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*Ilkka Pietilä*

# THE ROLE OF DKK1 AND WNT5A IN MAMMALIAN KIDNEY DEVELOPMENT AND DISEASE

*UNIVERSITY OF OULU GRADUATE SCHOOL;*
*UNIVERSITY OF OULU,*
*FACULTY OF BIOCHEMISTRY AND MOLECULAR MEDICINE;*
*BIOCENTER OULU;*
*OULU CENTER FOR CELL-MATRIX RESEARCH*

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# THE ROLE OF DKK1 AND WNT5A IN MAMMALIAN KIDNEY DEVELOPMENT AND DISEASE

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***Abstract***

This thesis focuses on mammalian kidney development and in particular on the question of how two Wnt signalling pathway genes, an antagonistic *Dkk1* and an agonistic ligand *Wnt5a*, regulate the process.

Wnts are secreted ligands that are involved in many developmental processes, including gonadal differentiation and kidney development, but also in various diseases and malformations. Wnts form a large signalling family containing 19 different glycoprotein ligands in mammals. Wnt signalling occurs via two different intracellular pathways. A canonical pathway proceeds via beta-catenin, and a non-canonical pathway utilizes other signalling molecules. Dkk1 is an antagonist of the canonical pathway and Wnt5a is considered a ligand that activates the non-canonical signalling pathway.

As part of the thesis, I have studied the role of Dkk1 in kidney morphogenesis using a conditional mouse model, in which the gene is deleted in a cell specific manner from the collecting ducts. Dkk1 deficiency increased renal papilla growth and the risk of hydronephrosis. Research pointed out that the lack of Dkk1 in the collecting ducts increased cell proliferation and disturbed the balance of canonical Wnt signalling, which led to an overgrowth of renal papilla. This led to functional phenotypes including increased water reabsorption and changes in ion secretion/ absorption. These changes are most likely due to altered Wnt7b signalling.

The second part of the thesis examines the role of the non-canonical *Wnt5a* gene in kidney development with a conventional knock out mouse model. At the time work began on the thesis, no corresponding kidney phenotype had been published. The primary finding in kidneys lacking *Wnt5a* was an altered basement membrane organization of the collecting ducts and glomeruli. The phenotype is most likely the reason behind morphological phenotypes which vary from bilateral kidney agenesis to duplex collecting system. Notably, during the course of this study we found a mutation in the human *WNT5A* gene of a CAKUT patient. This is the first time Wnts have been shown to organize kidney development via basement membrane formation.

*Keywords:* basement membrane, cell polarity, cell proliferation, collagen, Dkk1, Kidney development, laminin, Wnt-signalling, Wnt5a

**Pietilä, Ilkka, Dkk1 ja Wnt5a:n roolit nisäkkään munuaisen kehityksessä ja taudeissa.**
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Biokemian ja molekyylilääketieteen tiedekunta; Biocenter Oulu; Oulu Center for Cell-Matrix Research
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## *Tiivistelmä*

Tämän väitöskirjan tarkoituksena on ollut tutkia munuaisen kehitystä ja kuinka kaksi Wnt-signalointireitin geeniä, signalointia estävä Dkk1 ja signalointia edistävä *Wnt5a* säätelevät sitä.

Wnt ligandit ovat eritettäviä signaalimolekyylejä, jotka ovat osallisina monissa kehitysbiologissa prosesseissa kuten sukupuolen määräytymisessä ja munuaisen kehityksessä. Myös monissa taudeissa on havaittu muuntuneita Wnt geenien tuottotasoja. Wnt-geenit muodostava suuren signalointimolekyyliperheen, johon lukeutuu 19 jäsentä nisäkkäillä ja Wnt-signointi on jaettu perinteisesti kahteen signalointiryhmään. Dkk1 on kanonisen Wnt-signaloinnin estäjä ja Wnt5a:ta pidetään pääsaantiöisesti ei-kanonisena Wnt-ligandina.

Väitöskirjassani olen tutkinut *Dkk1* geenin toimintaa kohdennetussa *Dkk1*-poistogeenisessä hiiressä, jossa geenin toiminta on poistettu spesifisesti munuaisen kokoojaputkista. Dkk1:n puutos johtaa munuaisen papillan kasvuun ja lisää riskiä hydronefroksen muodostumiseen. Tutkimukset osoittivat että Dkk1:n puutos aiheuttaa lisääntynyttä solujakautumista kokoojaputkissa, jolloin Wnt-signaloinnin muutos aiheuttaa papillan ylikasvua. Ylikasvusta seuraa lisääntynyttä veden takaisin imeytymistä ja muutoksia ionien erittämisessä ja takaisin imeytymisessä. Todennäköisimmin muutokset johtuvat muuntuneesta Wnt7b signaloinnista, jota Dkk1 normaalisti säätelee.

Väitöskirjan toisessa osassa tutkittiin ei-kanonisen reitin Wnt5a ligandin roolia munuaisen kehityksessä käyttäen poistogeenistä hiirimallia, jossa Wnt5a:n roolia munuaisenkehityksessä ei ollut julkaistu työn aloituksen aikaan. Wnt5a:n puutoksen havaittiin vaikuttavan tyvikalvon järjestymiseen kokoojaputkissa ja munuaiskeräsessä. Tyvikalvon häiriö on todennäköisin syy morfologisiin muutoksiin, jotka vaihtelevat molempien munuaisen puuttumisesta kaksois-kokoojatiehyen muodostumiseen. Työssä osoitetaan ensimmäistä kertaa kuinka Wnt-signalointireitin proteiinit säätelevät munuaisen kehitystä tyvikalvon muodostuksen kautta.

*Asiasanat:* Dkk1, kollageeni, laminiini, munuaisen kehitys, solun jakautuminen, solun polariteetti, tyvikalvo, Wnt-signalointi, Wnt5a

*To my family*

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Oulu, Finland, December 2014 Ilkka Pietilä

# Abbreviations

| | |
|---|---|
| APC | Adenomatous polyposis coli |
| AQP2 | Aquaporin 2 |
| BM | Basement membrane |
| βTrCP | β-transducing repeat-containing protein |
| Ca | Calcium |
| CAKUT | Congenital Anomaly of Kidney and Urinary Track |
| CamKII | Calmodulin-dependent protein kinase II |
| CD | Collecting duct |
| CK1 | Casein kinase 1 |
| CM | Cap mesenchyme |
| CRD | Cysteine-rich domain |
| Cys | Cysteine |
| Daam1 | Disheveled-associated activator of morphogenesis 1 |
| DEP | Domain of Dishevelled |
| Dkk | Dickkopf homolog |
| Dvl | Dishevelled |
| E | Embryonic day |
| ECM | Extracellular matrix |
| ER | Endoplastic reticulum |
| Eya1 | Eyes absent homolog 1 |
| Fgf | Fibroblast growth factor |
| Fz | Frizzled |
| GBM | Glomerular basement membrane |
| Gdnf | Glial cell-derived neurotrophic factor |
| GFP | Green fluorecent protein |
| Gly | Glycine |
| GSK3 | Glycogen synthase kinase 3 |
| Hox11 | Homeobox gene 11 |
| HSPG | Heparin sulphate proteoglycan |
| IM | Intermediate mesenchyme |
| JNK | Jun kinase |
| kDa | kilodalton |
| Krm | Kremen |
| Lef-1 | Lymphoid enhancer-binding factor-1 |
| LG | Laminin globular-domain |

| | |
|---|---|
| LN | Laminin N-terminal |
| LRP | LDL receptor-related protein |
| MET | Mesenchyme epithelial transition |
| MM | Metanephric mesenchyme |
| MN | Mesonephros |
| NB | Newborn |
| NC | Nephrogenic cord |
| NC1 | Noncollagenous domain |
| Pax | Paired box |
| PCP | Planar cell polarity |
| PDZ | Domain of Dishevelled |
| PI3K | Phosphoinositide 3-kinase |
| PKC | Protein kinase C |
| PLCγ | Phospholipase-C gamma |
| PTA | Pretubular aggregate |
| Ras | Ras p21 (encode by Ras gene) G protein |
| Rho | Ras homolog gene family |
| RKT | Receptor tyrosine kinase |
| ROCK | Rho-associated kinase |
| Ror2 | Receptor tyrosine kinase-like orphan receptor 2 |
| RV | Renal vesicle |
| Ryk | Ryk receptor-like tyrosine kinase |
| Sall1 | Spalt-like transcription factor 1 |
| Scnn1b | Sodium channel, nonvoltage-gated 1, beta |
| Ser | Serine |
| sFRPs | Secreted Frizzled-related proteins |
| Slc12a1 | Solute carrier family 12, member 1 |
| TCF/LEF | T-cell factor/lymphoid enhancer-binding factor |
| Thr | Threonine |
| UB | Ureter bud |
| VEGF | Vascular endothelial growth factor |
| WD | Wolffian duct |
| WIF-1 | Wnt-inhibitory factor-1 |
| Wnt | Wingless related MMTV integration site |
| Wt1 | Wilm tumor 1 |

# List of original papers

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Pietilä I, Ellwanger K, Railo A, Jokela T, Barrantes Idel B, Shan J, Niehrs C, Vainio SJ (2011) Secreted Wnt antagonist Dickkopf-1 controls kidney papilla development coordinated by Wnt-7b signalling. Dev Biol. 2011; 353:50-60.

II Ilkka Pietilä, Kirsten Y. Renkema, Renata Prunskaite-Hyyryläinen, Susanna Kaisto, Nayia Nicolaou, Albertien M. van Eerde, Sanna Junttila, Antti M. Salo, Ilkka Miinalainen, Ernie M. Bongers, Wourt F. Feitz, Nine V. Knoers, Johanna Myllyharju & Seppo J. Vainio. (2014). Wnt5a Is Involved in Kidney Development, Disease and Control of Basement Membrane Assembly. Manuscript.

# Contents

# 1 Introduction

The kidney is a critical organ for the elimination of metabolic products, the maintenance of blood volume, the composition and pressure of the blood, bone density, and it is involved in hormone production. The kidney duct system is composed of two distinct cell populations, one forming the filtering units, called nephrons, and the other forming the ureteric bud-derived collecting duct system (Hughson *et al.* 2003). A nephron is composed of glomeruli that filter the blood to the Bowman´s capsule and tubular structures that reabsorb and concentrate primary urine. The collecting duct is a Wolffian duct-derived epithelial tube that concentrates and collects urine and transfers it via the renal pelvis into the urinary pool.

The secreted Wnt proteins activate various intracellular pathways that can be categorized into canonical and non-canonical Wnt pathways. Canonical signalling leads to the stabilization of β-catenin and induces the translocation of cytoplasmic β-catenin into the nucleus (Pinson *et al.* 2000, Reya & Clevers 2005, van Amerongen & Nusse 2009). Non-canonical pathways (PCP- and $Ca^{2+}$-pathways) do not utilize β-catenin but use other signalling molecules, including small GTPases and $Ca^{2+}$ sensitive molecules (Taki *et al.* 2003). Through these signalling pathways Wnt proteins have a variety of important roles in embryonic development including cell growth, migration, differentiation, and cell polarity generation (Logan & Nusse 2004). Malfunction of Wnt signalling leads to a diverse array of diseases including cancer, osteogenesis imperfecta, Mullerian-duct regression, and infertility (Biason-Lauber *et al.* 2004, Laine *et al.* 2013, Prunskaite-Hyyrylainen *et al.* 2014).

Dickkopfs (Dkk 1-4) are a secreted protein family with the capacity to inhibit the β-catenin mediated canonical Wnt-pathway. Dkk1 is known to be a critical inducer of head development in frogs and mice. It was first identified in the amphibian head induction (Glinka *et al.* 1998). Later research demonstrated that in mammals the Dkks are involved in the morphogenesis of limbs, bones, vertebrae, the craniofacial skeleton, the skin, the heart, and the eyes, and in certain diseases such as cancer, blindness, and bone disorders (Lin *et al.* 2010, Niehrs 2006). Even though many Wnts utilizing the canonical pathway are expressed during kidney organogenesis, the role of canonical Wnt antagonists such as Dkk1 had not been studied in kidney development before.

Wnt5a performs a critical role in embryonic development. It controls the growth of limbs and tail out from the body axis, aids face morphogenesis, and is

involved in the development of the gastrointestinal tract (Cervantes *et al.* 2009, Yamaguchi *et al.* 1999). Recent publications have demonstrated that Wnt5a is active during kidney organogenesis (Nishita *et al.* 2014, Yun *et al.* 2014). *In vitro* studies have indicated a dual role for Wnt5a in the canonical pathway: it can activate or inhibit Wnt signalling, depending on the receptor context (Mikels & Nusse 2006). Wnt5a is known to participate in developmental processes by stimulating cell fate, migration, and proliferation (Kikuchi *et al.* 2012).

The aim of the work conducted as part of this thesis was to gain a better understanding of how the two well-known Wnt-pathway components, Dkk1 and Wnt5a, regulate kidney development. To clarify the role and mechanisms involved of specific factors in kidney development and in kidney malformations. Knockout mice deficient of *Dkk1* or *Wnt5a* genes and *in vitro* models were studied in various ways.. The development and branching of the ureteric tree of the kidneys turned out to be a target of both genes.

# 2 Review of the literature

## 2.1 *Wnt* gene family

Wnts form a large group of secreted and extracellular matrix associated signalling proteins. They act via multiple receptor types and co-receptors such as the seven-pass transmembrane receptor Frizzled (Fz) (Bhanot *et al.* 1996), its co-receptor LRP5/6 (LDL receptor-related family member) (Tamai *et al.* 2000, Wehrli *et al.* 2000), and recently identified receptors Ryk (Ryk receptor-like tyrosine kinase) and Ror2 (receptor tyrosine kinase-like orphan receptor 2) (Hikasa *et al.* 2002, Lu *et al.* 2004). Wnts have pleiotropic biological activities including developmental induction, adult tissue homeostasis, and aging processes. They also play a crucial role in the development of certain diseases such as cancer and SERKAL syndrome (Angers & Moon 2009, Mandel *et al.* 2008, van Amerongen & Nusse 2009). During embryonic development Wnt ligands play a variety of important roles in cell growth, migration, differentiation, and the generation of cell polarity (Logan & Nusse 2004).

Wnt genes encode a large family of highly conserved glycoprotein molecules with 19 members in mammals (Wodarz & Nusse 1998). The Wnt proteins are approximately 40 kDa in size and composed of 350–400 amino acids, which include a signal sequence for secretion. Following the signal sequence, they carry a conserved pattern of 22–24 cysteine residues and more than 100 conserved residues evenly distributed across the whole sequence. The highly conserved spacing of cysteine suggests the involvement of intramolecular disulphide bonds in protein folding (Mikels & Nusse 2006). Other post-translational modifications for Wnts include one or more N-glygosylations and palmitoleic acid addition to highly conserved Cys-77 and Ser-206 residues (Lorenowicz & Korswagen 2009, Takada *et al.* 2006, Willert *et al.* 2003).

According to their capacity to induce secondary body axes in *Xenopus*, Wnts are historically divided into two functionally different groups: canonical (*Wnt-1, -3a, -8* and *-8b*) and non-canonical (*Wnt-4, -5a, -6* and *-11*) Wnts (Kuhl *et al.* 2000). Recently it has become increasingly clear that this kind of classification of Wnts is not sufficient, since many of them can signal either via the canonical or the non-canonical pathway depending on the cellular or receptor context (Bikkavilli *et al.* 2008, Kofron *et al.* 2007, Mikels & Nusse 2006).

## 2.2 Wnt signalling

After the identification of the first Wnt ligand (Nusse & Varmus 1982), Wnt research has focused on the β-catenin-dependent canonical signalling, which currently is the most studied and best understood Wnt signalling pathway. In recent years the understanding of the non-canonical Wnt pathway has increased significantly, but many gaps in our understanding regarding the mechanisms involved still exist (Angers & Moon 2009, van Amerongen & Nusse 2009).

Genetic and biochemical data have demonstrated that the Fz proteins are the primary receptors for the Wnts (Bhanot *et al.* 1996). Fzs are seven transmembrane receptors with a long N-terminal extension called a cysteine-rich domain (CRD), where Wnt-ligands directly bind (Bhanot *et al.* 1996, Dann *et al.* 2001, Hsieh *et al.* 1999). In addition to Wnt/Fz interactions, Fz mediated signalling requires the presence of a single-pass transmembrane molecule of the low-density lipoprotein receptor-related protein (LRP) family, *LRP5* or *6* (Pinson *et al.* 2000, Tamai *et al.* 2000). LRP5/6 co-receptors and the number of different Fz-receptors (Fz1-10 identified in humans) (Fredriksson *et al.* 2003) form a large set of ligand-receptor complexes, thus allowing cells to deliver a large variety of signals and responses (Wu & Nusse 2002). Fz activation during canonical signalling requires ligand binding (Bhanot *et al.* 1996, Rulifson *et al.* 2000).

### *2.2.1 Canonical Wnt signalling*

The canonical Wnt signalling is highly conserved between different animal species. It utilizes β-catenin (Chien *et al.* 2009). In the absence of the Wnt ligand, β-catenin is phosphorylated by a multipotent complex which contains Axin, casein kinase 1 (CK1), adenomatous polyposis coli (APC) and glycogen synthase kinase 3 (GSK3) (Fig. 1). The phosphorylation of four sites (Ser-45, Ser/Thr 41, 37 and 33) in β-catenin leads to ubiquitination mediated by β-transducin repeat-containing protein (βTrCP) and a subsequent degradation of β-catenin by the proteasome pathway. As a result the nuclear canonical pathway transcription factors (TCF and LEF) remain inactivated and the target genes are not expressed (Anastas & Moon 2013, Barker & Clevers 2006, Logan & Nusse 2004, McDonald & Silver 2009).

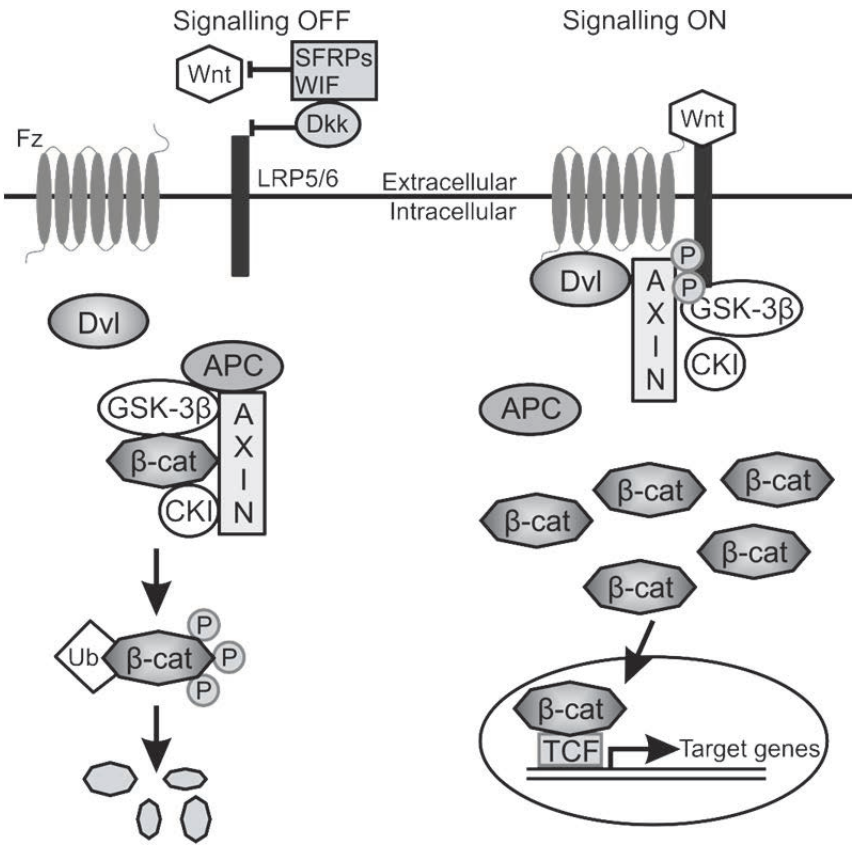


**Fig. 1. A schematic representation of canonical Wnt signalling. In the absence of a Wnt ligand, the β-catenin destruction complex is activated and after phosphorylation and ubiquitination the β-catenin is degraded (left side). When Wnt-ligand binds to Fz/LRP receptors, the Dvl mediated phosphorylation of LRP leads to Axin and GSK-3β binding to it. The β-catenin destruction complex cannot be formed and β-catenin accumulates in cytosol, translocates to the nucleus, and interacts with transcription factors.**

Binding of Wnt ligand to the Fz and LRP5/6 co-receptor complex initiates an intracellular signalling cascade in which the Wnt/Fz/LRP complex activates the Dishevelled (Dvl) to phosphorylate the LRP5/6 cytoplasmic tail. This leads to the interaction of the Axin complex with the receptor complex. As a result the β-catenin destruction complex is unable to phosphorylate β-catenin and β-catenin becomes stabilized. The process leads to an increase in free cytosolic levels of β-catenin (Anastas & Moon 2013, Logan & Nusse 2004, McDonald & Silver 2009)

which translocate into the nucleus. Binding to the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) leads to the activation of the target gene transcription (Angers & Moon 2009).

### *Inhibition of the canonical Wnt pathway*

Wnt signalling is regulated by a number of evolutionary conserved inhibitors and activators. The small Wnt signalling regulators are of great developmental importance. They can for instance control the fine-tuning of Wnt signalling during patterning of the anterior-posterior axis, somitogenesis, angiogenesis, vasculogenesis, and during the development of limbs, bones, teeth, and eyes. A common feature of the inhibitors is to antagonize Wnt signalling by preventing the ligand-receptor interaction or the maturation of Wnt receptors (Cruciat & Niehrs 2013). The six families and members of the secreted inhibitors include: Dickkopfs (Dkks), secreted Frizzled-related proteins (sFRPs), Wnt-inhibitory factor-1 (WIF-1), Wise/SOST, Cerberus, and insulin-like growth-factor binding protein 4 (IGFBP-4). There are also four types of transmembrane Wnt antagonists: Shisa, Wnt-activated inhibitory factor 1 (Waif1/5T4), adenomatosis polyposis coli down-regulated 1 (APCDD1), and Tiki1 (Cruciat & Niehrs 2013).

Dkks form a conserved family of four secreted glycoproteins. Dkk1, 2 and 4 are more related to each other than to Dkk3 (Glinka *et al.* 1998). Dkk1, 2 and 4 bind to the LRP co-receptor and inhibit canonical Wnt signalling by preventing the formation of the Wnt-Fz complex (Semenov *et al.* 2001). Dkk3 does not bind to LRP and cannot antagonise the Wnt-pathway. Dkk3 has been shown to regulate the FGF and Activin/nodal signalling in Xenopus (Pinho & Niehrs 2007). In addition to their interaction with LRPs, Dkks can also interact with another class of receptor called Kremen (Krm). Kremen is a single-pass transmembrane protein (Mao *et al.* 2002). When Kremen is present the Dkk1 can form a ternary complex with Krm and LRP, which induces a rapid endocytosis and the removal of LRP from the plasma membrane. This prevents the formation of a Fz-LRP-Wnt-complex (Mao *et al.* 2002).

### *Dkk1*

Dkk1 is composed of 266 amino acids of which the 3D structural organization is known (Ahn *et al.* 2011). After translation Dkk1 is glycosylated at several sites and is then secreted to the extracellular space (Semenov *et al.* 2001). Expression

of DKK1 is related to numerous diseases in adults and to abnormalities originating prenatally. In adults, Dkks are implicated in bone formation and bone disease, cancer, and Alzheimer disease (Aguilera *et al.* 2006, Caricasole *et al.* 2004, Li *et al.* 2006).

### *2.2.2 Non-canonical Wnt signalling*

The two non-canonical pathways, Wnt/PCP and Wnt/$Ca^{2+}$, do not require β-catenin for their signal transduction (Fig. 2). Over recent years the knowledge of these pathways has increased significantly, but many main events and components are still poorly described. The non-canonical Wnt pathways are involved in the control of cell polarity, cell movements during gastrulation, orientation of the hair bundle in the inner ear sensory cells, neural tube closure, cilia motility in the embryonic node, organogenesis of the kidney, and of many related processes (Belotti *et al.* 2012, Chien *et al.* 2009, Habas & Dawid 2005, Karner *et al.* 2009, Simons & Mlodzik 2008).

The non-canonical Wnts activate the PCP pathway by binding to the Fz receptor on the cell membrane. This is followed by the binding of Dishevelled to the C-terminal site of the Fz. In the non-canonical pathway this is suggested to take place independently of LRP5/6 (Boutros *et al.* 1998, Gao & Chen 2010). Dishevelled activates two parallel but independent pathways via small GTPases Rho and Rac (Dale *et al.* 2009, Habas & Dawid 2005, Kikuchi *et al.* 2012). Disheveled-associated activator of morphogenesis 1 (Daam1) is required for the Rho-mediated but not Rac-mediated PCP signalling. Daam1 has to bind to the Fz linked Dishevelled on its PDZ domain to activate the pathway. In the end of the pathway Rho-associated kinase (ROCK) becomes activated and mediates cytoskeletal reorganization, which enables cell movement. An alternate PCP pathway is activated by the binding of Rac to the DEP domain of the Dishevelled. The final steps in the Rac mediated PCP pathway include the activation of Jun kinase (Jnk) (Chien *et al.* 2009, Habas & Dawid 2005, Kikuchi *et al.* 2012, Taki *et al.* 2003).

The Wnt/$Ca^{2+}$ pathway was discovered when injection of Wnt5a or Wnt11 into a single cell stage zebrafish embryo resulted in an increase of $Ca^{2+}$ levels in the blastocyst embryo (Kohn & Moon 2005). The Wnt/$Ca^{2+}$ pathway is activated when Wnt ligand binds to the Fz receptor and recruits Dishevelled and G-proteins, which leads to an increase of free intracellular $Ca^{2+}$ and the activation of two $Ca^{2+}$ -dependent enzymes: protein kinase C (PKC) and calmodulin dependent

protein kinase II (CamKII)(Habas & Dawid 2005, Kohn & Moon 2005, Taki *et al.* 2003). In the end of the long and to this day poorly understood signalling cascade, Wnt/$Ca^{2+}$ pathway activation triggers cytoskeleton reorganization and/or calcium-mediated transcription (Gao *et al.* 2011, Yang *et al.* 2011).

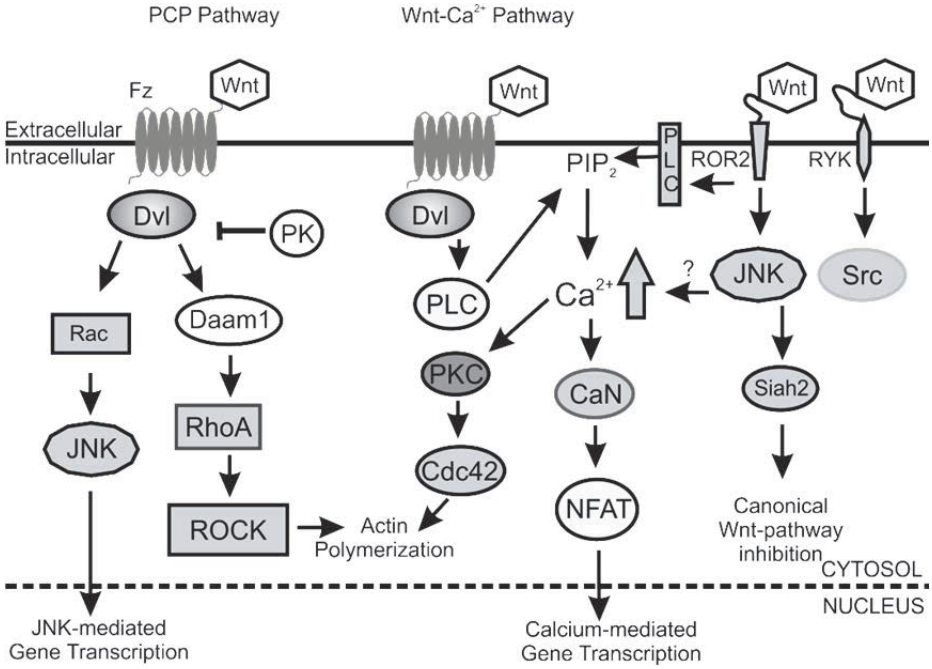


**Fig. 2. A schematic representation of non-canonical Wnt signalling. Non-canonical Wnt signaling can be initiated by different Fz receptors or recently identified alternative receptors. In the Wnt/PCP pathway the activation of small GTPases Rac and Rho leads to target gene transcription or cellular events such as cytoskeletal rearrangement. The Wnt/$Ca^{2+}$-pathway becomes activated when a Wnt ligand binds to Fz, which leads to elevated intracellular $Ca^{2+}$ levels causing the activation of calsium-sensitive proteins and in the end target gene translation and/or actin polymerization. Non-canonical Wnt signalling can also act via the newly identified Ror2 and Ryk-receptors.**

Recently two additional non-canonical signalling pathways were reported. Wnt-ligand may bind either to a tyrosine kinase-like orphan receptor 1/2 (Ror 1/2) to activate JNK or to the tyrosine kinase receptor (RYK) (Kikuchi *et al.* 2012, Kohn & Moon 2005). Ror2 mediated Wnt5a signalling has been shown to inhibit canonical Wnt signalling (Mikels & Nusse 2006) needed to regulate the

intermediate mesoderm extension and thus inhibits kidney development (Yun *et al.* 2014). RYK has also been shown to regulate the position of the metanephric mesenchyme (MM) by controlling the cell proliferation in the intermediate mesoderm (Nishita *et al.* 2014). In axon guidance Ryk- mediated signalling transmits the Wnt signal through the Ras oncoprotein (Schmitt *et al.* 2006).

Non-canonical Wnts are known to have the capability to inhibit the canonical Wnt signalling. Wnt5a has been the first "non-canonical" Wnt shown to be able to activate the canonical signalling pathway *in vitro* when *frizzled-4* and its co-receptor *LRP5* are over-expressed (Mikels & Nusse 2006).

## 2.3 Extracellular matrix

The extracellular matrix (ECM) is a combination of secreted molecules which provide biochemical and structural support to the surrounding cells (Michel *et al.* 2010). The ECM can be divided into the interstitial matrix and the basement membranes (BMs). The interstitial matrix is composed of polysaccharides and fibrous protein gels that fill the interstitial space and act as a compression buffer against the physical stress. BMs are sheet-like depositions of ECM on which various epithelial and endothelial cells, Schwann cells, muscle cells, and fat cells rest (Frantz *et al.* 2010, Timpl & Brown 1996).

### *2.3.1 Basement membrane*

Basement membranes are layered cell-adherent ECMs that form part of the tissue architecture and contribute to both embryonic tissue and organ differentiation and the maintenance of adult functions (Yurchenco 2011). The BM follows the plasma membrane, protects tissue against disruptive physical stress and provides an interactive interface between cell and surrounding environment. BMs can mediate local and distant signals within and between the cellular compartments (Yurchenco 2011).

In BMs two layers can be discerned using electron micrographs: an electron dense lamina densa and a more transparent lamina lucida (Miosge *et al.* 2001). Most of the BMs are 50–100nm thick but some special BMs, like the glomerular basement membrane (GBM), are thicker. Laminin, collagen IV, nidogen, and the heparin sulphate proteoglycans (HSPGs) perlecan and agrin are the main components of the BM (Timpl & Brown 1996).

The laminin and collagen IV isoforms show differences in their assembly, receptor binding, and subsequent cross-linking. This allows for variations in the final structure, signalling properties, and stability of the BM. BMs can also contain variable amounts of collagen XVIII, XV, and VI, fibronectin, usherin, bamacan, nephronectin, papilin, netrins, and other components adding additional receptor-binding activities and/or modulatory structures (Yurchenco 2011).

### *Laminins*

All laminins in mammals are heterotrimers composed of one of the five α chains, one of the three β chains, and one of the three γ chains. Out of all possible combinations, a total of 16 laminin isoforms have been characterized biochemically (Aumailley *et al.* 2005). Trimers, depending on the αβγ chain composition, resemble Y-shaped, or rod-shaped structures (Fig 3). Each chain has a laminin coiled-coil domain (Paulsson 1992) and interactions among three coiled-coil domains and limited interchain covalent bonding contribute to the formation of the long arm of all laminin trimers (Ekblom & Timpl 1996, Paulsson 1992).

The short arm is formed from the remaining segment of each chain and contains α laminin N-terminal (LN) domain, which plays an essential role in polymerization of trimers to form a functional network (Cheng *et al.* 1997, Yurchenco & Cheng 1993). Laminin α chains differ from the β and γ chains. The α chains contain a C-terminal laminin globular (LG) domain, which is composed of five tandem subdomains important for cell-matrix interactions (Timpl *et al.* 2000).

In BMs LG domain links laminin trimers to neighbouring cells by serving as a ligand for two major cellular receptors: dystroglycan and the integrins (Colognato & Yurchenco 2000, Henry & Campbell 1999). Dystroglycan binds to the LG-domain of the α chain, but studies have shown that deletion of dystroglycan in most of the kidney cells does not result in visible kidney defects (Jarad *et al.* 2011). On the other hand, the deletion of the main integrin expressed in podocytes (α3β1) leads to severe developmental kidney defects (Kreidberg *et al.* 1996). Integrins are transmembrane heterodimers important for cell-to-ECM signalling and direct cell-cell signalling (Hynes 2002).

After secretion to extracellular space laminin trimers self-polymerize to form a laminin network by interaction through their LN domains (Cheng *et al.* 1997). This polymerization step is a reversible, calcium-dependent process that requires

a critical concentration of laminin trimers to form an initiating complex (Yurchenco & Cheng 1993). This initial step is facilitated by the binding of laminin trimers to laminin receptors via the α chain LG domain (Colognato *et al.* 1999). This leads to an increase in local laminin trimer concentration and triggers the laminin polymerization. After the laminin polymerization and the formation of a primary network other BM components (Type IV collagen, nidogen, and sulfated protegycans) interact with the complex and initiate the assembly of the proper BM (Poschl *et al.* 2004).

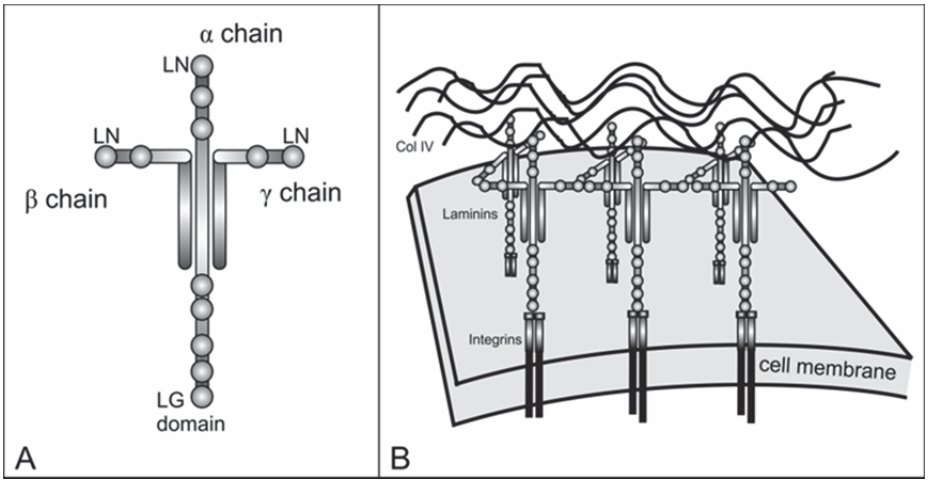


**Fig. 3. Schematic drawing of laminin and the laminin BM. A) Laminin molecule composed of αβγ-chains. The chains interact with each other within the LN domains and the LG domain of the α-chain binds to cell receptors. B) On a cell surface the accumulation of laminin molecules triggers their autopolymerisation and the formation of the primary BM, where for instance type IV collagen can bind.**

### *Type IV collagen*

Polymerized laminin forms the primary BM with an average pore size of 30 nm (Yurchenco *et al.* 1992). It is then that type IV collagen, the most abundant BM protein, starts to assemble in the network. Type IV collagen is also a trimeric protein composed of three α chains. There are altogether six α chains (α1 – α6). Of these only three folded trimer compositions, called protomers, have been identified in the BM; α1α1α2, α3α4α5, and α5α5α6. The most common variant

found in nearly all BMs consists of two α1 subunits and one α2 subunit (Timpl *et al.* 1981, Yurchenco & Furthmayr 1984). As opposed to fibril-forming collagens in the type IV collagen molecule, the GLY-X-Y repeat regions have multiple interruptions. The interruptions make the protomer more flexible than fibrillar collagens. This is an important feature enabling the dynamic structure of the BM (Timpl *et al.* 1981).

Each α chain of type IV collagen has a conserved structure containing three domains: the N-terminal 7S domain, the Gly-X-Y repeats, and the noncollagenous domain (NC1) at the C-terminus. The collagens are assembled inside ER and secreted into the extracellular space, where they self-polymerize into a network through hexameric and dodecameric interactions involving the NC1 and 7S domains. The collagen network becomes heavily cross-linked through disulphide bonds and lysyloxidase- mediated crosslinks (Hudson 2004, Vanacore *et al.* 2009). One of the key enzymes in the post-translational modifications of collagen is prolyl-4-hydroxylase. The enzyme plays a crucial role in collagen biosynthesis, because the 4-hydroxyproline residues produced in the modification are an absolute requirement for the folding of the newly synthesized procollagen polypeptide chains into triple-helical molecules (Pajunen *et al.* 1989).

Although knock out of the genes encoding the α1 and α2 subunits does not prevent BM assembly in most mouse tissues, it does cause lethality at E10,5–11,5 apparently due to the loss of BM stability (Poschl *et al.* 2004).

### *Nidogens*

Nidogen1 and 2 are homologous glycoproteins containing three globular-like domains separated by two rod-like domains (Kohfeldt *et al.* 1998). Studies have revealed that nidogen-1 binds both to laminin γ1 and type IV collagen. This has led to the hypothesis that nidogens act as a bridge linking the separate laminin and collagen networks in the BM. (Miosge *et al.* 2001).

The deletion of either nidogen-1 or -2 has not been shown to cause any significant developmental anomalies, but the double knockout mice exhibit perinatal lethality due to lung and heart malformations. Although BMs are forming normally in the absence of nidogen-1 and -2, the data suggests that nidogens are providing extra stability to the BMs under unusual stress even though they are not required for the initial formation of the BM (Bader *et al.* 2005, Miosge *et al.* 2001).

*Heparan sulphate proteoglycans (HSPGs)*

Heparan sulphate proteoglycans are composed of sulphated glycosaminoglycan side chains linked to a core protein. Perlecan and agrin are the two main HSPG in BMs. While perlecan is more common in the BM and in the mesangial matrix, agrin is the major HSPG found in the GBM (Groffen *et al.* 1998). Studies have shown that the sulfated glycosamionoglycan side chains of the agrin molecule give it a highly negative charge for the GBM (Harvey *et al.* 2007, Rennke *et al.* 1975). The N-terminal domain of the agrin binds to the LN-521 long arm and its C-terminus domain can bind to cell surface receptors such as dystroglycan and integrins (Bezakova & Ruegg 2003). These studies suggest that agrin could have an important role in mediating charge selectivity within the glomerular filtration barrier and in linking the GBM to the adjacent cells. Heparan sulphate proteoglycans can also bind growth factors such as VEGF in GBM (Eremina *et al.* 2003, Park *et al.* 1993).

### *2.3.2 Glomerular basement membrane*

The glomerular basement membrane (GBM) is a 250 nm to 400 nm thick meshwork of ECM components. It forms an interface between the fenestrated endothelial cells and podocyte foot processes (Miner 2011). Both endothelial cells and podocytes produce BM components which fuse and form the GBM. The three components form a glomerular filtration barrier. The mature GBM is composed primarily of four ECM molecules: laminin-521, type IV collagen α3α4a5, the HSPG agrin, and nidogen (Miner 2011).

The GBM serves multiple functions. It provides structural support for the glomerular capillaries and harbors ligands for the receptors on the surface of the adjacent endothelial cells, podocytes, and mesangial cells (Miner 2005, Yurchenco & Patton 2009). The GBM also restricts the passage of plasma proteins across the glomerular filtration barrier. Mutations in the major GBM proteins are known to cause human diseases such as Alport´s and Pierson syndrome (Kruegel *et al.* 2013, Matejas *et al.* 2010), as part of which proteins leak into urine. However, mutations are not the only mechanism causing the changes in the GBM composition. For example in the diabetic nephropathy, microenvironmental changes lead to a dramatic thickening of GBM and an increase in membrane pore size (Jefferson *et al.* 2008).

## 2.4 Kidney development (pro-, meso-, and metanephros)

During mammalian kidney development embryos have two transient kidneys (the pro- and mesonephros) before the permanent kidney (the metanephros) develops. These remnants of mammalian evolution represent functional kidneys in more primitive life forms (Saxen & Sariola 1987).

The classic work of Saxén indicated that kidney organogenesis is regulated by sequential and reciprocal cell and tissue interactions. These interactions occur for the most part between the epithelial Wolffian duct (WD) -derived ureteric bud (UB) and the metanephric mesenchyme (MM). Both of these tissue types are derived from the intermediate mesoderm (IM), which lies between the paraxial and lateral plate mesoderm and specializes during gastrulation (Obara-Ishihara *et al.* 1999). The kidney vasculature is thought to originate from the IM but also from the angioblasts and the endothelial cells that migrate to the kidney organ primordial at the start of organogenesis (Hyink & Abrahamson 1995).

Initiation of primitive kidney development is characterized by the formation of the WD from the dorsal and anterior IM cells. This step requires the expression of *Sim-1* and *Pax-2* in the IM, which is induced by BMP4 secreted from the surface ectoderm (Fig. 4) (Obara-Ishihara *et al.* 1999). As these specialized mesenchymal cells epithelialize, they start to form the nephric duct. In association with this process the mesenchyme cells at the ventral intermediate mesoderm construct the “nephrogenic cord” (NC) (Obara-Ishihara *et al.* 1999, Torres *et al.* 1995).

In mammals interactions between the NC and the WD generate the first transient kidney, the pronephros. In fish and amphibians the pronephros represents the permanent kidney and it remains functional throughout adulthood. In mammals however, the WD elongates caudally and induces formation of a second kidney, the mesonephros (MN). The MN serves as the main excretory organ in aquatic vertebrates. The MN represents a transient organ rudiment in reptiles, birds, and mammals. Although it is transient, the MN is critical in mammals for the formation of the cortical cells in the developing adrenal gland and the somatic cells in the developing male gonad. Once the WD has reached the rostro-caudal extreme of the mammalian embryo, the WD makes a dorsal outgrowth and forms the UB. The UB induces nephrogenesis in the adjacent predetermined MM. By this stage the kidney has evolved gradually from its primitive form and has contributed not only to the development of other

urogenital system derived organs, namely the gonad, and adrenal gland, but also to hematopoiesis (Costantini & Kopan 2010)

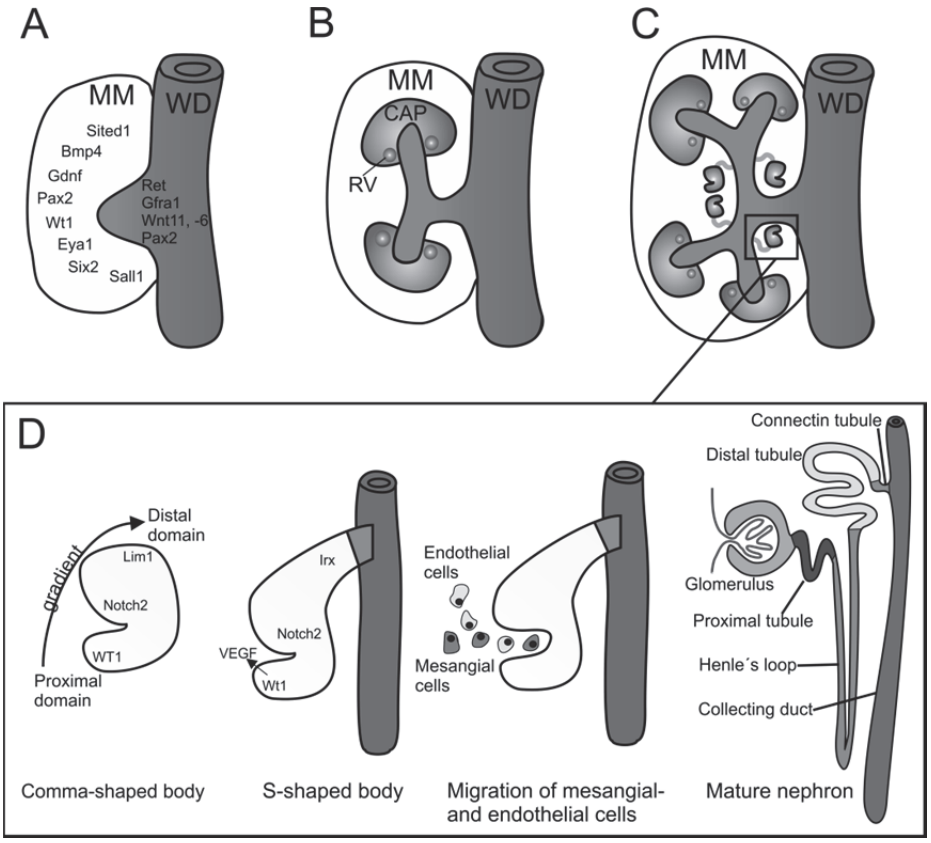


**Fig. 4. Development of the metanephros. A) An interplay between secreted signals and transcription factors both in the UB and MM is essential for the initiation of organogenesis. B) The UB enters to the kidney mesenchyme and makes the first T-type bifurcation. At the same time the UB induces condensation of the MM cells to form a CAP mesenchyme. The CAP cells contain the progenitor/stem cells of the nephrons. C) The epithelialized RV cells aggregate and initiate nephron formation. D) Proximal and distal specific genes form a gradient and polarise the comma-shaped body prior to the nephron becoming segmented. This leads to the specification of the glomerulus, proximal tubules, loop of Henle, distal tubule and connecting tubule. The collecting duct drains the concentrated urine to the renal pelvis and from there via the ureter to bladder.**

### 2.4.1 Formation of ureteric bud and induction of the metanephric mesenchyme

The mammalian kidney is organized into cortex and medulla. While the cortex is the place for primary filtration, the medulla is responsible for the concentration of the primary urine (Yu *et al.* 2009). The medulla contains the collecting ducts, loops of Henle and interstitium (Fig 5) (Little *et al.* 2007). In mice the development of the metanephros begins at E10.5–11 when the UB grows from the Wolffian duct into the MM in response to inductive signals mediated by growth factors (Reidy & Rosenblum 2009). The first visible morphological changes in UB development are visible at E10-10.5 by the broad swelling of the Wolffian duct at the level of the highly restricted MM. During the UB formation the Wolffian duct cuboidal epithelium has a short transient face of pseudostratified epithelium before returning back to the simple cuboidal epithelium of the UB (Bort *et al.* 2006, Costantini & Kopan 2010). The signals that induce the pseudostadified epithelium formation are largely unknown, but they seem to originate from the adjacent MM. It has been shown that mice lacking Osr1, which is expressed in the MM, fail to form the transient epithelial pseudostadification (Mugford *et al.* 2008).

At E11 the UB begins to emerge from the swollen Wolffian duct and grows dorsally into the MM. The UB is an epithelial tube with a continuous lumen and it is surrounded by a basement membrane (Chi *et al.* 2009, Meyer *et al.* 2004). As the UB elongates, it differentiates into two domains that differ in patterns of gene expression and developmental fates. The distal tip of the UB will begin to branch and give rise to the collecting duct system, while the trunk of the initial UB will give rise to the ureter and pelvis (Costantini & Kopan 2010, Shakya *et al.* 2005). The Gdnf/Ret pathway is a critical regulator of UB outgrowth and branching. Gdnf, secreted by the MM, activates a Gfra1/Ret receptor tyrosine kinase complex in the Wolffian duct, initiating a signalling cascade that up-regulates expression of the Ret receptor and triggers outgrowth of Ret positive cells from the WD towards the GDNF signal (Costantini & Kopan 2010). Loss of any of the genes mentioned above leads to almost complete penetrant failure of UB outgrowth and resulting kidney agenesis in mouse development (Jain *et al.* 2006).

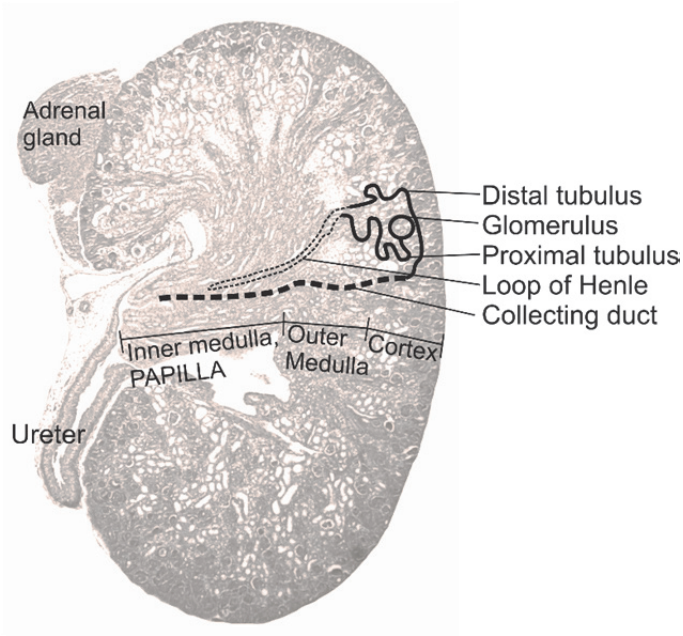


**Fig. 5. Schematic drawing of nephron components and their localization in the kidney. The kidney cortex contains glomeruli, proximal tubules, the main part of the distal tubules, and the connecting tubule. The medulla contains the interstitial stroma, part of the distal tubules, Loops of Henle, and collecting ducts.**

### *2.4.2 Formation of medulla and collecting duct system*

In the development of the collecting ducts, continuous dichotomous branching and elongation are the two main processes (Cebrian *et al.* 2004, Yu *et al.* 2009). The UB undergoes many iterations of branching morphogenesis to create the intrarenal collecting system composed of the collecting ducts (CD), calyces, and the pelvis, as well as forming the ureter and the bladder trigone (Smeeton *et al.* 2010).

After the UB has invaded the MM, it goes rapidly through nine cycles of branching events between E11.5 and E15.5, making about 350 to 500 tips (Cebrian *et al.* 2004, Short *et al.* 2010). The first UB branching event is always bifurcation, usually followed by trifurcation at each of the first two tips (Majumdar *et al.* 2003). The branching then proceeds via bifurcation. Each tip

first swells to form a rounded ampulla, which is then remodelled to form two or three tips that extend to generate new branches.

From the branching initiation the UB tips and trunks exhibit different patterns of gene expression, and the trunk cells differentiate (Michael *et al.* 2007, Mikels & Nusse 2006, Pepicelli *et al.* 1997, Short *et al.* 2010). After E15.5 the branching rate slows down and the trunk elongates extensively, generating the collecting duct, renal medulla and papilla. Generation of new nephrons continues until postnatal day 4 and the branching likely stops at the same time (Hartman *et al.* 2007).

#### *Mechanisms in the Ureter bud branching*

Branching occurs by growth and remodelling of the existing epithelium, which retains a continuous lumen throughout its length (Davies *et al.* 1995, Meyer *et al.* 2004). In this regard the branching is similar to that of the lung but different from the salivary gland, the mammary gland, the pancreas, or blood vessel formation (Lu *et al.* 2006).

Our understanding of the cellular mechanisms that underlie UB branching has remained very limited. Cell proliferation clearly contributes to the growth of the UB tree, which increases vastly in size during kidney development. During the rapid branching phase, cell proliferation is highest in the tips and lowest in the trunk, suggesting that the tip cells are the main progenitor cell population for UB growth. Time-lapse studies with GFP labelled tip cells indicate that these cells give rise to new tip cells and also contribute to the production of new daughter cells for the trunk elongation (Shakya *et al.* 2005). Tip cells remain relatively undifferentiated, while the trunk cells begin to express genes characteristic of the mature collecting ducts (Caruana *et al.* 2006, Schmidt-Ott *et al.* 2005).

### *2.4.3 Molecular signals that control ureteric bud growth and branching*

The discoveries that MM induces the UB growth and branching and the reciprocal ability of the UB to induce nephrogenesis were some of the earliest and most important findings in the field of kidney development (Saxen *et al.* 1986, Saxen & Sariola 1987). The MM further imposes a pattern on the UB branching, possibly by the local expression of stimulatory or inhibitory growth factors. According to this model the MM cells surrounding the UB tips produce factors

promoting growth and branching, while nephron epithelial and stromal cells surrounding the UB trunks may promote elongation and suppress branching (Sweeney *et al.* 2008). Many signals form the MM that influence UB growth and branching are the same factors known to control budding from the ND (e.g. GDNF, FGFs, BMPs), but there are also factors that do not play any role in the initial budding (Chi *et al.* 2011, Hartwig *et al.* 2008, Perantoni *et al.* 2005).

### *GDNF/RE-signalling in kidney development*

GDNF/RET signalling is highly important during primary bud formation and continues to be important in the UB branching throughout kidney development (Costantini & Shakya 2006). It has been shown that the same genes that control *Ret* expression in ND also act in the UB tips. Numerous studies have shown that Ret signals through many pathways including ERK MAP kinase, PI3K, and PLCγ pathways (Ihermann-Hella *et al.* 2014, Watanabe & Costantini 2004). Inhibition and knockout studies have shown that they all contribute to common UB branching (Fisher *et al.* 2001, Jain *et al.* 2006, Watanabe & Costantini 2004). A major role of GDNF/RET signalling is to upregulate UB tip target genes including *Ertv4, Etv5, Wnt11, Spry1, Met,* and *Mmp14* (Basson *et al.* 2005, Lu *et al.* 2009, Pepicelli *et al.* 1997). Among these known GDNF/RET target genes, only *Etv4* and *-5* are required for the UB outgrowth from the ND (Lu *et al.* 2009).

### *Wnt signalling pathway*

The Wnt signalling family is strongly present in the developing kidney. Wnts are expressed both in the ureteric bud (*Wnt5a, -5b, -6, -9b* and *-11*) and in the metanephric mesenchyme, (*Wnt2b, -4* and *-5a*). Wnt signalling between these cell types is known to induce and regulate organ growth and nephrogenesis (Carroll *et al.* 2005, Itaranta *et al.* 2002, Kispert *et al.* 1996, Kispert *et al.* 1998, Lin *et al.* 2001). Wnt5a is the most recently recognized member of the Wnt family involved in kidney development (Li *et al.* 2013, Nishita *et al.* 2014, Yun *et al.* 2014).

*Wnt4* is expressed in the condensing mesenchyme and the comma- and S-shaped bodies. *Wnt4* is compulsory for the epithelialization of condensed mesenchyme (Stark *et al.* 1994). Later research showed that *Wnt4* is expressed in the nephron precursor cells and that it is important for their development into nephrons (Kispert *et al.* 1998).

*Wnt7b* is expressed in the collecting duct epithelium from E13.5 onward. *Wnt7b* has a unique role in cortico-medulla axis formation. In the absence of *Wnt7b* the cortical epithelial development is normal, but the medullary zone fails to form, and urine fails to concentrate normally. *Wnt7b* is also essential for the coordinated growth of the loop of Henle, which elongates parallel to the collecting duct epithelium (Yu *et al.* 2009). Wnt7b activity is essential for orientation of the cell cleavage plane in the collecting duct epithelium and for normal mitogen activity in the loop of Henle. The role of the interstitial mesenchyme in the process is not well known, but it may be a critical mediator of these actions (Yu *et al.* 2009).

*Wnt9b* is expressed in the inductive epithelia of the UB and is crucial for the development of the transient mesonephric and permanent metanephric tubules and the extension of the Müllerian duct. *Wnt9b* signalling is implicated as a ureteric bud-derived inducer of nephrogenesis. *Wnt9b* is required for the first inductive response in the metanephric mesenchyme i.e. the formation of the pretubular aggregate (Carroll *et al.* 2005).

### *Wnt5a-ligand*

WNT5A has a 35 peptide long signal sequence, which is followed by a 25 amino acids long propeptide. The protein itself has 318 amino acids and has two known isoforms (Clark *et al.* 1993), but the reason for the variants is unknown. From the sequence similarity with better identified proteins, it has been deduced that Wnt5a goes through post-translational modifications including multiple palmitoylation and glycosylations (Clark *et al.* 1993).

The importance of *Wnt5a* gene function in mice has been shown for the development of the skeleton (Yamaguchi *et al.* 1999), midbrain (Andersson *et al.* 2008, Andersson *et al.* 2013), mammary gland (Roarty & Serra 2007), uterus (Mericskay *et al.* 2004), prostate (Huang *et al.* 2009), intestinal elongation (Cervantes *et al.* 2009), male spermatogonial stem cells (Yeh *et al.* 2011), and ovarian follicles by Naillat and excellent colleagues (Naillat *et al.* 2010). Recent publications have indicated the importance of Wnt5a for kidney development (Nishita *et al.* 2014, Yun *et al.* 2014). Wnt5a has been shown to inhibit type II collagen expression in chondrocytes (Yang *et al.* 2003). *Wnt5a* expression has also been demonstrated in many kidney cancers (Rao *et al.* 2010). The best described disease caused by Wnt5a mutation is Robinow syndrome, which is

characterized by short-limb dwarfism, costovertebral segmentation defects, and abnormalities of the head, face, and external genitalia (Person *et al.* 2010).

### *Elongation of the ureteric bud into collecting duct*

During late embryonic development the UB trunks elongate extensively, forming the CDs of the renal medulla and papilla. This process, together with the concurrent elongation of the loops of Henle from nephrons in the cortex, forms the cortico-medullary axis of the kidney along which the CDs and nephrons are patterned.

Orientation of the mitotic spindle is an important mechanism of CD elongation during development of the medulla and papilla (Yu *et al.* 2009), and also during postnatal growth of the kidney (Fischer *et al.* 2006, Karner *et al.* 2009). The plane of cell division is controlled by Wnt7b and Wnt9b (Karner *et al.* 2009, Yu *et al.* 2009). Normally, most mitotic spindles are oriented approximately parallel to the long axis of the duct. The separation of daughter cells during cytokinesis drives the ductal elongation with little or no increase in the diameter as a result. In mice lacking Wnt7b, the kidney medulla and pelvis fail to develop, and the CDs that normally form them are abnormally short and wide. Most likely this is due to a change in orientation of the cell division plane. *Wnt7b*, which is expressed in the UB trunks, is thought to signal to the adjacent interstitial cells via the canonical Wnt pathway. Via an unknown mechanism interstitial cells may signal back to the UB to promote oriented cell division (Yu *et al.* 2009). *Wnt9b*, also expressed in the UB trunks, is required for the normally oriented cell division during the development of late fetal and postnatal CDs. The action is mediated through a non-canonical WNT/PCP pathway involving RHO and JNK (Karner *et al.* 2009).

A second cellular mechanism of CD elongation during late fetal development apparently includes convergent extension. This was concluded from changes in the orientation of elongated cells in the CDs of Wnt9b mutants (Karner *et al.* 2009).

Many studies have shown the importance of the cell survival signals provided by Wnt7b, Hgf, Egf, and laminin in the formation of the medullary and papillary CDs (Kreidberg *et al.* 1996, Liu *et al.* 2009, Wu *et al.* 2009, Yu *et al.* 2009). Mice lacking either Wnt7b (Yu *et al.* 2009) or Egfr (Zhang *et al.* 2010) display increased apoptosis in the papillary CDs. The importance of ECM in the CD survival was studied by analysing the mutants for the integrin genes (Itga3 and

Itgb). ECM gene Lam*a*5, and ECM-cell signal transducer MET suggest that ITGA3/ITGB1 integrin acts together with MET to maintain Wnt7b expression, and thus promotes CD cell survival (Kreidberg *et al.* 1996, Liu *et al.* 2009, Wu *et al.* 2009).

### *2.4.4 Nephron progenitor cells and Nephrogenesis*

Nephron development is driven by epithelial-mesenchymal interactions between stem/progenitor cells within the ureteric bud epithelium and the adjacent cap mesenchyme (CM) (Costantini & Kopan 2010) (Fig. 4). As indicated earlier, GDNF and FGF signals secreted by the CM induce repetitive branching of the ureteric epithelium, elaborating the renal collecting duct network (Park *et al.* 2012). The ureteric epithelium secreted Wnt9b induces CM progenitors to differentiate into epithelial renal vesicle (RV) cells (Carroll *et al.* 2005). The process triggers sequential morphological and gene expression changes. First, a clustered pretubular aggregate (PTA) undergoes epithelisation into the circular renal vesicle (RV). Next, polarization of the RV forms the comma and S-shaped body and segmentation into specialized glomerular podocytes, the distal convoluted tubule, loop of Henle, and the proximal convoluted tubule of the nephron follows (Fig 4) (Little *et al.* 2007).

#### *Nephron progenitor cells*

At the tips of the branched UB nephron progenitor cells locate adjacent to the tip and cluster to form pretubular aggregates before their transition into epithelial RV. MM cells outside the tip area and towards the cortex remain undifferentiated. The CM receives signals from the UB and is triggered to adopt one of two independent fates: to undergo self-renewal to maintain the progenitor pool or to initiate a mesenchymal-epithelial transition (MET) to differentiate into the segmented nephron (Basta *et al.* 2014). The maintenance of CM progenitors ensures continued ureter branching through the production of branching factors and the cellular templates for new waves of nephrogenesis until the progenitor population is exhausted in the prenatal or early postnatal period (Park *et al.* 2012).

The MM contains a set of nephron progenitor cells that express a number of factors with integral roles in kidney development. These factors include: *Six1, Cited1, Pax2, Hox11*, *Osr1, Sall1, Six2, Eya1,* and *Wt1*. The depletion of each of them in the mammalian kidney results in insufficient kidney development.

Deletion of other genes than *Six2* and *Sall1* leads to the loss of progenitor cells and total lack of nephrons (James *et al.* 2006, Kobayashi *et al.* 2008, Kreidberg *et al.* 1993, Nishinakamura *et al.* 2001, Self *et al.* 2006, Torres *et al.* 1995, Wellik *et al.* 2002, Xu *et al.* 1999). In contrast, the entire nephrogenic mesenchyme of *Six2* and *Sall1* mutants commit to the nephron formation at the onset of kidney development. This will prematurely terminate the nephrogenic program with only a small number of renal vesicles. This indicates that *Six2* and *Sall1* have a unique role in promoting the self-renewal of the progenitor population (Basta *et al.* 2014, Kobayashi *et al.* 2008, Self *et al.* 2006). *Six2* is autoregulated through a proximal enhancer (Park *et al.* 2012). Gene knockout studies have shown that *Sall1* is expressed upstream of *Six2* and might be needed to maintain an appropriate level of expression in the CM so that at each UB tip only a subset of cells are committed to differentiation with the remainder undergoing self-renewal (Basta *et al.* 2014).

### *Nephrogenesis and the formation of the renal vesicle*

Nephrogenesis is initiated when the CM progenitor cells receive an inductive Wnt9b signal from the UB tip (Fig. 4) (Carroll *et al.* 2005). Downstream of Wnt9b acts another Wnt pathway component, Wnt4 and a member of the FGF family Fgf8 during transition of the induced CM into RVs (Grieshammer *et al.* 2005, Perantoni *et al.* 2005, Stark *et al.* 1994). Genetic analyses of these two genes pointed out that Fgf8 locates upstream of Wnt4 (Grieshammer *et al.* 2005, Perantoni *et al.* 2005). Wnt4 is required specifically for the activation of the LIM homeobox 1 (Lhx1), a transcriptional determinant of the distal nephron fates (Kobayashi *et al.* 2005). Canonical Wnt signalling directed by β-catenin is necessary and sufficient to mediate the essential early inductive actions of Wnt9b and Wnt4. Genetically elevated expression of β-catenin in the pretubular aggregate can mimic *Lhx1* actions (Park *et al.* 2007), but constituent canonical pathway activation blocks MET. In the final phase of epithelialization Wnt4 likely utilizes an alternative mechanism. This conclusion is supported by the analysis of the calcium/NFAT pathway in the process (Burn *et al.* 2011, Dudley & Robertson 1997, Godin *et al.* 1998, Park *et al.* 2007, Tanigawa *et al.* 2011). After the mesenchymal cells have started to aggregate, Bmp7 is expressed in the CM differentiating nephron progenitor cells and in the ureter epithelium (Dudley & Robertson 1997, Godin *et al.* 1998).

Soon after birth mouse kidneys go through the final wave of nephrogenesis and by postnatal day 3 all *six2* positive cells are lost. Currently it is not known what causes the cessation of nephrogenesis (Hartman *et al.* 2007).

### *Nephrogenesis at comma shape stage*

The epithelial RV forms a lumen (Stage I nephron) and begins to 'unwind' to form comma-shaped and S-shaped bodies (Stage II) (Fig. 4). The forming nephron vascularises at the proximal end (Stage III) and matures to nephron (Stage IV). The formed nephron fuses with the ureteric tip by a still poorly understood mechanism that generates a continuous luminal interconnection between the two epithelial networks (Georgas *et al.* 2009).

Directly after the formation of the RV it polarises in respect to gene expression in proximal-to distal direction (domains) (Georgas *et al.* 2009, Kobayashi *et al.* 2005, Nakai *et al.* 2003). Many of the identified genes are critical for the specification of nephron segments. Notch2 signalling is critical for the elaboration of proximal nephron fates (podocytes and proximal tubules) (Cheng *et al.* 2007), whereas Lhx1 regulates patterning of distal structures (Kobayashi *et al.* 2005). Pou3f3 (Brn1) expression is present in the distal and mid-regions of the S-shaped body where its actions are essential to form loop of Henle (Nakai *et al.* 2003). The localized cell cycle target gene expression is an early feature of the emerging proximal-distal polarity (Georgas *et al.* 2009), preceding elongation and folding of the tubular epithelium. Interestingly, also the UB secreted Wnt9b has been shown to have a role in the morphogenesis and lengthening of the proximal tubule region through convergent extension processes (Karner *et al.* 2009) .

The glomerulus, the filtrating unit of the nephron, develops from the proximal RV. The first gene specific to podocyte commitment in this region is Foxc2, which is expressed from the comma-shaped body stage (Takemoto *et al.* 2006). Wilm´s tumour 1 (*Wt1*) is expressed in the proximal nephron compartments and later its highest peaks of expression are observed in developing and postnatal podocytes, which are the specialized epithelial cells of the glomeruli (Little *et al.* 2007). Already at the s-shaped body stage, podocyte precursors and parietal epithelial cells can be differentiated (Takemoto *et al.* 2006). The parietal epithelial cells that form the Bowman´s capsule begin to express high levels of *Vegfa*, which attracts angioblast population from the surrounding interstitium to form glomerular capillaries (Eremina *et al.* 2003, Eremina *et al.* 2007).

Glomerular formation is also regulated by *Bmp4* in podocytes, and the *PdgfB/Pdgfrb* pathway controls mesangial cell recruitment (Lindahl *et al.* 1998). Full maturation and differentiation of podocytes is a multifactorial and highly regulated process. *Wt1* seem to be an organizer in the process because it is expressed in the podocytes throughout life (Srichai *et al.* 2004).

The above-mentioned developmental processes and the final structures of renal corpuscle, convoluted tubules (proximal and distal), and loop of Henle form the nephron and the cortex of the mammalian kidney (Little *et al.* 2007).

### *2.4.5 Polarization in the kidney development*

Almost all epithelial cells show polarity along two distinct axes. The first, known as apical/basal polarity (A-P), defines the orientation of the cells related to the underlying extracellular matrix. The second, called planar cell polarity (PCP), is orthogonal to the A-P axis, thus forming the polarity within the plane of the tissue (Carroll & Das 2011).

#### *Apical-basal polarity of the kidney epithelial cells*

In the mammalian kidney apical-basal polarization of the epithelia lining renal tubules is essential for the unidirectional vectorial transport of ions and fluids, which provides the basis for the appropriately regulated reabsorptive and secretory function of the normal kidney. During embryonic kidney development epithelial cell polarity is created by the localization of transporters, channels, matrix, and growth factor receptors into the structurally and biochemically distinctive apical or basolateral surface plasma-membrane domains (Wilson 2011).

The apical-basal polarity requires formation of specific cell junctions in lateral a basal membranes. The first junction to appear, called the adherent junction, forms on lateral membranes between neighbouring epithelial cells. The junction`s main function is to divide the plasma membrane into two separate portions: the apical and basolateral domains. After the adherent junctions have rapidly formed, a second junction, the tight junction, forms proximal to the adherent junctions. Together these form the apical junctional complex. A third important junction, called focal adhesion, forms between the basal cell membrane and the ECM. One of the main tasks of these structures is to organize the

recruitment of structural, signalling, actin-binding proteins and cystoproteins (Charron *et al.* 2000, Israeli *et al.* 2010, Wilson *et al.* 1999).

Adherent junctions contain calcium-dependent interactions between E-cadherin molecules of the neighboring cells. On its intracellular face, E-cadherin binds to p120-catenin, β-catenin and α-catenin forming a complex that binds to the actin cytoskeleton (Hartsock & Nelson 2008, Hartsock & Nelson 2012, Yamada *et al.* 2005). The formation of adherent junctions triggers the polarized delivery of basolateral membrane protein and apical polarization in the cytoplasm of both the Golgi complex and the microtubule organization centre (Dupin *et al.* 2009, Nejsum & Nelson 2007). In renal epithelial cells, adherent junctions have a role in signalling by triggering the canonical Wnt/βcatenin pathway. This process requires the recruitment of polycystin- 1 and -2 and nephrocystin-2 to the E-cadherin complex (Schlaepfer *et al.* 1999, Simons *et al.* 2005).

At the basal membrane, the attachment of cell to ECM is driven by clustering of a heterodimeric (αβ)-integrin receptor transmembrane protein. The focal adhesion complexes form when the cellular matrix receptors bind to the acting cytoskeleton of the epithelial cell (Israeli *et al.* 2010, Wilson *et al.* 1999). Intracellular signalling is mediated via the focal adhesion complex proteins FAK, c-src, and paxillin and results in the activation of intracellular targets ERK, JNK, and the MAPKs (Schlaepfer *et al.* 1999). These signalling pathways have a direct impact on cell proliferation, survival, spreading, and migration (Li *et al.* 2002). Abnormalities of the adherent and focal adhesions lead to formation of polycystic kidney disease. (Israeli *et al.* 2010).

### *Planar cell polarity*

Planar cell polarity (PCP) describes the coordinated polarization of tissue cells in a direction that is orthogonal to their apical/basal axis. Several studies have shown that Wnt signalling has a significant role in establishing PCP, but the precise role Wnt signalling plays in this process is complicated and not well understood (Carroll & Das 2011).

Oriented cell division during tissue growth is one of the most important functions of PCP. Defects in this process have been shown to lead to severe developmental defects (Fischer *et al.* 2006). Since the discovery that defects in oriented cell division were present prior to cystogenesis, many genes have been shown to have the same correlation, including *Fat4* and *Dchs1* (Mao *et al.* 2011, Saburi *et al.* 2008). Both of these genes are expressed in the stromal cell

population and most likely act via the same pathway to regulate the tubule diameter (Babayeva *et al.* 2011, Li *et al.* 2009, Luyten *et al.* 2010).

Two Wnt ligands, Wnt7b and -9b, are also required for proper oriented cell division during kidney development (Karner *et al.* 2009, Yu *et al.* 2009). *Wnt7b* is expressed in the distal collecting ducts of the embryonic and adult kidney. The deletion of Wnt7b from embryonic tissue results in a shortened renal medulla. Although the lack of *Wnt7b* does not result in the formation of cysts, dilation of the collecting ducts caused by disturbed oriented cell division is apparent in mutants. (Yu *et al.* 2009). Since Wnt7b is signalling to the medullary stroma via beta-catenin, it was speculated that the PCP phenotype might be an indirect consequence of a failure to activate *Wnt5a* and *Wnt11* expression in the stroma adjacent to the area of Wnt7b activity (Yu *et al.* 2009). Both Wnt5a and -11 have been shown to regulate PCP by acting through a non-canonical pathway that utilizes the Rho GTPases and Jnk (Marlow *et al.* 2002, Mikels & Nusse 2006, Oishi *et al.* 2003). In addition to Wnt7b, *Vangl2* deletion also leads to defects in the renal pelvis and to mild tubular dilation. The mutant kidneys also branch less and have glomerular defects, but the precise cellular cause of these phenotypes is unknown (Yates *et al.* 2010). Another interesting study has shown that Wnt5a can regulate the subcellular localization of Vangl2 by interacting with the co-receptor Ror2 (Gao *et al.* 2011).

### *Directed cell Movements*

Directed cell movement is another type of renal tubule elongation during morphogenesis. This process, also known as convergent extension, is regulated by PCP and non-canonical Wnt signalling (Wallingford *et al.* 2002). During embryogenesis kidney epithelial cells have planar polarity. They are elongated and the axis of elongation is vertical to the proximal-distal axis of the tubule. Studies have shown that Wnt9b is required for this orientation and for determining the tubule diameter. This was independent of any changes in the rates of cell proliferation or death. During tubule diameter formation Wnt9b acts via β-catenin independent or non-canonical pathways (Karner *et al.* 2009). As previously described in chapter 2.2.2, non-canonical Wnt signalling activates the small GTPases Rho and Rac and the c-Jun N-terminal kinase (Jnk1). In Wnt9b mutants the activated Rho and Jnk2 were reduced (Karner *et al.* 2009). In line with these results, studies using Rho kinase (Rock) inhibitors found that tubule branching is reduced and that the diameter of the epithelia appears increased in

similar manner to the Wnt9b mutant phenotype. This suggests Rho signalling may lie downstream of Wnt9b (Meyer *et al.* 2006).

### *2.4.6 The role of ECM in kidney development*

The kidney as a branching organ consists of two main cell populations: the contiguous epithelium, which undergoes the branching process, and the surrounding mesenchyme. Between these cell populations spatiotemporally regulated reciprocal interactions are essential to build the functional organ (Rozario & DeSimone 2010). A large set of ECM molecules are synthesized and secreted during kidney morphogenesis and their distinct patterns of expression and deposition are tightly regulated for normal development. New research data has disproven the old hypothesis of ECM as a passive physical support for the growing organ. Currently it is known that ECM is composed of a variety of functional and dynamic components that can serve as a physical support for tissue architecture, a reservoir for growth factors and other diffusible signals, and a substratum on which to adhere through receptors such as integrin. In other words the ECM provides a microenvironment that can influence cell behavior including proliferation, differentiation, change of shape, and establishment of polarity (Rozario & DeSimone 2010).

The importance of ECM components in kidney development has been studied with several mouse models. The role of laminin in ureteric branching morphogenesis has been studied with mice lacking the laminin-α5 (*Lama5*$^{-/-}$) and laminin-γ1 *(Lamc1*$^{-/-}$) chains. The deletion of laminin-α5 leads to a decrease in UB branching and results in unilateral or bilateral renal agenesis in about 20% of the mice (Miner & Li 2000). Mice with a deleted *Lamc1*-gene showed a lack of BM, which resulted in failure of development after E5.5 (Smyth *et al.* 1999). Selective inactivation of the floxed-*Lamc1* gene with Hoxb7-Cre, (UB specific deletion) showed defects in UB growth and branching, reduced BM, and disorganized epithelial cells in the ampulla (Yang *et al.* 2011). In organ culture studies the blocking of the laminin-γ1-binding site of nidogen leads to malformation of the BM assembly and branching (Ekblom *et al.* 1994). The mice deficient in the nidogen-binding site of the laminin-γ1 chain mainly fail to form the UB (Willem *et al.* 2002).

The laminin-binding receptors such as α3β1 and α6β4 integrin have been shown to be vital for UB branching. *In vivo, in vitro,* and isolated UB culture

models have shown that inhibiting the formation of these complexes reduces UB branching (Zent *et al.* 2001).

Integrin α8 and its ligand nephronectin are also shown to be important for kidney development (Brandenberger *et al.* 2001, Muller *et al.* 1997). Over 50% of integrin α8 knock out mice fail to develop ureters or develop kidneys without any significant defects in other organs (Muller *et al.* 1997). Both of these mutant mice showed delays in UB invasion and frequent renal agenesis or hypoplasia. These mutations do not affect the BM formation but it has been shown to lead to transient decrease of in the expression of GDNF at E11.5 (Linton *et al.* 2007).

## 2.5 Congenital Anomalies of the Kidney and Urinary Tract

Congenital anomalies of the kidney and urinary tract (CAKUT) encompasses a spectrum of anomalies that result from aberrations in spatio-temporal regulation of genetic, epigenetic, environmental, and molecular signals at key stages of urinary tract development (Davis *et al.* 2014). CAKUT commonly causes progressive chronic kidney disease (CKD) and is the most frequent cause of end-stage renal failure (CKD Stage 5) and renal replacement therapy in childhood (Harambat *et al.* 2012, Mansoor *et al.* 2011). Studies have shown that CAKUT represents approximately 30% of all prenatal malformations (1 in 500 fetal ultrasound examinations) and is among the most frequent malformation detected *in utero* (Loane *et al.* 2011).

CAKUT has a variety of phenotypes from asymptomatic abnormalities like hypoplasia, duplex collecting system, unilateral renal dysplasia, renal fusion, and vesicoureteral reflux (VUR) to lethal phenotypes like renal aplasia and bilateral dysplasia (Renkema *et al.* 2011). Currently, more than 20 genes causing monogenic symptoms of CAKUT have been identified in humans. Many of those genes are known to have a critical role in kidney development including RET, ROBO2, GDNF, BMP4, TCF2, and WNT signalling components (Vivante *et al.* 2014). Understanding the causes and developmental mechanisms of CAKUT is essential for adequate disease stratification and prognosis.

# 3 Outline of the present work

The aims of this study were to reveal the roles of Dkk1 and Wnt5a in mammalian kidney organogenesis and in the developmental human kidney disease (CAKUT, congenital anomalies of the kidney and urinary tract). Even though Wnt-signalling has been the subject of kidney development studies for decades, several important aspects remain unresolved. These include the role of Wnt-signalling antagonists, like Dkk1, in nephrogenesis, and the function of Wnt5a, a recently found nephrogenic factor, in kidney development. The specific aims of this work were:

1. To study the role of Dkk1, an antagonist of the canonical Wnt signalling, in kidney development using a conditional gene inactivation mouse model.
2. To identify the expression pattern and role of Wnt5a in kidney organogenesis using a total knockout mouse model.
3. To elucidate the role of WNT5 gene in CAKUT disease in humans.

# 4 Material and methods

The detailed description of the material and methods used in this thesis are found in the original articles I-II.

**Table XX. Methods used in the original publications.**

| Method | Original publication |
|---|---|
| Mouse lines and genotyping | I, II |
| Tissue preparation | I, II |
| RNA extraction and real time PCR | I, II |
| Whole mount and section *in situ* hybridization | I, II |
| Immunohistochemistry | I, II |
| Histological staining | I, II |
| Urine collection and analysis | I |
| Wnt reporter assay | I |
| Cell proliferation assay | I |
| β-galactosidase staining | I |
| Transmission electron microscopy | II |
| Optical projection tomography (OPT) | II |
| Prolyl-4-hydrozylase activity measurements | II |
| *In silico* modeling | II |

## 4.1 Optical projection tomography (OPT)

Optical projection tomography (OPT) produces high-resolution 3D images of both non-fluorescent and fluorescent biological specimens with a thickness of up to 15mm and 20mm in length. With an OPT scanner it is possible to map the tissue distribution of RNA extractions and protein localization in intact embryos or organ systems. These features make OPT an ideal instrument for research in developmental biology or gene function (Sharpe *et al.* 2002).

The dissected kidneys (E15.5-16.5) were fixed with 4% paraformaldehyde and stained as whole mount with the Troma-1 antibody (Hybridoma bank, Iowa USA) or with a Wnt-11 *in situ* probe. Due to the large size of the samples the immunostaining protocol was specifically optimised for OPT (Alanentalo *et al.* 2007, Alanentalo *et al.* 2008). After staining the kidneys were mounted to 1% low melting point agar. After the agar had set, it needed to be cut in a diamond shape, because 90 degree angles in the block disturb the path of light and impair the quality of the scan. The samples were dehydrated overnight (o/n) in methanol at

RT. After the two exchanges of methanol, the samples were cleared o/n at RT with benzyl alcohol and benzylbenzoate (1:2) and imaged with the OPT Scanner 3001M (Bioptonics Microscopy, UK). For the chlorophores white light and infrared light were used and for the fluorophores UV wavelengths were used. The Imaris software (Bitplane, Zurich Switzerland) was used to analyse the OPT data. The branching morphology was assessed using the filament tracing plug in.

# 5 Results

## 5.1 Dkk1 controls the elongation of the renal papilla (I)

Wnt ligands are known to play a critical role during kidney development and both the canonical and non-canonical pathways are needed. A lot of factors have been described which regulate Wnt signalling during organogenesis, but the role of direct canonical pathway antagonist Dkk1 has remained poorly understood.

### *5.1.1 Dkk1 is expressed widely during kidney development and the Pax8Cre mediated gene knock-down targets the developing papilla*

The presence of *Dkk1* in the developing kidney at E14.5 was published in the open database gene paint earlier (http://www.genepaint.org/cgi-bin/mgrqcgi94), but to understand the regulative role of Dkk1 we needed to establish the expression profile for *Dkk1* also during later stages of kidney development.

Fom E10.5 to new-born (NB) *Dkk1* expression was identified in MM cells and later in their derivatives using *in situ* hybridization. At NB *Dkk1* is expressed in the cells of the epithelial UB, in the tubular structures, in the developing collecting duct, and at the tip of the kidney papilla.

Consistent with the mRNA distribution, the Dkk1 protein is present in the cells of the collecting duct depicted with Aquaporin 2 (AQP2) expression and the loop of Henle. This was confirmed using double immunostaining. The stromal cells did not produce Dkk1.

As *Dkk1* is expressed widely in developing kidney, we chose three Cre-lines for Dkk1–LoxP crossings. *Pax3Cre*, *Wnt-4Cre* and *Pax8Cre* mouse lines were used, which all target Cre-recombinase in the kidney from the early developmental stages and on (Bouchard *et al.* 2004, Engleka *et al.* 2005, Shan *et al.* 2009). Pax3Cre-mediated inactivation of Dkk1 function truncated the anterior portion of the embryos. This phenotype resembles the head defects of a complete Dkk1 knockout (Mukhopadhyay *et al.* 2001) and is the likely reason for the frequent perinatal deaths of *Pax3Cre;Dkk1*$^{n/c}$ embryos. The kidneys of the *Pax3Cre;Dkk1*$^{n/c}$ embryos appeared normal and the development of the kidney compartments was indistinguishable from that of the wild-type controls. From the

NB histological analysis we observed the most severe kidney phenotype in *Pax8Cre;Dkk1*$^{n/c}$ mice.

The efficiency of *Pax8Cre*-mediated *Dkk1* inactivation was estimated by staining wild-type and *Pax8Cre;Dkk1*$^{n/c}$ embryonic kidneys with an antibody against Dkk1. Pax8Cre inactivated *Dkk1* expression was observed in the emerging medulla tubules, including the papilla. *Dkk1* expression was lost both from the collecting duct and the loops of Henle cells judging from double immunostaining with AQP2, a collecting duct marker. We did not observe a difference in the production of Dkk1 in the kidney cortex.

Overgrowth of renal papilla and hydronephrosis were observed following Pax8cre specific Dkk1 deletion. *Pax8Cre*-induced inactivation of Dkk1 function leads to two characteristic phenotypes in the medulla of the kidney. Two out of ten kidneys studied from the *Pax8Cre;Dkk1*$^{n/c}$ NB mice had developed hydronephrosis. The other typical phenotype with high penetrance was overgrowth of the renal papilla in proportion to the rest of the kidney. In such cases the papilla in the kidney medulla had become extended over the lateral edge of the kidney capsule and had reached the lumen of the ureter outside the kidney. An extended papilla was observed in both kidneys of seven out of ten analysed knockout mice, whereas the kidneys of the stage-matched wild-type mice that served as controls did not show such a phenotype.

An additional characteristic feature in the kidneys of the *Pax8Cre;Dkk1*$^{n/c}$ mice was the hypertrophic collecting duct epithelial cells observed at the tip of the extended renal papilla. The corresponding cells of the kidneys of wild-type controls were cuboidal and arranged in a row in respect to each other in the epithelial lining of the papilla tip.

### *5.1.2 Dkk1 deficiency stimulates cell proliferation and leads to reduced expression of ion pump genes in papilla*

*Dkk1* deficiency enhanced the amount of mitotic cells in papilla when compared with wild-type controls. Studies revealed that the most proximal papilla region contained the highest amount of mitotic cells in both the wild-type and *Dkk1*-deficient kidneys, but cell proliferation was notably enhanced in the *Dkk1* deficient mice in comparison with controls ($p<0.005$). The number of actively proliferating cells decreased towards the papilla tip region in both genotypes, but the *Dkk1*-deficient papilla still showed enhanced proliferation in each of the analysed papilla segments ($p<0.1$ for the middle and $p<0.05$ for the tip region).

*Dkk1* deficiency by *Pax8Cre* did not change the rate of cell proliferation in the kidney cortex. The proliferation rate was comparable with wild-type controls.

Expression of the loop of Henle marker, *Slc12a1*, and the collecting duct marker, *Scnn1b*, were both reduced based on their intensity in comparison with the wild-type controls. Hence, *Pax8Cre*-mediated *Dkk1* deficiency reduces marker gene expression in the loop of Henle and collecting duct, suggesting that Dkk1 deficiency also affects these structures and may therefore have an impact on the functional performancy of the kidney.

### 5.1.3 Renal papilla malformation causes functional defects

The kidneys of the 1-year-old *Pax8Cre;Dkk1*$^{n/c}$ mice produced clearly less urine than the kidneys of the wild-type control mice during the analysed 24 hour time period. Moreover, the analysis of the constituents of the urine obtained from the *Pax8Cre;Dkk1*$^{n/c}$ mice revealed changes in the amount of ions in comparison with the wild-type controls. Sodium, potassium, urea, creatinine (CreUr), phosphate, glucose and protein/Uprot amounts were all decreased. In contrast, the amount of chloride and calcium were increased in the urine produced by the *Pax8Cre;Dkk1*$^{n/c}$ mice when compared with the corresponding values of the wild-type mice.

We also analysed potential changes in urinary proteins. Proteins with a size of around 100 kDa, which were detected in the urine of the wild-type mice, were not present in the urine of the *Pax8Cre;Dkk1*$^{n/c}$ mice. MALDI-TOF sequencing of this component of urine revealed notable amino acid sequence similarity to the major urinary proteins (Cavaggioni & Mucignat-Caretta 2000) that normally are synthesised in the liver, submaxillary glands, and mammary glands, and secreted to serum, which is excreted via urine (Shaw *et al.* 1983).

### 5.1.4 Dkk1 deficiency increases stromal canonical Wnt target Lef-1 synthesis

We next addressed whether *Pax8Cre*-mediated *Dkk1* deficiency would change gene expression of genes that could be relevant for the growth of the papilla. *Wnt7b* has been implicated in medulla formation, because *Wnt7b* deficiency in the collecting duct leads to compromised papilla and pelvis development (Yu *et al.* 2009).

In wild-type kidneys *Wnt7b* is expressed specifically in the collecting duct cells of the papilla (Yu *et al.* 2009). *Wnt7b* remained expressed in the collecting duct cells irrespective of *Dkk1* deficiency induced by *Pax8Cre*. However, we found increased numbers of cells that expressed canonical Wnt signalling of the target gene *Lef-1* in the stroma of the medulla. Dkk1 likely regulates Wnt7b signalling from the collecting duct cells to the stromal cells during kidney papilla development.

Next we used CHO-KI cells to study whether Dkk1 can inhibit Wnt7b signalling. We found that the recombinant Dkk1 protein (100 ng/ml) efficiently inhibited both Wnt7b mediated activation and the control Wnt3a mediated activation of the Wnt reporter gene in the TOP Flash assay.

## 5.2 Wnt5a has a critical role in ureteric bud basement membrane formation (II)

*Wnt5a* gene function has been shown to be important in many developmental processes. We became interested in the role of Wnt5a during kidney development because a previous study (Naillat *et al.* 2010) showed that a lack of *Wnt4* induced the *Wnt5a* expression during gonad development.

### *5.2.1 Wnt5a expression characterization and observed morphological phenotypes*

We studied the *Wnt5a* expression pattern using *in situ* hybridization in embryonic kidneys of mice. Expression was present in the epithelial UB cells at E11.5, when organogenesis had just initiated. Furthermore, *Wnt5a* gene expression occurred in the mechanically separated uninduced and induced MM. Metanephric kidney cells express *Wnt5a* from an early developmental stage in the UB and MM cells.

At E12.5/13.5 *Wnt5a* expression remains present in the UB. *Wnt5a* expression became weakly present in the stromal cells from E14.5, whereas at E16.5 *Wnt5a* transcripts had accumulated in the tip of the CD, while the medulla region expressed *Wnt5a* weakly. Glomerular cells expressed *Wnt5a* as well. *Wnt5a* expression seemed to depend on Wnt4 function as *Wnt5a* expression in the stromal cells was reduced in *Wnt4* deficient kidneys at E14.5. However, *Wnt5a* expression was noted to reappear in the kidney of NB *Wnt4*$^{-/-}$ mice. Collectively, the presence of *Wnt5a* in developing kidney, i.e. its UB and MM derivatives, and stromal cells points towards a potential role in UB and/or MM cell differentiation.

To address whether Wnt5a signalling participates in the control of kidney organogenesis, we analysed *Wnt5a*$^{-/-}$ mice (Yamaguchi *et al.* 1999). The absence of *Wnt5a* function led to four major categories of kidney abnormalities: 1) formation of duplex collecting system, 2) renal agenesis, 3) kidney hypoplasia due to altered branching morphogenesis and CD dilatation, 4) anomalous kidney bifurcation of UB causing multilobular kidney formation. Additionally, renal pelvis formation in all *Wnt5a*$^{-/-}$ mice was altered. The common nominator for all these characteristic abnormalities was considered to be deregulation of UB tree morphogenesis.

Renal histology of the *Wnt5a*$^{-/-}$ mice at E15.5 depicted that the diameter of the CD and the overall degree of bifurcation of the individual UB branches were enlarged when compared with controls. The lumen in the Bowman's capsule was also dilated when compared with controls. Morphometric analysis of the captured optical projection tomography (OPT) images and the assembled movies of the kidneys at E15.5 indicated a 30% reduction and at E16.5 a 30-40% reduction in the total number of the terminal UB tips when compared to the controls. OPT data also revealed that the branching angle of the UB tree, as suggested by the histological inspection, were indeed increased ($p < 0.03$) in *Wnt5a*$^{-/-}$ mice when compared to the controls. Since the *Wnt5a*$^{-/-}$ mice die during the prenatal period (E18.5), we examined the adult *Wnt5a*$^{+/-}$ mice and observed an increased number of kidney dysplasia.

### 5.2.2 Ultrastructural analysis reveals malformed basement membrane in the ureteric bud

At the initiation of organogenesis (E11.5) the kidneys of *Wnt5a*$^{-/-}$ mice showed severe changes in the BM, including loop formation and BM dilatation. At E16.5 the BM was more loosely organized, less electron dense, and the distance between the UB cells and the BM was higher than in the controls. The BM thickness of the *Wnt5a*$^{-/-}$ mice remained increased in comparison to the controls at E16.5, and in certain regions the BM was up to five times thicker than in the controls. It was also more diffusely organized with occasional loops.

### 5.2.3 Lack of Wnt5a changes ECM gene expression and production

Consistent with the findings in the EM, type IV collagen and laminin of the CD tubular epithelium BM and the glomerulus demonstrated weak and fragmentary

immunohistochemistry stained expression, which was not the case in controls. The noted changes in the glomerular endothelial cells and effacement of the podocyte foot processes may collectively compromise the filtration barrier of the Wnt5a$^{-/-}$ kidneys, which could cause dilation of Bowman's capsule.

These studies indicate that while the expression of genes encoding *laminin α2, β1, γ2* and *γ3* chains are downregulated in the absence of *Wnt5a* signalling the expression of *laminin β2* becomes upregulated. Also *Col4a1*, *Col4a2,* and *Col4a3* gene expression were downregulated in the absence of *Wnt5a* when compared to control. Such differences were not detected in the expression of *Col4a4* and *Col4a6* or in the genes encoding another BM collagen, type XVIII collagen, or the non-BM collagens, type I and II. The data shows that Wnt5a is involved in the control of the expression of laminin and type IV collagen isoforms.

Our next step was to examine the roles of Wnt5a by a gain of function approach using virus-mediated overexpression of *Wnt5a* in a MK3 cell line derived from embryonic kidney. A lentivirus that contained a *Wnt5a-IRES-GFP* construct was transduced into the MK3 cells, while GFP served as the control for successful transduction. Out of the analysed laminin and collagen genes the *Wnt5a-IRES-GFP* lentivirus induced the expression of genes encoding the *laminin γ1* and *collagen α1(IV)* and *α2(IV)* chains, while expression of the gene encoding *laminin β2* was decreased when compared with controls. No changes were seen in the other analysed laminin or collagen IV genes. The data confirms that Wnt5a regulates the expression of certain laminin and collagen IV genes and thus controls the synthesis of specific components of the BM during kidney morphogenesis.

### *5.2.4 P4ha1 enzyme may compensate for decreased ECM production*

Given that *Wnt5a* deficiency caused defects in the assembly of the BM and control of expression of the BM components laminin and collagen IV, we wondered whether changes in the enzymes required for collagen post-translational modification could be involved. We studied the expression levels of *P4ha1, which* encodes the catalytic subunit of collagen prolyl 4-hydroxylase I (C-P4H-I), a critical enzyme in collagen synthesis (Holster *et al.* 2007), and the corresponding amount of C-P4H activity in *Wnt5a*$^{-/-}$ and wild-type mice. The results indicated that expression of the *P4ha1* gene is upregulated in *Wnt5a*$^{-/-}$ mice and that the amount of C-P4H activity increased significantly (26%) in the *Wnt5a*$^{-/-}$ E16.5 kidney lysates compared with controls.

We speculated that the noted induction in C-P4H-I expression might be a compensatory mechanism to diminish the harmful effect of down-regulation of collagen IV synthesis in *Wnt5a*$^{-/-}$ mice. To study this we analysed whether a decrease in C-P4H-I activity would aggravate the effect of reduced *Wnt5a* function on BM assembly. We utilized a *P4ha1*$^{-/-}$ mouse line that is embryonic lethal at E10.5 because of loss of collagen IV assembly (Holster *et al.* 2007) and generated compound heterozygous *Wnt5a*$^{+/-}$;*P4ha1*$^{+/-}$ mice. No differences in the BM structure between the CD epithelium and the mesenchyme were observed in the *Wnt5a*$^{+/-}$ and *P4ha1*$^{+/-}$ single heterozygous E16.5 embryos with respect to the controls. In contrast, the kidneys of the compound *Wnt5a*$^{+/-}$;*P4ha1*$^{+/-}$ mice demonstrated severe BM defects at E16.5 similar to those observed in the *Wnt5a*$^{-/-}$ mice. PAS staining of E16.5 *Wnt5a*$^{+/-}$;*P4ha1*$^{+/-}$ kidneys revealed local hyperplasia in the CD epithelium like in the *Wnt5a*$^{-/-}$ mice. In 7-month-old mice the renal dysplasia was increased also in *P4ha1*$^{+/-}$, *Wnt5a*$^{+/-}$ and in compound heterozygote when compared to WT littermates. In adult mice glomerular defects, including invasion of mesangial cells between the glomerular cells indicating proliferative glomerulonephritis, were observed in the *P4ha1*$^{+/-}$ and *Wnt5a*$^{+/-}$ mice, but the defects were markedly more severe in the *Wnt5a*$^{+/-}$;*P4ha1*$^{+/-}$ mice.

### 5.2.5 Wnt5a crosstalk with other Wnts during kidney development

Since Wnt4, -7b and -9b control the UB and MM formation in developing kidney, we considered that Wnt5a might contribute to UB development also by influencing the expression of these Wnts. To address this we studied whether the expression of these Wnts changes in the absence of Wnt5a signalling, or alternatively when Wnt5a expression is enhanced *in vitro*.

Our data suggests a synergistic role between Wnt4 and Wnt5a. However, *Wnt4* expression was not affected by the absence of *Wnt5a*, whereas *Wnt7a* and *Wnt9b* expression appeared elevated when compared with controls. In a reverse experiment *Wnt5a* overexpression induced *Wnt7a, -7b* and *-9b* expression but no *Wnt4* expression.

Since the expression of *Wnt4* and *Wnt5a* were both induced in an experimentally induced kidney mesenchyme, we wondered whether these genes were synergistic in the kidney development. To find an answer we created double knockout embryos. Our data revealed that Wnt4 and Wnt5a signals do indeed have a synergistic role in the stromal cells. At the E14.5 *Wnt4*$^{-/-}$;*Wnt5a*$^{-/-}$ kidneys form a clear stroma population but the amount of stroma was notably reduced in

the kidneys of double knockout mice, presenting only a thin stromal cell layer surrounding the poorly developed CD in the *Wnt4*$^{-/-}$;*Wnt5*$^{-/-}$ kidneys. Collectively we consider this as evidence that besides controlling the BM integrity and by that way the patterned UB development, Wnt5a coordinates the read out of the other Wnts controlling kidney development also.

### 5.2.6 Wnt5a variant discovered in CAKUT patient and in silico modelling of mutant indicates an increase in receptor binding pocket

Our findings that Wnt5a serves as a novel critical signal for kidney development in mice made it a candidate gene for human CAKUT. We therefore performed a mutation screening by Sanger sequencing of *WNT5A* (NM_003392) in a cohort of 129 CAKUT patients. The patients had a duplex collecting system, renal agenesis, horseshoe kidney, or Alport syndrome with an unknown genetic cause.

A novel heterozygous *c.779G>A WNT5A* variant was found in a patient with a unilateral duplex collecting system. The discovered variant was not found in an in-house database that contains >100 exomes, dbSNP, the NHLBI exome variant server, nor in 189 healthy control individuals. The father of this *WNT5A* mutation carrier patient was also identified as a carrier of the variant. However, at present it is not known if the father has a CAKUT disease phenotype. The mother was diagnosed at the age of 11 with CAKUT, but the mother did not have a mutation in the Arg260. The mother's genome has not been screened for the known CAKUT genes either. The *c.779G>A* variant locates in exon 5 of *WNT5A* and leads to a p.R260H amino acid change in a conserved region of the protein. The PhyloP conservation score was 6.26 and software-based predictions classified the discovered variant as deleterious by SIFT (score 0), disease-causing by Mutation Taster (score 1.0), and probably damaging by PolyPhen-2 (score 0.999).

To study whether the *R260H* hWNT5A could indeed be causal for CAKUT development we first depicted its putative influence on the three-dimensional structure of the WNT5A protein. For this purpose we took advantage of the recently solved structure of the xWnt8 protein (Janda *et al.* 2012) and *in silico* modeling. The study revealed that the average width of the receptor-binding site of wild-type WNT5A was 3.06 nm (SD 0.437 nm), while the corresponding distance in the *R260H* WNT5A was notably wider, extending up to 3.74 nm (SD 0.53 nm), which amounts to an distance increase of about 22%. The simulated model of the WNT5A structure predicted that the loop region of the wild-type

WNT5A protein oscillates in the range of 1.5-4.3 nm. The corresponding value of oscillation of the chain due to the R260H mutation was 2.5-5.18 nm. The data suggests that the point mutation would probably decrease the affinity of WNT5A to its receptor. This in turn would reduce WNT5A signalling, promoting the development of CAKUT.

To address the putative functional consequences of the R260H mutation we generated recombinant viruses expressing wild-type and R260H Wnt5a. The influence of this mutation to the efficiency of Wnt signalling was studied by testing the capacity of the Wnt5a to inhibit canonical Wnt signalling. The canonical Wnt pathway was initiated by the Wnt3a and the Wnt reporter activity (TOP Flash) was measured as the readout. Indeed the results indicated that the R260H mutation decreases the Wnt5a mediated inhibition capacity of Wnt3a signalling by about 17% ($p < 0.05$) when compared with wild-type Wnt5a. Hence, the identified R260H mutation in WNT5A reduces the respective signalling and promotes the conclusion that the mutation may indeed lie behind the diagnosed CAKUT in the patient.

# 6 Discussion

## 6.1 Dkk1 controls renal papilla development by increasing canonical Wnt regulated cell proliferation (I)

The extended papilla phenotype observed in *Pax8Cre;Dkk1$^{n/c}$* mice is likely caused by enhanced proliferation of the kidney papilla cells, the collecting duct, and the loop of Henle cells. Typically, reduction of Dkk function is connected to enhanced proliferation of cancer cells (Hirata *et al.* 2011, Niehrs 2006, Zhou *et al.* 2010), and this is in line with our findings. We conclude that Dkk1 function is connected to the maturation of the papillary epithelial cells, the ureteric bud-derived collecting duct, and the loop of Henle cells.

*Wnt7b* is expressed in the collecting duct epithelial cells of the developing papilla region, but the knockout also influences the loop of Henle development, making Wnt7b a signal that also coordinates loop of Henle development via the stromal cells. We demonstrated that the Dkk1 protein inhibits Wnt7b induced activity of a canonical Wnt signalling pathway reporter when the receptor for Wnt7b is expressed in the target cell. We further showed that expression of canonical Wnt signalling targeted gene Lef-1 was increased due to Dkk1 deficiency in the stromal cells. We cannot exclude at present that Dkk1 may also have an independent role in the embryonic loop of Henle cells.

Since *Pax8Cre;Dkk1$^{n/c}$* kidneys exhibit an extended papilla phenotype and inhibition of collecting duct and loop of Henle marker gene expressions, we propose that Dkk1 in the kidney papilla regulates kidney development by controlling the activity of Wnt7b in the stromal cells. By binding to the Frizzled/LRP complex in the stromal cells, Dkk1 may fine-tune the activity of Wnt7b, thereby also influencing loop of Henle growth and maturation (Glinka *et al.* 1998, Niehrs 2006).

### *6.1.1 Renal papilla specific deletion of Dkk1 causes renal function complication (I)*

We have shown that Dkk1 knock out by Pax8Cre reduced expression of loop of Henle and collecting duct marker genes. Of these, the *Slc12a1* gene encodes a $Na^+$–$K^+$–$2Cl^-$ pump in the loop of Henle, and the *Scnn1b* gene encodes a sodium channel non-voltage-gated 1, β in the collecting duct. The respective proteins take

part in the tubules to control the reabsorption of ions, calcium, and magnesium from the urine into the body and the maintenance of the volume of extracellular fluid (Mount *et al.* 1999). The reduction in *Slc12a1* expression is in line with the increase in calcium that was observed in the urine of the Dkk1-deficient mice.

The reason for the reduced overall urine production noted in the Dkk1 mutant mice may be due to the elongated papilla with an extended loop of Henle, the collecting duct tubules and a defect in their maturation. These changes likely alter the balance of the ion channels that are involved in absorption of ions and fluids from primary urine. By controlling development of the papilla and expression of the associated genes, Dkk1 is involved in setting up the functional capacity of the mature kidney.

## 6.2 Wnt5a coordinates basement membrane formation and disruption in Wnt5a signalling may lead to development of CAKUT (II)

We have provided evidence that Wnt5a is functional in the embryonic kidney and that it has a novel and important role in kidney organogenesis. Our results show that Wnt5a controls patterning of the UB derived tree development via BM formation. We also report a novel heterozygous WNT5A variant from a human duplex collecting system patient.

Lack of Wnt5a in the developing mouse kidney *in vivo* was found to lead to renal agenesis, multilobular kidneys or duplex collecting systems, all of which pointed towards a critical role for Wnt5a in UB development.

### *6.2.1 Wnt5a has a role in ureteric bud basement membrane formation*

We found in an early embryonic kidney (E11.5) that when organogenesis had just initiated, there already was a severe defect in the $Wnt5a^{-/-}$ BM between the UB and the MM. The BM of the $Wnt5a^{-/-}$ UB was split and had folds and loops. The lamina densa was also wider and the whole BM was more distant from the plasma membrane than in the controls (E16.5). By regulating the expression of *collagen IV α1-3* and *laminin α2, β1-2, γ2-3* chains, *Wnt5a* may promote formation of the BM sheet between the tissue layers that influence the pattern of UB morphogenesis during its branching. These findings point to the conclusion that Wnt5a contributes to kidney development by controlling the integrity of the BM.

Indeed we noted that the kidney phenotypes caused by the absence of Wnt5a signalling resembled those caused by the lack of the ECM component laminin γ1, which also leads to kidney agenesis or duplex collecting system formation (Yang *et al.* 2011). Because laminin is a critical initiator of BM formation (Smyth *et al.* 1999), our gene deletion affects the primary stages of the embryogenesis and organogenesis (Smyth *et al.* 1999, Yang *et al.* 2011). Since the growth factors that mediate the reciprocal tissue interactions in all organ systems also need to exert their effect through the BM, the role of Wnt5a may point to a more general role of the Wnt signals to coordinate organogenesis via the control of BM dynamics. Disturbances in the ECM and BM would be expected to influence the mode of cell–matrix signalling via the respective cell surface receptors or by having an influence on the diffusion and binding properties of the growth factors that mediate the tissue interactions (Kuo *et al.* 2012) and ultimately the kidney morphogenesis (Sorokin *et al.* 1997). As in the human ECM mutations, the effect of malformed BM presents a large set of disorders through the body such as Alport´s syndrome and HANAC disease (Plaisier *et al.* 2007, Plaisier *et al.* 2010).

The noted changes in the *P4ha1* expression and the morphological defects in the $Wnt5a^{+/-};P4ha1^{+/-}$ double knockout embryos further support the conclusion that Wnt5a signalling is connected to ECM integrity. We suggest that Wnt5a provides a signal that maintains the functional BM to correctly bind and localize Wnt5a function in the UB and promote its development.

### 6.2.2 Lack of Wnt5a changes collecting duct epithelial cell polarisation

On the other hand, the characterized BM phenotype may result as a secondary effect of disturbed polarisation of the developing collecting duct epithelia. Studies have shown that deletion of apico/basal and planar cell polarity genes leads to a decrease of ureter branching, and that increases in medullar cyst formation in adulthood and Wnt signalling play a significant role in establishing planar cell polarity in adults (Carroll & Das 2011). I*n vitro* it has been shown that Wnt5a controls the planar cell polarity mediated cell proliferation and migration. Our results indicate that this might be the case also in the total knock out mouse model. We observed increased cyst formation in 1-year-old $Wnt5a^{+/-}$ mice, as the total knock out leads to prenatal lethality. It would be interesting to study the conditional collecting duct specific Wnt5a knock out mice, for example with

Hoxb7-Cre-mediated deletion and stain the epithelial cells with polarisation markers.

Our scanning electron microscopy analysis showed thigh junction phenotype in polarised endothelial cells, indicating that in strongly *Wnt5a* expressing endothelial cells the polarisation seems to be disrupted also *in vitro*. Another strong evidence of a Wnt5a knock out mediated polarisation defect is the down regulation of integrin receptor alpha 1 (*Itga1*), which is an important component of the ECM binding focal adhesion. Itga1 binds directly to the collagen and laminin. Because focal adhesion between ECM and cells mediates the signals via FAK, c-src, and paxillin activating ERK, JNK, and MAPKs, the disruption of this complex would affect cell proliferation, survival, and migration. Our data shows increased cell proliferation and apoptosis in *Wnt5a*$^{-/-}$ kidneys.

Disruption of oriented cell division may also cause some of the phenotypes we have observed in our mouse model. Our data shows that lack of Wnt5a changes the expression levels of *Wnt9b* and -*7a*. At least Wnt9b is known to play a critical role in PCP controlled oriented cell division and *in vitro* studies with other genes involved in oriented cell division have shown the correlation with cystogenesis. Altered Wnt9b expression may also change the directed cell movement, which is known to have a critical role in collecting duct lumen diameter formation and is regulated by PCP. Lack of Wnt5a increases the lumen diameter, even though the proliferation has not specifically increased in the collecting ducts.

These above mentioned results and their correlation with published data makes the polarisation defect a plausible cause for the observed phenotypes.

### *6.2.3 Wnt5a variant found in CAKUT patient leads to decreased Wnt functions by broadening the receptor binding pocket*

We describe a novel heterozygous WNT5A variant in a patient with duplex collecting system, which is part of the CAKUT disease spectrum. The mutation changes the p.Arg 260 to His in a highly conserved domain of the WNT5A protein. *In silico* based simulation of the WNT5A protein structure by using the existing xWnt8 crystal structure as a representative model suggested that the R260H mutation broadens the WNT5A receptor-binding pocket.

It is very likely that such broadening decreases the effectiveness of the WNT5A receptor binding and therefore the effectiveness of WNT5A signal transduction. To study this possibility we engineered the discovered Arg260His

mutation to the corresponding mouse Wnt5a protein. The mutant Wnt5a protein indeed considerably reduced the capacity of Wnt5a to inhibit the Wnt3a mediated induction of Wnt signalling. Based on this data and on the fact that the Wnt5a knockout also leads to a CAKUT type of developmental anomaly in the mouse kidney, we consider WNT5A as one causal factor in the development of CAKUT in humans.

### *6.2.4 Wnt signalling network in kidney*

Another restricted role of Wnt5a according to our data is to promote stromal growth in synergy with the Wnt4 signal. This idea is based on the histological findings that the differentiated stromal cells are compromised when both Wnt4 and Wnt5a signalling are impaired. In single Wnt5a knock outs the cortical layer of the undifferentiated mesenchymal cells was broader than in wild-type and in Wnt4 knock outs. Besides Wnt4, we found that some other Wnt genes that also encode critical renal developmental signals and mediate the tissue interactions, namely Wnt7a and -9b, appeared to be elevated in the absence of Wnt5a function.

# 7 Summary and Conclusion

The target of this study was to explore the roles of two Wnt-pathway genes, *Dkk1* and *Wnt5a,* in kidney development and kidney disease. Many Wnt-pathway components and ligands are known for their critical role in kidney organogenesis, but Dkk1 and Wnt5a remained poorly characterized.

This study identified a novel role for the canonical Wnt pathway antagonist Dkk1 in kidney development. It plays the role of a regulator of cell proliferation in renal papilla development and acts as an organizer of CD ion pump production. A lack of Dkk1 in the collecting duct cells increases the proliferation in the collecting ducts and loop of Henle, which leads to renal papilla overgrowth and kidney functional abnormalities. When these results are combined with other published data, Wnt7b signalling emerges as the primary target of the Dkk1 inhibition. The studies also indicate that disruption in Dkk1 mediated fine-tuning of Wnt signalling may lead to the development of hydronephrosis.

The second gene of interest, *Wnt5a,* proved important for the ureteric budding from the WD and the ureteric branching. Studies revealed that the variable phenotypes identified in Wnt5a KO mice were due to a malformed BM surrounding the collecting duct, which impairs the signalling between CD epithelial cells and MM cells. The data indicates that Wnt5a influences the BM formation by regulating the ECM gene expression and also by affecting the ECM components' post-translational modification. These results suggest that Wnt5a may be a candidate gene for development of CAKUT in humans.

Another possible mechanism for Wnt5a in kidney development is the regulation of epithelial cell polarization. Changes in cell polarity are known to lead to severe developmental disorders.

The development and branching of the ureteric tree turned out to be a mutual target of both genes. My thesis pointed out that these genes are involved in UB and CD development from the initiation of ureteric budding to the final elongation, maturation of renal papilla, and the function of CD. The disorders in these gene expressions can lead to a variety of diseases.

Further studies are required to identify the exact mechanism of Dkk1 inhibition in renal papilla development and also to better describe the role of Dkk1 in the cortex. Before Wnt5a can be regarded as a CAKUT causing gene, more patients with Wnt5a mutation need to be characterized, and Wnt5a target genes need to be identified during different stages of kidney development.

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# Original articles

I Pietilä I, Ellwanger K, Railo A, Jokela T, Barrantes Idel B, Shan J, Niehrs C, Vainio SJ (2011) Secreted Wnt antagonist Dickkopf-1 controls kidney papilla development coordinated by Wnt-7b signalling. Dev Biol. 2011; 353:50-60.

II Ilkka Pietilä, Kirsten Y. Renkema, Renata Prunskaite-Hyyryläinen, Susanna Kaisto, Nayia Nicolaou, Albertien M. van Eerde, Sanna Junttila, Antti M. Salo, Ilkka Miinalainen, Ernie M. Bongers, Wourt F. Feitz, Nine V. Knoers, Johanna Myllyharju & Seppo J. Vainio (2014). Wnt5a Is Involved in Kidney Development, Disease and Control of Basement Membrane Assembly. Manuscript.



Original publications are not included in the electronic version of the dissertation.

1265. Manninen, Anna-Leena (2014) Clinical applications of radiophotoluminescence (RPL) dosimetry in evaluation of patient radiation exposure in radiology : determination of absorbed and effective dose

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