td>Hes6</td>
<td>(Vasiliauskas and Stern, 2000)</td>
<td>I</td>
</tr>
<tr>
<td>Hey1 and -2</td>
<td>(Leimeister et al., 2003)</td>
<td>I</td>
</tr>
<tr>
<td>HeyL</td>
<td>(Leimeister et al., 2000)</td>
<td>I</td>
</tr>
<tr>
<td>hJag1</td>
<td>1.1 kb fragment of the 5’end of the hJagged1 gene</td>
<td>I</td>
</tr>
<tr>
<td>Jag1</td>
<td>(Mitsiadis et al., 1997)</td>
<td>I</td>
</tr>
<tr>
<td>Lef1</td>
<td>(Travis et al., 1991)</td>
<td>II, III</td>
</tr>
<tr>
<td>Lim1</td>
<td>(Kobayashi et al., 2005)</td>
<td>II, III</td>
</tr>
<tr>
<td>Numb</td>
<td>(Zhong et al., 1997)</td>
<td>I</td>
</tr>
<tr>
<td>Pax2</td>
<td>(Dressler et al., 1990)</td>
<td>I, III</td>
</tr>
<tr>
<td>Pax8</td>
<td>1.43 kb cDNA</td>
<td>III</td>
</tr>
<tr>
<td>Pea3</td>
<td>(Lin et al., 1998)</td>
<td>II, III</td>
</tr>
<tr>
<td>Six2</td>
<td>a gift from Dr RL Maas</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Tcf1</td>
<td>(James et al., 2006)</td>
<td>II, III</td>
</tr>
<tr>
<td>Ret</td>
<td>(Pachnis et al., 1993)</td>
<td>I, II</td>
</tr>
<tr>
<td>Vsnl1</td>
<td>IMAGE-clone 5687661</td>
<td>II</td>
</tr>
<tr>
<td>Wnt4</td>
<td>(Stark et al., 1994)</td>
<td>III</td>
</tr>
<tr>
<td>Wnt11</td>
<td>(Kispert et al., 1996)</td>
<td>II</td>
</tr>
<tr>
<td>Wt1</td>
<td>(Kreidberg et al., 1993)</td>
<td>I, II, III</td>
</tr>
</tbody>
</table>
### Table 3. Methods used and described in the articles I-III.

<table>
<thead>
<tr>
<th>Method</th>
<th>Described in</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunohistochemistry</td>
<td>I, III</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Generation of new transgenic mouse lines</td>
<td>I</td>
<td>I, III</td>
</tr>
<tr>
<td>Cloning of transgenic constructs</td>
<td>I, III</td>
<td>I, III</td>
</tr>
<tr>
<td>Mice breeding</td>
<td>I, II, III</td>
<td>I, II, III</td>
</tr>
<tr>
<td>PCR genotyping</td>
<td>I, II, III</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Quantitative Real-Time PCR</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Histological analysis</td>
<td>I, II</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Organ culture</td>
<td>I</td>
<td>I, II, III</td>
</tr>
<tr>
<td><em>In situ</em> hybridization</td>
<td>I</td>
<td>I, II, III</td>
</tr>
<tr>
<td>LacZ staining</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Western blotting</td>
<td>II, III</td>
<td>II, III</td>
</tr>
</tbody>
</table>

### Table 4. Mouse strains used in thesis studies

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Generated</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hoxb7-Jag1</em></td>
<td>hJag1 cDNA was subcloned into <em>Hoxb7</em>-promoter vector containing splice sites and polyA signal</td>
<td>I</td>
</tr>
<tr>
<td><em>Hoxb7-Cre-GFP</em></td>
<td>(Zhao et al., 2004)</td>
<td>II</td>
</tr>
<tr>
<td><em>Gdnf</em> knockout</td>
<td>(Pichel et al., 1996)</td>
<td>III, unpublished</td>
</tr>
<tr>
<td><em>GATA3</em> knockout</td>
<td>(Hendriks et al., 1999)</td>
<td>III</td>
</tr>
<tr>
<td><em>Ret</em> knockout</td>
<td>(Schuchardt et al., 1994)</td>
<td>II, unpublished</td>
</tr>
<tr>
<td><em>Six2-Cre</em></td>
<td><em>Six2</em>-promoter cDNA was subcloned into pBluescript and followed by Cre recombinase cDNA and polyA signal</td>
<td>III</td>
</tr>
<tr>
<td><em>β-catenin</em>&lt;sup&gt;xon3&lt;/sup&gt;</td>
<td>(Harada et al., 1999)</td>
<td>II, III</td>
</tr>
<tr>
<td><em>β-catenin</em>&lt;sup&gt;xon2.6&lt;/sup&gt;</td>
<td></td>
<td>II</td>
</tr>
<tr>
<td>NMRI</td>
<td></td>
<td>I, II, III</td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td>I, III</td>
</tr>
</tbody>
</table>
4. RESULTS AND DISCUSSION

First, the experiments and results studying the function of Jag1-activated Notch signalling (4.1-4.3) and canonical Wnt pathway (4.4-4.8) in the control of ureteric branching morphogenesis will be described. Nephron induction studies will be discussed lastly in the chapters 4.9-4.11.

4.1 Overexpression of Jag1 in Wolffian duct derivatives activates Notch signalling and causes renal aplasia or hypodysplasia (I)

The analysis of potential Notch functions in early renal differentiation was started by studying the expression of Notch pathway genes in developing kidney. Prior to the ureteric budding, high Jag1 expression was detected in the mouse mesonephric tubules, and also in the cranial Wolffian duct (unpublished data). Slightly later Jag1 was expressed both in the Wolffian duct and ureteric bud (I, Fig. 1A). The expression was thereafter confined to the tip of the ureteric bud being simultaneously up-regulated in the metanephric mesenchyme (I, Fig. 1B), where it was later detected in early epithelialising nephrons (data not shown and Leimeister et al., 2003). Interestingly, at T-bud stage Jag1 was highly expressed in the peritubular mesenchyme around the Wolffian duct, but was absent from the close proximity of the ureteric stalk, leaving a gap in the Jag1 expression exactly at the site where Bmp4 is normally detected. The dynamic expression pattern suggested that Jag1 may participate in the control of kidney induction at several stages. A possible function of Jag1 in the primary ureteric budding was supported by the finding that the gene expression was up-regulated in the supernumerary ureteric buds, which were induced by exogenous GDNF (I, Fig. 1C-D). However, GDNF-activated Ret signalling is not required for the expression of Notch pathway genes since Jag1 as well as Notch down-stream target Hes1 were both expressed in E11 Ret-deficient Wolffian duct and ureteric bud epithelium (unpublished data).

Deletion of Jag1, Dll1 or Notch1-2 in mouse causes embryonic lethality prior to the onset of organogenesis (Hrabe de Angelis et al., 1997; Jiang et al., 1998; Xue et al., 1999; Gale et al., 2004; Hellstrom et al., 2007). In order to study Jag1-activated Notch signalling in the kidney development transgenic mice expressing human Jag1 in Wolffian duct and its derivatives were generated with the help of Hoxb7-promoter (Kress et al., 1990; Vogels et al., 1993). Transgenic mice were born but ~46% of newborns died within 24 - 48h of birth. Macroscopic examination and histology of the
Transgenic animals revealed a range of renal malformations, which included mild hypoplasia, hydroureters, hydropelvises, tubular cysts and unilateral or bilateral aplasia (I, Fig. 3A-F). Unilateral aplasia was often accompanied by a contralateral hypodysplastic kidney, which had glomerular and tubular cysts and only few well-differentiated glomeruli. The hypoplastic kidneys in general were histologically normal exhibiting only occasionally tubular cysts (I, Fig. 4A-F). Measurement of the relative transgene copy numbers by quantitative real-time PCR (qRT-PCR) showed that the most severe renal defects were in the high copy number animals indicating that the gene dosage influenced the phenotype.

Interestingly, some of the transgenic animals who survived the first two days after birth, died between 2-9 months of ages suggesting a deficiency in renal function, potentially similar to that seen in hypomorphic Notch2 and compound heterozygous Jag1/Notch2 animals (McCright et al., 2001; McCright et al., 2002). Indeed, when the urine of such transgenic mice was examined, it was proteinuric (Fig. 8) showing that the misexpression of Jag1 also affected nephron function.

However, no gross defects could be observed in the nephrogenesis of such transgenic kidneys or in the nephrogenic competence of Jag1 transgenic mice in general. Transgenic metanephric mesenchymes expressed Pax2, Wt1 and Eya1, and were induced to nephrogenesis by the heterologous inducer (I, Fig. 6A-B). These findings suggest that structural defects causing proteinuria arise during the maturation of nephrons (eg. nephron segmentation or glomerular capillary tuft differentiation). As development is very sensitive to changes in the dosage of Notch activity (McCright et al., 2002; Duarte et al., 2004; Krebs et al., 2004), and due to the regulation of kidney development by inductive interactions, it is possible that diminishing (McCright et al., 2001; McCright et al., 2002) or increasing signalling activity (see below) results in similar defects.

Figure 8. SDS-PAGE analysis of adult urines indicates protein load in urine of transgenic (TG) mice, which died at the age of 3 months. WT lane shows proteins in the urine of wild type littermate.
Both qRT-PCR and in situ hybridization were used to evaluate potential changes in Notch activity caused by overexpression of Jag1 in the Wolffian duct derivatives. The activity was measured by analysing the expression level of Notch downstream target Hes1 from wild type and Jag1 transgenic kidneys at E12 (n=4). Hes1 expression level was in average 1.9 times higher in the transgenic than in wild type kidneys (p < 0.005, Student’s T-test) suggesting that ectopic Jag1 expression in the ureteric epithelium overactivates Notch pathway. As the ureteric bud epithelium constituted only a fraction of the whole kidney volume, the fold-change is most probably very significant due to the local expression in the epithelium (see below).

At E11, Notch2, encoding a putative receptor for Jag1, was expressed in the Wolffian duct and mesenchymal cap condensates, which also expressed down-stream targets Hes1, Hes6 and SuH suggesting that Notch2 mainly signals in the cap condensates. Several genes known to regulate ureteric branching are expressed in the cap condensates, and its formation is essential for the progress of branching. In the transgenic kidneys, where the bud was present at E11, Notch2 expression remained normal but the expression of Hes1 and -6 was shifted from the cap condensates towards the ureteric bud tips. Transgenic metanephric mesenchyme without a ureteric bud failed to up-regulate Notch2 and the down-stream targets, which were highly expressed only in the Wolffian duct (I, Fig. 7A-H). Therefore, it is reasonable to conclude that Notch activity in Jag1 transgenic kidneys was shifted from cap condensate to the Wolffian duct and ureteric bud epithelium, at least in the high transgene copy number animals. Thus, the renal malformations can result from intrinsic alterations in the epithelium or potentially from down-regulated signalling in the mesenchymal cap condensate. Most likely both contribute to the defects seen in Jag1 transgenic kidneys.

4.2 Changes in the GDNF/Ret signalling disturb the ureteric budding and branching in Jag1 transgenic mice (I)

The renal malformations causing the mortality in newborn Jag1 transgenic mice point to the defects in the ureteric branching morphogenesis. This possibility was studied by culturing E11 transgenic urogenital blocks in vitro up to three days. This revealed that in 42% of the transgenic explants ureteric budding occurred but showed either retarded branching (I, Fig. 5A-D) and/or spontaneous supernumerary ureteric budding (Fig. 9A-B). The lack of, or formation of very rudimentary ureteric bud was
observed in 29% of transgenic kidneys, and neither branching nor formation of secretory nephrons was observed in such kidneys (I, Fig. 5E-F).

GDNF-activated Ret signalling is both necessary and sufficient for ureteric budding and branching (reviewed in Costantini, 2006). At E11, the expression of Gdnf in the transgenic mesenchyme was undetectable by in situ hybridisation (I, Fig. 8A-B), and qRT-PCR confirmed Gdnf down-regulation, which was in average 3.5-fold (n=6, p<0.001, Student’s T-test). However, similarly to Notch2, Gdnf expression was normal in transgenic kidneys at E12 if renal development proceeded to this stage (data not shown). Reduced Gdnf levels alone could explain why ureteric budding often failed in transgenic animals. Moreover, the expression of GDNF receptors Ret and GFRα1, which is normally confined to the ureteric tips at T-bud stage, persisted in the transgenic Wolffian duct and ureteric stalk (I, Fig. 8C-F). This reflects either epithelial immaturity, a general delay in kidney development or lack of the signals that would normally down-regulate Ret and Gfrα1 outside the ureteric tips. As it is known that GDNF up-regulates its receptors and vice versa (Costantini and Shakya, 2006), it is possible that lack of Gdnf and misexpression of its receptors are partially consequences of each other. It is still equally possible that normal Notch signalling in the cap condensates is required for Gdnf up-regulation.

In conclusion, the data show that disruption of normal Notch signalling changes Gdnf/Ret expression and causes failure in the induction of ureteric budding and branching. Accordingly, exogenous GDNF rescued the ureteric budding in all transgenic kidneys, and also the branching was restored (I, Fig. 9A) in 71% of the samples suggesting that defects in Jag1 transgenic kidneys indeed arise from the reduced GDNF/Ret signalling. Furthermore, exogenous GDNF induced more extra ureteric buds in transgenic than in wild type explants, but failed to confine Ret expression to the ureteric bud tips in transgenic samples (I, Fig. 9B). The increase in extra ureteric buds induced by GDNF most probably reflects the persistent Ret/GFRα1 expression in the transgenic Wolffian duct. This prolonged GDNF receptor expression suggests that Notch signalling could actually regulate the receptor expression, or alternatively unknown signals that are required for Ret/GFRα1 repression outside the tip. By this way Jag1-activated Notch signalling may contribute to the determination of ureteric tip identity. The expression of Hes1 and Ret co-localised in the epithelium of both wild type and transgenic kidneys, and Ret promoter has the binding site for Hes1 (nucleotides 3099-3105 in genebank accession number AY255629). Thus, the data from
Jag1 transgenic kidneys suggest that Notch signalling in the Wolffian duct and ureteric epithelium may directly regulate the expression of GDNF receptors. However, studies in MDCK cell line showed that endogenous expression of Notch pathway molecules is not enough to activate Ret expression in these cells (unpublished data), but it is still possible and tempting to speculate that Hes1 promotes Ret transcription although the mechanism remains unknown.

Chemical inhibition of Notch signalling by gamma-secretase inhibitor DAPT decreases ureteric branching (Cheng et al., 2003) and suggests that Notch signalling participates in the guidance of ureteric branching morphogenesis. However, the mechanism for reduced ureteric branching in gamma-secretase inhibited kidneys remained unidentified. It may be secondary effect of severely disturbed nephrogenesis but as well a direct consequence of inhibited Notch signalling. The experiment showing that gamma-secretase inhibition does not inhibit GDNF-activated Ret-signalling in Neuro2A cells does not prove that DAPT-treatment in kidneys could not interfere with Gdnf/Ret expression, or Ret signalling. Genetic deletion of enzymes responsible for gamma-secretase, Psen1 and -2, does not affect the expression of Gdnf (Wang et al., 2003) but its effect on Ret-signalling remains unknown. Our experiments clearly suggest that at least Jag1-activated Notch signalling participates in the control of ureteric branching by interacting with GDNF/Ret signalling.

4.3 Suppression of Bmp4 by BAMBI may explain spontaneous extra ureteric budding in Jag1 transgenic kidneys (unpublished)

Spontaneous extra ureteric budding (Fig. 9A-B), which gives rise to duplex ureters and pelvises (I, Fig. 4E), was occasionally observed in Jag1 transgenic kidneys. Both genetic and in vitro evidence indicate that Bmp signalling negatively regulates ureteric budding. Bmp4 is expressed by the smooth-muscle-type mesenchymal cells around the Wolffian duct and it is down-regulated at the budding site (Miyazaki et al., 2000). Increased Bmp4 levels also inhibit primary ureteric bud formation in vitro (Raatikainen-Ahokas et al., 2000). Bmp4 expression pattern was normal in the Jag1 transgenic mice but Bambi, an antagonistic Bmp pseudo receptor (Onichtchouk et al., 1999), was highly up-regulated in the cells surrounding the transgenic Wolffian ducts (Fig. 9C-D). This raises the possibility that up-regulation of Bmp antagonists rather than down-regulation of the ligand itself antagonises Bmp signalling at the budding site thus providing an
alternative mechanism for the regulation of ureteric bud formation. Both antagonistic (Machold et al., 2007) and synergistic action (Nobta et al., 2005) of Notch and Bmp signalling have been reported in other organ systems but how these pathways potentially interact in renal morphogenesis remains to be elucidated.

Taken together, the experiments aiming to understand the role of Notch signalling in the kidney development revealed that Jag1-activated Notch signalling participates in the regulation of primary ureteric budding and subsequent branching by interacting with GDNF and its receptors. The results from Jag1 transgenic mice imply that a strict spatio-temporal control of Notch activity is needed for normal ureteric morphogenesis during the early steps of kidney development, and suggest that Notch signalling may contribute to the specification of ureteric tip identity through Ret regulation.

4.4 Canonical Wnt activity and β-catenin localisation suggest dual function for
Wnt signalling in renal development (II, III)

Reporter mice studies (Maretto et al., 2003; Moriyama et al., 2007, and II, Fig. 1C-E) and β-catenin localisation (II, Fig. 1A-B) indicated activation of canonical Wnt
pathway specifically in the ureteric epithelium of the developing kidney. This suggests function for Wnt signalling in the ureteric branching morphogenesis but very little is known about the role of β-catenin in kidney development, or Wnt signalling in the ureteric morphogenesis. On the other hand, the role of Wnt signalling in the induction of nephron differentiation is established. Wnt4 may activate planar cell polarity pathway and thereby regulate renal progenitor cell population size in isolated kidney mesenchymes (Osafune et al., 2006) but the intracellular pathways utilized in nephron induction have remained unknown. The present study showed first that β-catenin, a mediator of canonical Wnt signalling, was localized to early nephron structures of developing kidney (II, Fig. 1A-B), and also that Wnt-reporter activity was detected in pretubular aggregates (II, Fig. 1E).

4.5 Activation of canonical Wnt pathway through stabilisation of β-catenin arrests ureteric branching and induces nephron formation (II, III)

Wnt binding to Fz/LRP receptor complex turns on canonical Wnt pathway by activating Dsh, which then by an unknown mechanism inhibits the key enzyme of the pathway, GSK3. This inhibition allows stabilisation of cytosolic of β-catenin leading to its nuclear translocation and binding to Tcf/Lef transcription factors (Huelsken and Behrens, 2002). Two structurally unrelated GSK3 inhibitors, lithium and 6-bromoindirubin-3’-oxime (BIO) were used to activate the canonical Wnt pathway in whole kidneys or isolated metanephric mesenchymes. GSK3β inactivation was demonstrated in the kidney mesenchymes by a phospho-specific antibody after 30 min of BIO-treatment. Inactivation led simultaneously to the increased cytosolic β-catenin levels (III, Fig 3A lower). Accordingly, BIO stabilized β-catenin also in kidney rudiments dissected free of majority of the mesenchymal cells (II, Fig. 5C and supplementary Fig. 2A). These findings indicated that BIO may activate the canonical Wnt pathway, which was further confirmed by studying the transcriptional activation of β-catenin in isolated renal mesenchymes and whole kidneys. The expression of canonical pathway targets Lef1 and Tcf1 was highly up-regulated in GSK3-inhibited kidney mesenchymes (III, Fig. 3B-F) demonstrating activation of the canonical Wnt/β-catenin pathway. Similarly, transcriptional activation of β-catenin was confirmed in whole kidneys of BAT-gal reporter mice (II, Fig. 5A-B).
GSK3 mediates also other pathways than Wnt signalling including Insulin-like growth factor (IGF) in mammals and Hedgehog in *Drosophila*. It is theoretically possible that these pathways could contribute to the morphological responses seen in kidneys and isolated mesenchymes upon GSK3 inhibition by BIO/lithium (see below and chapter 4.9). The potential consequence of GSK3 inhibition on Sonic hedgehog (Shh) signalling was studied but no changes in the expression of *Gli1, -2* or -3 were detected in BIO-treated renal mesenchymes (III, unpublished observation). Additionally, both the biochemical and genetic evidence indicate that GSK3 inhibition by lithium/BIO during embryogenesis mimics the activation of Wnt signalling (Klein and Melton, 1996; Stambolic et al., 1996; Seufert et al., 1999; Gould and Manji, 2005) rather than IGF or Shh signalling. Therefore, based on our results and those published by others, it is unlikely that the other GSK3 targets would engage with the effect on kidney.

Previously it was shown that nuclear extracts isolated from LIF/TGFβ2-induced rat renal mesenchymal cells can bind to *Lef/Tcf* consensus sequence (Barasch et al., 1999). Although we found that Wnt down-stream target *Lef1* (III, Fig. 5A) was expressed in the metanephric mesenchyme of rat kidneys with the similar pattern as in mouse, its expression was not up-regulated by LIF, TGFβ2 or their combination either in rat (III, Fig. 5H-I) or mouse mesenchymes (III, Fig. 3G-H and unpublished data). Our finding that LIF/TGFβ2 does not up-regulate canonical Wnt targets in kidney mesenchymes does not rule out the possibility that they would still promote binding of nuclear extracts to *Lef1/Tcf1*. The failure to up-regulate *Lef1/Tcf1* apparently reflects the differences in the downstream targets through which LIF/TGFβ2 and Wnt signalling pathways operate in nephrogenesis (see below).

The potential function of GSK3 inhibition on renal morphogenesis was tested on E11 kidneys. Lithium- and BIO-treatment inhibited completely the ureteric branching but elongation of the epithelium still occurred to some extent. Unexpectedly condensates were formed without ureteric branching but they were peculiarly located in the periphery of kidney mesenchyme, apart from the ureteric epithelium (III, Fig 1A-D) suggesting that GSK3 inhibition may promote pretubular aggregate formation in whole kidneys similarly as reported in isolated mesenchymes (Davies and Garrod, 1995). To test this, the inhibitors were withdrawn from the culture at 48h, and the kidney explants were sub-cultured for two more days. In the kidneys transiently exposed to lithium (data not shown) or BIO, branching morphogenesis was still arrested but more abundant
tubular differentiation was observed (III, Fig. 1D) than in control kidneys (III, Fig. 1C). Apparently β-catenin stabilisation had dual effect on kidneys as it blocked ureteric branching but simultaneously induced mesenchymal differentiation.

4.6 In vivo manipulation of β-catenin specifically in the ureteric epithelium results in similar ureteric branching defect as its in vitro stabilisation

Next the canonical Wnt/β-catenin function in the ureteric epithelium was studied in vivo by two different genetic models generated with the help of Hoxb7-Cre; Gfp mice (Zhao et al., 2004). Briefly, transgenic mice in which exons 2-6 of β-catenin gene are flanked with loxP sites (Brault et al. 2001) were used to produce offspring with no functional β-catenin in ureteric epithelium (termed β-catenin deficient) and the mice in which exon 3 is flanked by loxP sites (Harada et al., 1999) were used to generate progeny with stabilized β-catenin in the ureteric bud lineage (termed, β-catenin stabilized). The effect of these genetic manipulations was first studied by investigating β-catenin expression in kidneys of mutant mice. This confirmed that Hoxb7-Cre; Gfp deleted the gene product and increased the protein levels specifically in the ureteric epithelium of deficient mice and stabilized mice, respectively (II, Fig. 1G-I).

Analysis of postnatal β-catenin mutant mice at P15 by genotyping revealed neither β-catenin deficient nor stabilized pups suggesting lethality either after the birth or during embryogenesis. Examination of litters at P0 or P1 identified 26.9% β-catenin deficient and 30.5% β-catenin stabilizes (n=27 and n= 36, respectively) pups demonstrating that in both genetic models, transgenic animals were born alive but they died postnatally (II, Table 1). Newborn β-catenin deficient pups exhibited bilateral renal aplasia (3/7) or bilateral renal hypodysplasia (4/7) (II, Fig. 2B). Similarly, the newborns with the stabilized β-catenin allele exhibited bilateral renal aplasia (5/11) or bilateral renal dysplasia (6/11) (II, Fig. 2C). Both β-catenin deficient and stabilized mice with bilateral renal dysplasia also showed bilateral hydroureter (II, Fig. 2B-2C). Accordingly, analysis of mutant mice at E14.5 revealed the similar renal defects as in newborns, and provided a more accurate estimate of the relative occurrence of renal aplasia and dysplasia. 20% of β-catenin deficient embryos were characterised by bilateral renal aplasia, while 80% showed bilateral renal dysplasia. β-catenin stabilized embryos exhibited bilateral renal aplasia in 17 % of the mutant embryos, while 83 % had bilateral renal dysplasia (II, Table 1). Together, these observations demonstrate that
both β-catenin deficient and β-catenin stabilized mice exhibit similar, severe renal phenotypes, which result in early postnatal death.

Histological analysis of embryonic kidneys at E12.5 suggested a decrease in kidney size in both β-catenin deficient and stabilized mice (II, Fig. 2D-4F). At E13.5, ureteric bud branching failed to progress beyond that observed at E12.5 in both types of β-catenin mutant mice (data not shown), and by E14.5, kidneys in both types of mutant mice exhibited a cystic dysplastic appearance (II, Fig. 2G-2I). All the above described phenotypes point to the defects in ureteric bud morphogenesis, which was confirmed by visualizing the ureteric bud branches with the GFP tag in Hoxb7-Cre construct (II, Fig. 2J-L). GFP revealed marked attenuation of ureteric branching in β-catenin deficient (II, Fig. 2K) and β-catenin stabilized mice (II, Fig. 2L) at E12.5. Together, these results demonstrate that both β-catenin deficiency and stabilisation in the ureteric bud lineage abrogates renal branching morphogenesis and causes severe renal dysgenesis.

4.7 Cellular mechanisms in epithelium specific β-catenin mutant mice

To determine cellular mechanisms that control abnormal kidney development in β-catenin mutant mice, we first investigated those events important to branching morphogenesis and generally controlled by β-catenin. In the developing limb, β-catenin deficiency or its stabilisation increases apoptosis (Hill et al., 2006). Since increased apoptosis has been shown to control decreased ureteric branching in Pax2-deficient mice (Dziarmaga et al., 2003), we determined the effect of β-catenin manipulation on the number of apoptotic cells in ureteric bud. TUNEL analysis of embryonic kidneys at E12.5 did not reveal any difference in the number of apoptotic epithelial cells in either β-catenin deficient or β-catenin stabilized mice (II, Fig. 3A, and Supplementary Fig, 1). Consistent with the known requirement for ureteric bud branching in rescuing the metanephric mesenchyme from apoptosis (Barasch et al., 1997), apoptosis was increased in metanephric mesenchymal cells 4.4-fold (P=0.03) and 5-fold (P=0.015) in β-catenin deficient and β-catenin stabilized mice, respectively. These results do not suggest a primary role for apoptosis in the arrest of branching morphogenesis in β-catenin mutant mice but highlight a deleterious role for apoptosis in the subsequent abnormal development of the metanephric mesenchyme.

Cell proliferation is a critical cellular event during branching morphogenesis (Shakya et al., 2005b). Since β-catenin in partnership with its Tcf transcription factors...
generally increases the transcription of genes that positively regulate cell proliferation, we measured rates of cell proliferation in β-catenin mutant mice. BrdU incorporation at E12.5 β-catenin deficient mice indicated a 1.8-fold decrease in ureteric bud cell proliferation in comparison to wild type ureteric epithelium (P=0.004) (II, Fig. 3A and Supplementary Fig. 1). In contrast, BrdU incorporation was slightly increased (1.3-fold) in E12.5 ureteric epithelial cells of β-catenin stabilized mice (P=0.02). These studies indicate that manipulation of β-catenin dosage in the ureteric bud modulates cell proliferation in a manner consistent with its positive regulatory function. At the same time, the stimulatory effects of β-catenin stabilisation on cell proliferation did not increase ureteric bud branching but rather inhibit it.

β-catenin regulates cell-cell adhesion by binding the cytoplasmic domain of cadherins (Takeichi, 1995). Therefore it was hypothesized that abnormal β-catenin expression may disrupt cell adhesion through E-cadherin. However, immunohistochemical analysis of renal tissues from E13.5 wild type and β-catenin mutant mice revealed similar patterns of E-cadherin in epithelial tubules despite the abnormalities in the tissue architecture of the β-catenin mutant kidneys (II, Fig. 3B-3D). Moreover, analysis of junctional complexes and adherens junctions by electron microscopy demonstrated that these structures are maintained in epithelial cells of mutant kidneys (II, Fig. 3E-3G). Together, these results suggest that β-catenin deficiency or stabilisation does not disrupt cell-cell adhesion in the embryonic kidney, and that other mechanisms disrupt branching morphogenesis in the mutant kidneys.

4.8 Ureteric epithelium specific β-catenin manipulation disturbs Ret-signalling and causes loss of ureteric tip identity

Next we investigated molecular mechanisms that could mediate the deleterious effects of β-catenin deficiency or stabilisation on renal development. The studies using Tcf-dependent reporter mice indicated activation of canonical Wnt/β-catenin in the ureteric bud lineage (see II, Fig. 1C-E). Thus, we hypothesized that β-catenin/Tcf activity may control genes required for guidance of branching morphogenesis. Survey of databases for genes expressed in the ureteric bud and required for branching morphogenesis revealed the presence of Tcf consensus binding sequences (CTTTG A/T A/T) in the 5’UTR of Ret making it a good candidate to mediate β-catenin effects. Normally Ret is expressed in the tips of ureteric buds but strikingly, it was undetectable
in the ureteric buds of β-catenin deficient kidneys (II, Fig. 4A-B). In contrast, Ret expression was maintained in the ureteric bud branches of β-catenin stabilized mice (II, Fig. 4C). Thus, the data suggest that β-catenin is required for Ret expression, and that the ureteric branching defects in β-catenin deficient mice may derive from the loss of Ret signalling. At the moment it is impossible to determine whether Ret expression is directly or indirectly regulated by β-catenin, but based on the expression patterns, it is unlikely that β-catenin would control spatial-temporal restriction of Ret expression to the tips of ureteric buds. One possible mechanism through which β-catenin could regulate Ret expression is via Pax2, which is expressed in the metanephric mesenchyme of the β-catenin deficient embryos but lacking from the ureteric bud (data not shown). It has been shown that Pax2 dose dependently regulates Ret expression (Brophy et al., 2001; Clarke et al., 2006) and this regulation could be defective in the β-catenin deficient kidneys.

The molecular mechanisms for ureteric morphogenesis defects in β-catenin stabilized kidneys remained unknown. Although the proliferation in the ureteric epithelial cells with genetically stabilized β-catenin is slightly altered (II Fig 3. and Supplementary Fig. 1), it cannot explain the branching morphogenesis defect in the mutant kidneys. The analysis of expression patterns of potential β-catenin targets in ureteric epithelium (III, Fig. 4C and 4F) could not explain the phenotype either. As stabilisation of β-catenin through GSK3 inhibition by BIO or lithium arrests ureteric bud branching (II, Fig. 5D-E and III, Fig. 1A-D) we took the advantage of in vitro stabilisation of β-catenin in kidney cultures in order to reveal molecular mechanisms leading to ureteric branching defect in β-catenin stabilized kidneys. Similarly to genetic stabilisation of β-catenin, activation of canonical Wnt/β-catenin in cultured kidneys did not affect the expression of Gdnf/Ret pathway molecules (II, Fig. 5F-I) but clearly blocked ureteric branching.

Since Ret-mediated signalling is critical for ureteric bud branching, we next investigated the possibility that stabilisation of β-catenin in the ureteric bud interrupts the signalling events down-stream of Ret, thereby abrogating ureteric branching. This was supported by the finding that Ret signalling targets Wnt11 (Pepicelli et al., 1997) and Pea3 (Haase et al., 2002 and personal communication by B. Lu and F. Costantini) were undetectable in ureteric bud tips of BIO-treated kidneys (II, Fig. 6D, F). Similarly, Wnt11 was lost also from the kidneys where β-catenin was genetically manipulated (II,
Kidney Induction

Results and discussion

Fig. 6H-I). Accordingly, ligand-dependent induction of Ret signalling, which involves phosphorylation of residues within the Ret tyrosine kinase domain and activation of downstream events, was disrupted as the level of phosphorylated Erk was markedly reduced in β-catenin stabilized kidneys (II, Fig. 6C). The decrease in signalling activity appears to be specific to Erk/MAPK pathway, which by others has been shown to regulate ureteric branching (Fisher et al., 2001), since no differences were detected in pAKT levels (data not shown). Thus, stabilisation of β-catenin in the kidney disrupts signalling that occurs downstream of Ret resulting in loss of Ret target gene expression in the tips of ureteric buds. The phenotype in β-catenin stabilized kidneys resembles molecularly to that reported from Six2-deficient mice in which Ret and Gdnf are normally expressed but Wnt11 is missing (Self et al., 2006). The expression of Six2 in β-catenin stabilized kidneys remained normal although its pattern was slightly expanded in BIO-treated kidneys (unpublished data). It will be interesting to reveal regulatory relationships of Six2 and β-catenin because of their obviously essential functions in nephron induction.

We next studied whether the exogenous GDNF could rescue the effect of β-catenin stabilisation in BIO-treated kidneys. Normally GDNF induces formation of several ectopic ureteric buds from the Wolffian duct (Sainio et al., 1997 and II, Fig. 6A) but simultaneous application of exogenous GDNF and BIO to kidneys failed to induce supernumerary budding. Accordingly, exogenous GDNF did not rescue ureteric branching in the presence of BIO (II, Fig. 6B). Not even high local concentrations of GDNF released from beads (Sainio et al., 1997) had an effect on ureteric morphogenesis if canonical Wnt/β-catenin pathway was simultaneously activated (II, Supplementary Fig. 2H and 2I). Similarly, application of GDNF after 24h of BIO treatment could not completely rescue ureteric branching although the epithelium responded to GDNF by swelling of the ureteric epithelium and formation of bud-like structures, which expressed Wnt11 (II, Supplementary Fig. 2J). Together, these results demonstrate that β-catenin stabilisation irreversibly blocks ureteric branching, and re-activation of Ret signalling cannot overcome this effect. This may be due to the effect of β-catenin stabilisation on the metanephric mesenchyme, which is induced to nephrogenesis (III) and may therefore block the growth of ureteric bud in a similar manner as suggested in Six2-deficient kidneys (Self et al., 2006).

Formation of ureteric tip is essential for branching morphogenesis (Sariola and Sainio, 1997; Shakya et al., 2005b; I). Consistently, the molecular identity of ureteric tip
cells is distinct from that of ureteric stalk cells. Indeed, ureteric tips express a subset of transcripts that differ from those expressed in ureteric stalk epithelium such as Arginase2, Sox9 and Wnt11 among others (Kispert et al., 1996; Schmidt-Ott et al., 2005; Caruana et al., 2006). To determine the effects of β-catenin stabilisation on the tip identity, we analysed the expression of tip-specific markers in BIO-treated kidney explants. The expression of Arg2 (Caruana et al., 2006 and II, Fig. 7A) and Sox9 (Pepicelli et al., 1997) is normally restricted to ureteric tips, but their expression was markedly decreased in β-catenin stabilized kidneys (II, Fig. 7B and data not shown). Remarkably, the localisation of DBA-lectin, which is restricted to ureteric stalks (Michael et al., 2007 and II, Fig. 7C), was mislocalised to the entire ureteric epithelium including the tip in β-catenin stabilized kidneys (II, Fig. 7D). Consistent with this finding, DBA-lectin was also expanded throughout the ureteric epithelium in Ret-deficient kidneys (II, Supplementary Fig. 2K), in which ureteric branching is clearly diminished (Schuchardt et al., 1994; Sanchez et al., 1996). Together, the data demonstrate that activation of canonical Wnt signalling disrupts ureteric tip-identity, which is replaced by stalk-identity. The loss of tip identity significantly contributes to the ureteric branching defect in stabilized kidney similarly to that in Ret-deficient kidneys. Also Jag1 overexpression in the Wolffian duct and ureteric epithelium (I) disrupts the tip identity (I, Fig. 8C-F) and contributes to the branching morphogenesis phenotype in this transgenic model. It is possible that similar competition of inhibiting and activating modes of functions as demonstrated for Wnt and Shh in chick neural tube patterning (Lei et al., 2006), are operating in normal ureteric bud epithelium to produce an interface between Ret and canonical Wnt/β-catenin pathways to establish distinct tip and stalk sub-regions (II, Fig. 8). On the other hand, Ret and Notch activity needs to be restricted to the ureteric tips, since if Notch activity is forced throughout the epithelium, the whole ureteric bud expresses Ret and gains tip-identity. This theory would suggest that canonical Wnt activity is required for ureteric stalk formation and Ret and Notch signalling determine the tip cells. Deletion of β-catenin (II) results in situation where neither of the identities are established due to the lack of Ret expression whereas β-catenin stabilisation (II) and thereby over-activation of canonical Wnt pathway causes expansion of stalk with the expense of the tip identity. It is likely that mesenchymal signals also participate in the patterning of ureteric epithelium into distinct sub-regions, but how these contribute to the formation of tip-stalk identities remain to be elucidated. Taken together, the work demonstrates for the first time that normal activation of
canonical Wnt/β-catenin pathway in ureteric bud epithelium is crucial for epithelial patterning and branching.

**4.9 β-catenin stabilisation triggers nephrogenesis in isolated mesenchymes**

The data on whole kidney explants (III, Fig 1A-D) suggested that canonical Wnt pathway activation may induce nephrogenesis. To study this, isolated kidney mesenchymes were transiently exposed to GSK3 inhibitors. Control kidney mesenchymes failed to express condensate (III, Fig. 1E) or pretubular markers (Fig. 1F), and as reported earlier (Barasch et al., 1997) died in few days while the mesenchymes cultured with BIO (data not shown) or lithium formed condensate-like structures expressing *Pax8* (III, Fig. 1G), *Pax2*, and *Wt1* (data not shown). Also pretubular aggregate markers (Carroll and McMahon, 2003) *Lim1* and *Wnt4* (III, Fig 1H and I) were up-regulated. When the GSK3 inhibitors were removed from the cultures at this point, the pretubular aggregates fully epithelialised into all main nephron structures during the following 4-5 days culture (III, Fig. 1J and Fig. 2). Transient GSK3 inhibition induced abundant tubulogenesis also in isolated rat mesenchymes (III, Fig. 5) suggesting, unlike previous experiments (Barasch et al., 1999), an evolutionarily conserved mechanism for nephron induction. However, the optimal exposure time of GSK3 inhibitors in rat was 24h while in mouse it was 48h.

Interestingly, when the mesenchymes and whole kidneys were exposed to the GSK3 inhibitors constantly, they became necrotic and showed no epithelial differentiation (data not shown) as reported previously (Davies and Garrod, 1995). Therefore, only transient exposure of kidney mesenchymes to BIO or lithium could trigger nephron differentiation suggesting that either the inhibitors are toxic in long term culture, or that GSK3 reactivation/β-catenin destabilisation must occur in order to allow the progress of the mesenchyme-to-epithelium transformation. The latter hypothesis is supported by the up-regulation of canonical Wnt signalling antagonists during nephron formation. For example, *sFRP* is a negative regulator and target of Wnt4 signalling in developing metanephros (Lescher et al., 1998). Therefore the endogenous inhibitors may suppress Wnt pathway during the normal epithelialisation of the nephron similarly to that suggested in tooth development (Järvinen et al., 2006). Constant β-catenin stabilisation by lithium or BIO is probably so robust that endogenous feedback mechanisms cannot overcome it. Also in *vivo* the nephron induction may be transient since a the mesenchymal cells giving rise to future nephron meet the inducer, the ureteric bud, only
briefly due to its continuous growth and branching. All in all, these experiments demonstrated for the first time that isolated mouse metanephric mesenchyme can be induced to full tubulogenesis without cell contacts with the inducer substance, and that the mechanisms triggering the induction are probably conserved in vertebrates.

4.10 Genetic stabilisation of β-catenin in mouse mesenchymes induces nephrogenesis

We generated transgenic mice, where Six2-promoter (Brodbeck et al., 2004) drives the expression of Cre-recombinase in the metanephric mesenchyme (III, Fig. 4A) to enable genetic stabilisation of β-catenin (Harada et al., 1999 and see chapter 4.9) specifically in the renal mesenchyme. Kidney mesenchymes isolated from Six2-Cre<sup>ex3fl/ex3fl</sup> embryos formed spontaneously pretubular aggregates, which in five days differentiated into secretory nephrons binding LT- and PNA-lectins (III, Fig. 4B-C). Mesenchymes isolated from the control embryos (III, Fig. 4D-E) showed no nephron development. Unfortunately Six2-Cre<sup>ex3fl/ex3fl</sup> embryos died around E11, which prevented us from the analysis of late embryonic kidneys. The cause for embryonic lethality remains unknown, but may be due to the Six2-Cre expression detected in embryonic heart (data not shown). However, genetic stabilisation of β-catenin in the kidney mesenchymes resulted in similar epithelialisation of isolated kidney mesenchymes as in vitro stabilisation indicating an in vivo role for β-catenin stabilisation in triggering the nephrogenesis. This is further supported by activation of canonical Wnt pathway only in pretubular aggregates of developing nephron (II, Fig. 1B and D). In conclusion, activation of canonical Wnt pathway by chemical or genetic means is sufficient for induction of nephrogenesis. Wnt inhibitors such as Cited (Plisov et al., 2005) and sFRPs (Yoshino et al., 2001), are up-regulated in early nephron structures and may suppress the pathway also in the mice with genetically stabilized β-catenin. This further endorses the requirement for transitory nature of nephron induction, but remains to be shown.

4.11 Establishment of nephrogenic competence does not require the ureteric epithelium

The data from whole kidneys transiently exposed to GSK3 inhibitors suggested that ureteric branching morphogenesis is not required for the induction of kidney
mesenchyme. This has been previously shown in another experimental setup, where ECM proteoglycans were depleted in the kidney cultures resulting in complete lack of the ureteric branching, which did not prevent mesenchymal differentiation (Davies et al., 1995). The metanephric mesenchyme expresses a set of nephrogenic marker genes already prior to the primary ureteric budding (Vainio and Lin, 2002), but the factors regulating this mesenchymal competence are not known. One possibility still is that the ureteric bud or the Wolffian duct epithelium provides cues for the nephrogenic competence. To test this, in vitro β-catenin stabilisation method was used in two genetic models where Wolffian duct or the ureteric bud development is disrupted. Gdnf-deficient mice show normal Wolffian duct development but often lack the ureteric bud (Pichel et al., 1996). When Gdnf-deficient isolated kidney mesenchymes were transiently exposed to GSK3 inhibitors, they differentiated into kidney tubules as extensively as wild type controls (III, Fig. 6A and data not shown). Similarly, activation of the canonical Wnt pathway induced nephron differentiation in Gata3-deficient mesenchymes (III, Fig. 6B), which have never been in contact with the epithelium due to disrupted Wolffian duct migration (Lim et al., 2000). The duct in Gata3 -/- mice reaches at its best the level of caudal mesonephric tubules but never the level of the presumptive metanephric mesenchyme (Grote et al., 2006). Thus, the nephrogenic competence in kidney mesenchymes is established independently of the influence of the epithelium. However, in vivo the formation of a ureteric bud is essential for nephron induction and subsequent differentiation since tubulogenesis does not take place in the genetic mouse models where the ureteric budding is disrupted (Vainio and Lin, 2002).

Induction by GSK3 inhibition resembles that by the spc, which is a potent inducer of nephrogenesis in vitro (Saxén, 1987). However, contrary to the studies with spc and Wnt-expressing cells, our results demonstrate that induction may be experimentally triggered in contact independent manner. Stabilisation of β-catenin, the hallmark of canonical Wnt pathway activation, is sufficient for triggering nephron formation. This makes chemical GSK3 inhibition a suitable method to study molecular mechanisms and intracellular signalling pathways of nephron induction and kidney development in general.
5. CONCLUDING REMARKS

Understanding the origin and pathogenesis of developmental defects is possible only if the regulation of normal embryogenesis is known. Much of our knowledge of how kidney differentiation is controlled was already available during the course of the experimental work for this thesis but more importantly several key aspects were missing. The role of GDNF/Ret signalling in the initiation of ureteric branching morphogenesis was established but how it possibly interacts with other developmentally important pathways was unidentified. It was also known that Notch signalling patterns the future nephron prior to its epithelialisation but nothing was known about its function during the early stages of kidney development. Furthermore the requirement for Wnt signalling in the formation of nephrons was evident while the down-stream pathways through which it induces the nephrogenesis were not defined. The potential role of the canonical Wnt pathway in the ureteric epithelium, where it is strongly activated, was a completely unexplored subject. The main reason for the lack of the answers for the above listed issues has been the lack of appropriate tools to study these questions. Generation and utilisation of new mouse models, which allow tissue-specific manipulation of gene activity specifically in distinct tissue compartments of the developing kidney, has been the basis of this thesis.

Here it was demonstrated that Notch and Wnt signalling pathways participate in the control of ureteric branching morphogenesis, and that activation of the canonical Wnt pathway is sufficient for nephron induction. Previous experiments had suggested that a decrease in the Notch activity reduces kidney size, potentially through regulation of branching morphogenesis, but the direct evidence and the mechanisms through which Notch would supervise branching were missing. Characterisation of Jag1 transgenic kidneys revealed that Notch signalling interplays with GDNF/Ret in the regulation of ureteric budding and potentially also during later branching events. Interestingly, although lateral inhibition is a key mechanism through which Notch signalling regulates, for example, neuronal and pancreatic differentiation, no evidence could be found for that in the development of the ureteric bud. It is still relevant to speculate about whether the Wolffian ductal cells fated to form a ureteric bud, are selected by the intrinsic mechanism within the epithelium, such as lateral inhibition or induction. Demonstration of the mode of Notch pathway function may be possible in future if new techniques enable manipulation of gene activities at the single cell level.
Although the canonical Wnt pathway is strongly activated in the ureteric epithelium, its function in these cells had remained unknown. The β-catenin manipulation experiments here demonstrated that canonical Wnt signalling regulates ureteric branching. Which of the Wnt ligands actually trigger the pathway in the ureteric epithelium remains unknown, and conditional deletion of at least two if not three of them may be required to indicate their necessity in the ureteric branching morphogenesis. The situation was opposite in the regulation of nephron differentiation, which was known to depend on the function of Wnt9b and -4, but the down-stream pathways through which they induce nephrogenesis had remained obscure. This thesis shows that activation of the canonical Wnt pathway is able to trigger nephron differentiation but the full mesenchyme-to-epithelium transition requires transient activation of the pathway. This requirement is supported by transient activation of canonical Wnt pathway in nephron intermediates as detected in kidneys of reporter mice and by up-regulation of the canonical pathway antagonists in late intermediates (pretubular aggregates). One important aspect of induction studies was that they demonstrated that isolated mouse mesenchymes can be induced to full nephrogenesis by chemical means. It is methodically significant finding, which will hopefully facilitate future nephron induction studies. However, neither the data from ureteric branching nor mesenchyme experiments exclude the potential involvement of the non-canonical pathways in these processes. A lack of the specific methodological tools to study β-catenin independent pathways makes it difficult to dissect their function in renal differentiation.
6. ACKNOWLEDGEMENTS

This study was carried out at the Institute of Biomedicine, University of Helsinki. I want to warmly thank Professor Olli Jänne, Director of the Biomedicum Helsinki, Professor Esa Korpi, the head of the Institute of Biomedicine, Professor Tomi Mäkelä, the head of Biochemistry and Developmental Biology at the Institute and Director of Helsinki Biomedical Graduate School, for providing excellent working facilities, high-quality education and financial support and Professor Irma Thesleff, the head of Center of Excellence (CoE), for keeping up the spirit of Finnish developmental biology.

I wish to express my deepest gratitude to my supervisors Professor Hannu Sariola and Docent Kirs Sainio, who have taught me the true meaning of research, and provided the lab of Circus Sariola with humane environment and excellent equipments. Your enthusiasms and attitudes are admirable - neither of you was ever too busy to welcome a discussion, whether it was scientific or not. I also want to thank Professor Seppo Vainio for introducing me to the fascinating world of developmental biology.

I want to acknowledge my thesis committee members Docents Päivi Miettinen and Marjo Salminen for their helpful comments and support towards my work.

I also want to thank the reviewers of my thesis, Docent Eero Lehtonen and Professor Årindam Majumdar, for careful reading and valuable commenting of the manuscript.

Today science is more than ever full of teamwork and I thank all my co-authors for their fruitful collaboration. I want to specially acknowledge Darren Bridgewater and Norm Rosenblum for combining their data with ours, Paul Riccio and Frank Costantini for providing their important know-how especially in the isolated ureteric bud cultures, my hands-on supervisor Reetta Vuolteenaho for teaching me the tricks of transgene construct cloning, my “brother” Anttu the Lynx and summer-boy Jouni for expertise in quantitative RT-PCR, Tiina Immonen for everlasting patience in answering my overwhelming questions. In addition to science, Anna Popsueva and Madis Jakobson are highlighted for their friendly companionship during the endless hours of lab work, and Kirmo Wartiovaara for long discussions dealing with everything under the sun.

I warmly thank all the previous and present members of the Circus Sariola, with whom I have had the privilege to delight in crayfish and fondue parties, outdoor activities and challenges of publishing – depressing rejections do sometimes lead to rare moments of success. I am deeply grateful for Agnès Viherä for her precious skills and the numerous experiments she has done for me, but more importantly, for her most valuable friendship. I want to emphasise Laura Kerosuo’s extraordinary sense of
humour and the way how she values life. I would like to thank Nina Perälä for shared trips, Roxana Ola for her eagerness to bench work, Heli Fox and Mariann Nymark for joining the “lunch-club”, Samer Hussein for helping with the English language, Anita Tuomainen and Eric Pedrono for the nice moments of SS, Jetta Kelppie for her warm friendship, and Elena Arighi for bringing in the glimmer of sunshine and excellent presents whenever visiting the lab. Marjo, Katja, Valtteri, Fares, Alex, Anastasia and CoE as well as DevRepro members are thanked for the nice time we have shared together.

Lea Armassalo and Virpi Syvälahti are acknowledged for their important technical help in laboratory work and Kirsi Salonen, Sanna Kauppinen, Eija Nissinen and all the others in the transgenic units in Oulu and Biomedicum Helsinki are acknowledged for their expertise. Kari “Ransu” Ojala, Maiju Aalto and the others are thanked for taking such good care of my research tools. Aija Kaitera is acknowledged for her valuable help in practical matters concerning my PhD-studies.

There were times during this thesis when I tended to forget that research does not equal life, and that success or failure in my work does not measure me as a human being. I am most fortunate to have honest and straight friends who have guided me back from the wrong tracks. The warm friendship with Hanna Rinne and Miia Tuikka begun when we were five years old and I want to thank the girls for walking through life with me. The chance to get to know Marika Kuusela and Tiina Seppänen made the years in Vainio group extremely precious. And so far, I have survived our adventures.

Without Marjo Kestilä the probability that this thesis would have never been completed is amazingly high – thanks for your ears, which are always there and your infinite suggestions how things could be dealt. The value of Mikko Kestilä cannot be overcome since he has made living here in Southern Finland tolerable by feeding me and my family numerous times with enjoyable food and company.

Mom and dad, without you I would not exist. Together with Eila and my parents-in-law, Armi and Viljo, you are always there to help.

There are no ways that I could compensate to my family the time, which this work has kept me away from them. Above all, Pekka, who made this thesis possible, together with Otto and Oula – you are my life.

Satu Kuure
Helsinki, 2007
References


References


Kidney Induction


Derived Neurotrophic Factor is Required for Bud Initiation from Ureteric Epithelium. *Development* 124, 4077-4087.


