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Nephrin – mutations in Congenital Nephrotic Syndrome of the Finnish type and cell lineage specific gene regulation

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На баба и дядо

Summary

Kidneys, the main excretory organs in vertebrates, play a central role in removing water-soluble metabolic waste products from the organism. Many acquired and inherited renal diseases in man lead to kidney dysfunction and nephrotic syndrome – life-threatening conditions affecting and increasing number of people. Congenital nephrotic syndrome of the Finnish type (CNF) is one such disease, which affects the renal filter. It is an autosomal, inherited disease characterised by development of nephrotic syndrome shortly after birth. In 1998 Kestilä et al. reported the positional cloning of the gene defective in CNF patients. The gene product was termed nephrin due to its nephron specific expression.

In Finnish patients two common mutations were identified, Fin_{major} and Fin_{minor}, which together account for about 94% of the CNF cases. In contrast, most of the patients outside Finland have unique, "individual mutations". This thesis work presents the summarized results from the mutation screening of more than 30 non-Finnish families. In the course of the study 20 novel disease causing nucleotide changes were identified including missense, nonsense, frameshift and splice mutations, as well as small deletions and insertions. Missense mutations are the most common and account for almost half of all reported CNF causing nucleotide changes in the nephrin gene.

Nephrin is a transmembrane protein belonging to the Ig super family. In man it is found only in podocytes, where it is localized in the slit diaphragm and has a central role for the organization and maintenance of the renal ultrafilter. In rodents nephrin is expressed not only in the kidneys, but also in the central nervous system and pancreas. One aim of the present work was to study the *cis*- and *trans*-regulatory elements directing this tissue specific expression of the gene in mice. For that purpose five different sequences from the nephrin upstream region were cloned in front of β-galactosidase as a reporter gene and the resulting constructs were used for generation of transgenic mice. Several independent transgenic lines were generated with each construct. The tissue expression of *LacZ* in each line was studied by histochemistry. The data from the transgenic mice indicated the presence of kidney and brain specific regulatory elements in the region -4 kb; -2.1 kb (where the first nucleotide of the ATG codon of nephrin is +1). The sequence between -1.9 and -1.2 kb was found to be important for spinal cord and pancreas expression.

Further studies of the -4; -2.1 kb podocyte specific enhancer region based on homology between mouse and man led to the identification of a region specifically recognized and bound by putative transcription factor(s) expressed in podocytes. A stretch of six guanines was shown to be essential for the binding of that protein(s) from podocyte nuclear extract. This, as yet unknown, transcription factor is a zinc-finger protein. It was found to be expressed by mouse podocytes and human embryonic kidney cells.

The transgenic mouse experiments also resulted in the identification of a novel nephrin isoform, expressed in rodent brains along with the regular nephrin. This brain specific nephrin has an alternative exon 1, exon 1B, situated approximately 1.5 kb upstream of the original first exon, now designated 1A. Exon 1B is spliced with exons 2 through 30, giving rise to a protein differing from the regular nephrin only in the sequence coding for the signal peptide. A detailed study of the expression of nephrin in brain of mice was carried out for the first time as a part of this thesis work.

The summarized results from the nephrin gene regulation studies presented in this thesis showed that the differential expression of nephrin is controlled by both tissue specific enhancer elements and alternative splicing in the 5' end of the gene, giving rise to alternative promoters.

Original publications and manuscripts

I. **Beltcheva O**, Martin P, Lenkkeri U, and Tryggvason K. Mutation spectrum in the nephrin gene (NPHS1) in congenital nephrotic syndrome. Hum Mutat 2001 May;17(5):368-73.

II. **Beltcheva O**, Kontusaari S, Fetissov S, Putaala H, Kilpeläinen P, Hökfelt T, Tryggvason K. Alternatively used promoters and distinct elements direct tissue-specific expression of nephrin. J Am Soc Nephrol 2003 Feb;14(2):352-8.

III. **Beltcheva O**, Hjörleifsdóttir E, Kontusaari S, and Tryggvason K. Characterization of a nephrin gene regulatory element active in podocytes. *Manuscript*

Abbreviations

ACTN4 – alpha actinin 4

AFP - α-fetoprotein

ATRA - all trans retinoic acid

bp – base pair(s)

CD2AP – CD2 associated protein

cDNA – complementary DNA

CNF – congenital nephrotic syndrome of the Finnish type

CNS – central nervous system

DPE – downstream promoter element

E – embryonic day

EM – electron microscopy

EMSA – electromobility shift assay

ER - endoplasmic reticulum

GBM – glomerular basement membrane

GTF – general transcription factor(s)

HIF – hupoxia inducible afctor

Ig - immunoglobulin

Inr – initiator element

kb – kilobase pairs

PAGE – poly acrilamide gel electrophoresis

PAN – puromycin aminonucleoside

PCR – polymerase chain reaction

RACE – rapid amplification of cDNA ends

RAR – retinoic acid receptor

RT-PCR – reverse transcriptase PCR

TAFs – TBP associated factors

TBP – TATA binding protein

VEGF – vascular endothelial growth factor

WT1 – Wilms' tumor 1

ZO1 – zonula occludence 1

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Review of literature

1 Kidney podocytes – origin, structure and function

Kidneys are bean-shaped excretory organs in vertebrates. As a part of the urinary system they remove water soluble metabolic waste products from the blood, help the body to maintain the electrolyte and acid-base balance of the serum, regulate the fluid homeostasis and the blood pressure. In addition, the kidneys secrete the hormones renin and erythropoietin, and play an important part in the vitamin D3 synthesis.

In kidney cross sections two distinct parts can be observed – cortex and medulla. The functional unit of the kidney is called nephron. It is composed of glomerulus and tubules – proximal tubule, thin limbs, distal tubule and collecting duct. The glomeruli, together with the proximal tubules and part of the distal tubules are located in the cortex and the rest of the tubular system is in the medulla.

In adult humans almost 180 litres of blood are filtered through the kidneys every 24 hours, forming 1-1.5 litres of urine. The main filtration unit of the kidney is the glomerulus. It is composed of the following cell types: parietal epithelial cells of the Bowman's capsule, endothelial cells of the capillary tuft, podocytes (visceral epithelial cells) and mesangial cells. Figure 1 shows a schematic drawing of the glomerulus structure. The blood enters the glomerulus via an afferent blood vessel, which branches into a capillary tuft. It is filtered through the filtration barrier forming the primary urine, which, in turn, is collected in the Bowman's capsule and later processed in the tubular system of the kidney.

The filter of the kidney is formed by the fenestrated endothelial cells, the glomerular basement membrane (GBM) and the podocytes (Fig. 2). Previously the main role for the filtration was assigned to the GBM – a 300 nm thick amorphous, extracellular, sheet-like structure. Its main components are type IV collagen, laminin, nidogen and proteoglycans (Tryggvason, 2001). The collagen forms the structural meshwork of the basement membrane. It is connected to the laminin network by the nidogen (Timpl and Brown, 1996). In the embryo and during early childhood the collagen is formed by α1 and α2 chains, which are later replaced by α3, α4 and α5. This change is essential for ensuring the strength and

stability of the GBM. Failure to replace the embryonic collagen leads to Alport syndrome, manifested by disruption of the GBM with consecutive haematuria (Hudson et al., 1993).

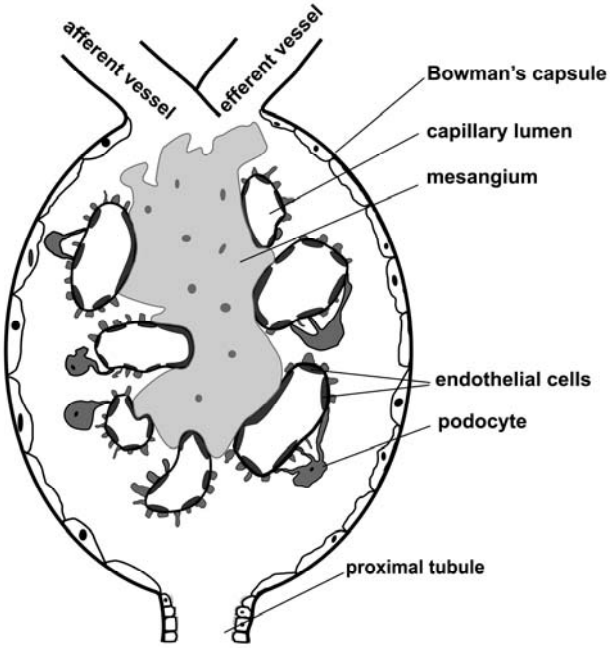

Fig. 1. Schematic presentation of glomerulus cross section.

Laminin is another component of the GBM. It has both a structural function and a role in cell adhesion and differentiation. As with the collagen, the embryonic laminin is replaced in adults with another isoform (Miner et al., 1997). Mice lacking the adult β2 laminin chain develop nephrotic syndrome despite the compensatory increase in the expression of the embryonic β1 isoform (Noakes et al., 1995). The phenotype observed in these animals shows the importance of laminin for the glomerular filtration and for maintaining the GBM stability.

The proteoglycans in the GBM, perlecan and agrin, are rich in anionic heparan sulphate moieties, and are thought to contribute to an electric barrier preventing plasma proteins from entering the filter (Groffen et al., 1999). However, in mice with heparan sulfate deficient perlecan the kidneys function normally and have no structural defects in the GBM (Rossi et al., 2003). This result does not preclude a role for the anionic molecules in the GBM, since other proteoglycans in the GBM might compensate for the perlecan defects.

Despite its importance for the filtration process, the GBM is neither the only nor the main macromolecular filter of the kidney (Tryggvason and Wartiovaara, 2005). Upon entering the glomeruli, the blood is first filtered through the openings of the fenestrated endothelium, which retains the blood cells and then passes through the GBM, which holds back the largest plasma proteins. Smaller proteins, the size of albumin or larger, are retained by the kidney ultrafilter – the slit diaphragm formed between the foot processes of the podocytes.

Podocytes are terminally differentiated cells. Their cell bodies are located in the urinary space and extend long processes towards the capillaries. On the surface of the blood vessels the processes branch, forming many thin, interdigitating foot processes, attached to the GBM through α3β1 and/or α2β1 integrins (Cybulsky et al., 1992). The narrow spaces between neighboring foot processes are bridged by a highly organized extracellular structure with a constant width of 300-450Å (Rodewald and Karnovsky, 1974). This structure represents the ultimate molecular sieve of the kidney - the slit diaphragm.

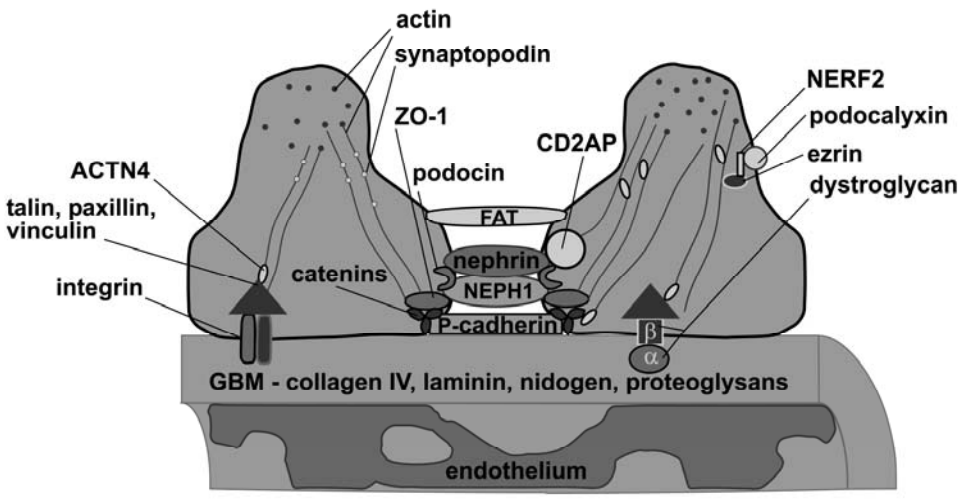

Fig. 2 Components of the glomerular ultra-filter. Cross section of podocyte foot processes with a slit-diaphragm. Nephrin, NEPH1, P-cadherin and FAT are presented in this way for simplicity, even though the former three do not span the entire width of the slit and the exact position of FAT is not yet determined.

The podocytes are highly specialized cells. They have a well developed Golgi apparatus, an abundant rough and smooth endoplasmic reticulum, numerous mitochondria and lysosomes. This is an indication for the high level of the metabolic activity in these cells,

necessary for sustaining the complex slit diaphragm structure and synthesizing most of the components of the GBM (Pavenstadt et al., 2003).

The unique shape of the podocytes with their long foot processes is maintained thanks to their distinctive cytoskeleton. Several specialized cytoskeleton proteins have been identified in podocytes. Synaptopodin has been shown to play a role in actin cytoskeleton organization and cell motility (Mundel et al., 1997). It is specifically expressed in dendrites of neurons and in podocytes. Alpha actinin 4 (ACTN4), an actin cross linking molecule is also important for cell motility (Honda et al., 1998). Mutations in both ACTN4 and synaptopodin have been shown to cause glomerular abnormalities (Kaplan et al., 2000; Srivastava et al., 2001). Podocalyxin has been recently found to participate in the organization of the actin cytoskeleton via its interactions with ezrin and NERF2 (Schmieder et al., 2004). This sialomucin located in the apical surface of the podocytes is also important for maintaining the urinary space open by means of its negatively charged ectodomain (Ohtsuka et al., 2001).

It is interesting to note the existence of common molecular structures in podocytes and neuron dendrites (Kobayashi et al., 2004). Both podocytes and neurons develop thick processes supported by microtubules, from which smaller and thinner, actin-based extensions branch out. The process formation is dependent on common intracellular traffic machinery, signal transduction, transmembrane transport and intercellular contacts. Some of the molecules common between podocytes and neurons are the actin associated proteins synaptopodin (Mundel et al., 1997) and densin (Ahola et al., 2003); signalling pathways molecules like the membrane protein-tyrosine phosphatase GLEPP1 (Thomas et al., 1994) and the receptor protein tyrosine phosphatases (RPTPs) (Beltran et al., 2003); amino acid transporters CAT3 and EAAT2 (Gloy et al., 2000); vesicle transport associated proteins like Rab3a and its effector rabphilin-3a (Rastaldi et al., 2003), as well as transmembrane proteins including nephrin in mouse and rat (Putaala et al., 2001), NEPH1 (Donoviel et al., 2001), FAT1 (Ciani et al., 2003) and kirrel2 (Sun et al., 2003).

1.1 Origin of the podocytes

During embryonic development in vertebrates kidneys originate from the intermediate mesoderm forming the nephric ridge. In mammals and birds, the kidney develops in three embryonic stages – pronephros, mesonephros and metanephros (Vize et al., 1997; Kuure et al., 2000). All three developmental forms have similar functional organization and differ mostly with regard to spatial organization, numbers and types of nephrons.

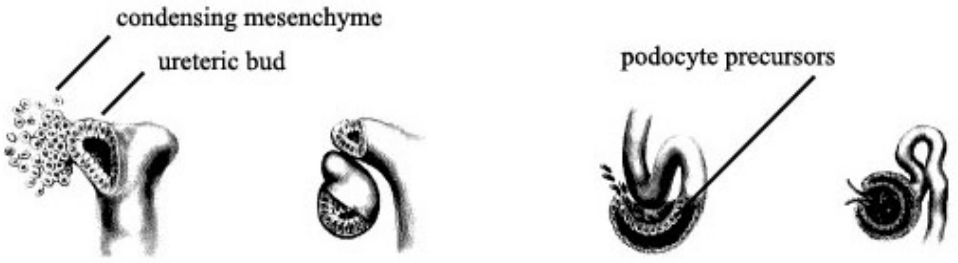

Fig. 3. Stages of metanephros development. A. Induction of condensation. **B.** Comma-shaped body. **C.** Capillary loop stage. **D**. Mature glomerulus. (Adapted from Saxén and Lehtonen, 1987)

The metanephros, the permanent adult kidney, starts to develop during gestation weeks 4-5 in man and E10.5 - E11 in mice (Fig. 3). Signals from the metanephric mesenchyme induce the formation of the ureteric bud from the Wolffian duct (the collecting duct in the pro- and mesonephros). The growing ureteric bud starts branching and forming the ureteric tree. It returns signals to the mesenchymal cells, converting them to epithelial phenotype and inducing them to condense and form the new metanephric nephrons. The mesenchyme aggregates and undergoes morphogenesis via several developmental stages. First, the condensed mesenchyme forms a hollow vesicle - renal vesicle, which consists of polarized epithelial cells surrounded by basement membrane. The next stages of the morphogenesis are the comma-shaped and S-shaped bodies. This is when the vascular cleft is formed and the epithelial cells start to differentiate, forming the Bowman's capsule (parietal epithelia) and the podocytes (visceral epithelia). At this stage, the future podocytes are simple polygonal cells, which multiply quickly. In the S-shaped bodies the vesicles join with the ureteric tree forming a continuous lumen. The beginning of the capillary loop stage is marked by the initiation of vascularization. The branching blood vessels form the capillary tuft with its fenestrated endothelium. During this last developmental stage, the podocytes acquire their specific morphology with long extensions enclosing the capillaries and slit diaphragms between the shorter foot processes. Podocytes and endothelial cells together synthesize the components of the GBM. By the end of this phase the mature metanephric glomerulus is established.

1.2 Molecular nature of the glomerular ultrafilter

In electron microscope sections the slit diaphragm is seen as wide intracellular space with a zipper-like pattern formed by periodic cross-bridges extending from the podocyte plasma membrane to a dense central filamentous line (Rodewald and Karnovsky, 1974). It is now known that multiple proteins are essential for formation, function and maintenance of the slit diaphragm. Among those are nephrin, ZO-1, NEPH1, podocin, CD2AP, FAT-1, P-cadherin, catenins and possibly some more as yet unidentified molecules. A schematic diagram of the structure of the slit diaphragm based on recent findings and hypothesis is shown on figure 2.

1.2.1 Nephrin

Nephrin was the first slit diaphragm protein to be identified. The NPHS1 gene coding for nephrin was cloned by Kestilä and co-authors in 1998. During embryonic development it is expressed in podocyte precursors in the late s-shaped bodies or the early capillary loop stage, in gestation weeks 13-23 in man and E14-E14.5 in mice (Li et al., 2000; Putaala et al., 2000). Mutations in the human gene cause congenital nephrotic syndrome of the Finnish type (Kestilä et al., 1998; Lenkkeri et al., 1999). In mice, inactivation of the nephrin gene causes massive proteinuria, and leading to death within 24 hours of birth (Putaala et al., 2001). This demonstrates the importance of nephrin for the slit diaphragm structure and function.

In mice nephrin, apart from kidney podocytes, is also expressed in the central nervous system (CNS), pancreatic β cells (Palmen et al., 2001; Putaala et al., 2001) and the testis (Liu et al., 2001a). The role of nephrin in these extra-renal tissues is still unknown. In man nephrin is expressed only in podocytes in the kidney glomeruli (Kestilä et al., 1998; Ruotsalainen et al., 1999; Kuusniemi et al., 2004). Extensive studies of human and porcine samples from embryonic or adult kidneys did not reveal nephrin expression outside kidney in embryo or adult (Kuusniemi et al., 2004).

Nephrin is a 180 kDa transmembrane protein of the immunoglobulin super-family (Fig. 4). It has an extracellular domain formed of eight Ig motifs and one fibronectin type III-like motif, a short transmembrane region and a cytoplasmic tail. N-linked glycosylation has been shown to be important for the proper folding and membrane localization of nephrin (Yan et al., 2002). Using immuno-electron microscopy it has been shown that nephrin is located in the slit diaphragm (Ruotsalainen et al., 1999). A model for the slit diaphragm has been suggested based on this finding. According to this hypothesis nephrin molecules from two adjacent foot processes reach out into the intracellular space and through homophilic interaction form the

molecular sieve of the slit (Ruotsalainen et al., 1999; Khoshnoodi and Tryggvason, 2001b; Tryggvason and Wartiovaara, 2005). This model has been supported by studies of nephrin homophilic interactions using surface plasmon resonance biosensor (Khoshnoodi et al., 2003) and a novel electron tomography technique (Wartiovaara et al., 2004).

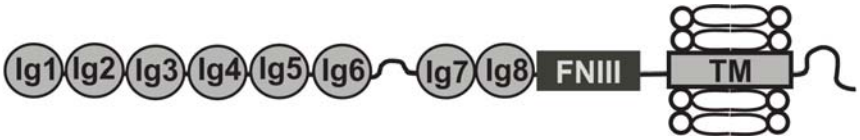

Fig. 4. Nephrin. Ig1-8 – immunoglobulin domains, FNIII – fibronectin type III like domain, TM transmembrane domain.

Nephrin is important for maintaining the cell-cell contacts and for organizing the cytoskeleton of the podocytes. This notion is supported by several recent reports. Nephrin has been shown to bind actin, possibly via CD2AP, thus providing a link between the cytoskeleton and the slit diaphragm (Lehtonen et al., 2002; Saleem et al., 2002; Yuan et al., 2002). In another study, nephrin has been found to co-precipitate with CD2AP, ZO-1, CASK, P-cadherin and p120 catenin in a large multiprotein complex - an indication of possible interaction with these scaffolding and cell-adhesion proteins (Lehtonen et al., 2004). Recent work from our lab shows that nephrin binds the putative regulator of cell–cell adherence junctions IQGAP1 (Liu et al., 2005).

There are indications that, apart from its structural function, nephrin also has a role as a signalling molecule (Benzing, 2004). The intracellular domain of the protein contains several tyrosine residues, which are potential targets for phosphorylation (Kestilä et al., 1998). Indeed, Fyn, a member of the Src family kinases has been shown to phosphorylate nephrin (Lahdenpera et al., 2003; Verma et al., 2003). This seems to be essential for the maintenance of the slit diaphragm, as Fyn deficient mice have been reported to develop massive proteinuria (Yu et al., 2001).

Recent reports show that nephrin may be involved in several signalling pathways. Expression of nephrin in HEK293 cells leads to 20-fold induction of AP-1 transcription factor (Huber et al., 2001). This induction is augmented by podocin and mediated by p38 and JNK

protein kinases. Fyn kinase seems to stimulate the interaction with podocin by phosphorylating the cytoplasmic tail of nephrin (Li et al., 2004).

It has been demonstrated that nephrin, upon phosphorylation (possibly by Src family kinases) and together with CD2AP, interacts with phosphoinositide 3-OH kinase (PI3K). This interaction results in recruitment of PI3K to the membrane and allows nephrin and CD2AP, together with podocin, to trigger a PI3K-dependant AKT signalling cascade (Huber et al., 2003a). One proposed effect of this nephrin-CD2AP-podocin induced activation of AKT is the inhibition of the detachment-induced apoptosis in podocytes.

Even though there is growing evidence for the involvement of nephrin in different signalling pathways, the biological function of these interactions is not completely clear and will require further studies.

1.2.2 ZO-1

ZO-1 is a 225 kDa protein of the membrane-associated guanylate kinase (MAGUK) family (Gonzalez-Mariscal et al., 2000). It contains three PDZ domains, and SH3, guanylate kinase and cytoplasmic domains. It has been shown that ZO-1 is subject to phosphorylation, which regulates its functions in the cells.

ZO-1 is involved in signal transduction and organization of the transmembrane complexes by binding cell junction (occludin and ZO-2) and cytoskeletal (actin) proteins. The PDZ domain of ZO-1 was recently shown to bind the C-terminus of NEPH1 (Huber et al., 2003b). It has been suggested that in this way ZO-1 may help in organizing the NEPH proteins and in recruiting signalling components to the slit. As mentioned earlier, ZO1 was shown to participate in a multiprotein complex with nephrin, P-cadherin, p120 catenin and CD2AP (Lehtonen et al., 2004).

ZO-1 is specifically enriched at tight junctions of epithelial and endothelial cells, but is also found in adherence junctions in certain cell types. It is located in the cytoplasm adjacent to the cell junction or in the nucleus. The nuclear localization of ZO-1 seems to be inversely related to the maturity of the cell contacts and may be an indication for a role in transcription regulation (Gonzalez-Mariscal et al., 2000). Indeed certain transcription factors have been shown to bind the SH3 domain of ZO-1 and shuttle together with it between the cytoplasm and the nucleus. ZO-1 has several known alternative splice isoforms, which differ in their C-termini (Gonzalez-Mariscal et al., 2000). Two of the splice variants are distinguished by the presence (+) or absence (-) of an 80 amino acid α domain. Most epithelial cells express both

α+ and α- isoforms with certain predominance of the former. The podocytes are the only known epithelial cell type where the α+ isoforms is absent. Endothelilal cells also express both α+ and α- isoforms but the latter is predominant. Interestingly, the glomerular endothelial cells only express the α- isoform. The two ZO-1 isoforms seem to have different roles in the cell. The α- isoform appeares to be related with junctional plasticity and is more typical for low-resistance tissue, such as podocytes and glomerular endothelium, characterised with higher permeability.

1.2.3 NEPH1

NEPH1 is a 90 kDa protein of the immunoglobulin family, recently shown to be expressed in kidney podocytes (Donoviel et al., 2001). Electron microscopy shows that it is localized in the slit diaphragm (Barletta et al., 2003). The NEPH1 knockout mice develop proteinuria and die within 8 weeks of birth (Donoviel et al., 2001). Absence of NEPH1 in these animals causes effacement of the foot processes, diffuse mesangial hypercellularity and increased mesangial matrix. No edema is observed in the affected mice. NEPH1 belongs to a family of three related proteins, shown to interact with the C-terminal domain of nephrin (Sellin et al., 2003). All three NEPH proteins share a common podocin binding motif. Similarly to nephrin, NEPH1 interaction with podocin activates AP1. This trans-activation is dependant on the Tec family kinases (Sellin et al., 2003). It was recently shown that NEPH1 and nephrin are involved in homodimeric and heterodimeric interactions with both their cytoplasmic and extracellular domains (Barletta et al., 2003; Gerke et al., 2003). It has been proposed that this interaction may be important for establishing the glomerular permeability (Liu et al., 2003). As mentioned above NEPH1 binds also to ZO-1 (Huber et al., 2003b).

1.2.4 Podocin

Podocin is a 42 kDa integral membrane protein belonging to the stomatin family (Boute et al., 2000). Immunoelectron microscopy reveals that it is located in the slit diaphragm and forms a hair-pin structure with both the N- and C-termini in the cytoplasm (Roselli et al., 2002). The gene coding for podocin, NPHS2, is found to be mutated in patients with autosomal-recessive steroid-resistant nephrotic syndrome (Boute et al., 2000). It is expressed in the metanephric kidney from the late S-shaped bodies stage (Roselli et al., 2002). Podocin-deficient mice die a few days after birth due to massive proteinuria (Roselli et al., 2004). The podocytes in these

animals have fused foot processes and lack a slit diaphragm. The expression of nephrin in the knockouts is downregulated, while CD2AP and ZO-1 appear to be upregulated. It has been proposed that podocin has a scaffolding function and helps in organizing the structure of the slit diaphragm (Roselli et al., 2002; Roselli et al., 2004).

As mentioned earlier podocin interacts with nephrin, CD2AP and NEPH1, thus augmenting the intracellular signalling (Huber et al., 2001; Schwarz et al., 2001; Sellin et al., 2003). Interestingly, mutations in the NPHS2 gene, not only cause misfolding and mislocalization of podocin itself, but they also affect the proper trafficking of nephrin (Nishibori et al., 2004).

1.2.5 CD2AP

CD2 associated protein (CD2AP) is an 80 kDa protein, with an actin binding site, a proline-rich domain and three SH3 domains. One of the SH3 domains binds the cytoplasmic domain of CD2 – a T- and natural killer cell membrane protein facilitating their adhesion to antigen presenting cells (Dustin et al., 1998). The discovery of CD2AP in podocytes came as a result from studies of CD2AP knockout mice. Apart from the defects in the immune system, the CD2AP deficient animals develop a severe renal phenotype and die at 6-7 weeks of age from kidney failure (Shih et al., 1999). Heterozygous animals develop glomerular defects at 9 months of age and show increased susceptibility to glomerular injury from nephrotoxic agents and immune complexes (Kim et al., 2003). EM studies reveal impaired trafficking in the podocytes of the affected animals and inability to clear plasma membrane proteins (Kim et al., 2003).

During metanephros development, CD2AP is first expressed at the capillary loop stage, shortly after nephrin (Li et al., 2000). It was shown to localize in the slit diaphragm region and interact with nephrin and podocin (Schwarz et al., 2001; Shih et al., 2001). These three proteins seem to be involved in regulation of the actin structures in the foot processes and in maintenance of the intracellular architecture (Li et al., 2000; Welsch et al., 2001; Saleem et al., 2002). As mentioned above, CD2AP also participates in nephrin-podocin induced intracellular signalling (Huber et al., 2003a) and interacts with ZO1, P-cadherin and p120 catenin (Lehtonen et al., 2004).

1.2.6 FAT1

FAT1 is a member of the cadherin family. It is a 516 kDa protein, which has 34 tandem cadherin and five EGF-like repeats, and laminin A-G domain (Dunne et al., 1995). In glomeruli FAT1 is expressed in podocytes and co-localizes with ZO-1 in the slit diaphragm (Inoue et al., 2001). It is involved in the organization of the complex actin cytoskeleton structures through its interaction with Ena/VASP proteins, which are implicated in actin-dependent motility processes (Kwiatkowski et al., 2003; Moeller et al., 2004; Tanoue and Takeichi, 2004).

FAT1 knockouts die before birth, possibly due to the renal abnormalities observed in the embryos -loss of slit diaphragm and fusion of foot processes (Ciani et al., 2003). It has been proposed that through its gigantic extracellular domain FAT1 plays role both as an adhesion molecule and as a spacer maintaining the width of the slit.

1.2.7 P-cadherin

P-cadherin is a 120 kDa transmembrane protein consisting of five cadherin domains in the extracellular part and a β-catenin binding site in the intracellular domain. During kidney development it is first expressed in the renal vesicles and later, during the S-shaped bodies stage, it co-localizes with nephrin and ZO-1 (Ruotsalainen et al., 2000). It has been proposed that P-cadherin bridges the slit diaphragm through homophilic interactions (Reiser et al., 2000). Since patients with mutations in the P- cadherin gene (Sprecher et al., 2001) and mice deficient of the protein do not develop any renal phenotype (Radice et al., 1997), this protein is not considered to be essential for the slit diaphragm formation.

2 Genetic defects of the renal ultrafilter. Congenital nephrotic syndrome of the Finnish type

Kidney filtration is affected in a large number of disease conditions in man. The renal injury may be due to genetic conditions, infections or exposure to toxins. Molecular defects in any part of the glomerular filtration barrier - fenestrated endothelium, GBM or podocytes with their slit diaphragm, can disturb the filtration process.

Nephrotic syndromes are defined by the presence of massive proteinuria, hypoalbuminemia and development of edema. This is a heterogeneous group of kidney

diseases, caused by defects in the structure and function of the podocyte foot processes and associated with increased permeability or loss of the glomerular ultrafilter (Khoshnoodi and Tryggvason, 2001a). Nephrotic syndromes can be classified by the age of presentation. Congenital nephrotic syndromes develop before 3 months of age. One well clinically defined type of these diseasse is the congenital nephrotic syndrome of the Finnish type (NPHS1 or CNF).

2.1 Clinical features and renal pathology

CNF is an autosomal, recessive, inherited disorder. The name of the disease derives from the fact that it was first diagnosed in the relatively isolated, homogenous, Finnish population, where it affects approximately 1 in 8,000 newborn children (Kestilä et al., 1994). Currently, however, CNF cases have been diagnosed worldwide, and in another isolated population, the Old Order Mennonites in Pennsylvania, it is even more common than in Finland with an incidence of 1:500 (Bolk et al., 1999). NPHS1 is characterised by heavy proteinuria already *in utero*, premature birth, a large placenta (more than 25% of the birth weight) and edema within a month after birth (Holmberg et al., 1996). It is often accompanied by complications like infections, thrombosis, and hypothyroidism, due to the immense loss of plasma proteins such as albumin, immunoglobulins, plasminogen, antithrombin III and thyroid hormones (Gavin et al., 1978; Panicucci et al., 1983; Harris et al., 1986). The CNF patients also have a high risk of developing atherosclerosis already during the first year of their life due to the loss of lipids and because of vascular pathology (Antikainen et al., 1992; Antikainen et al., 1994). All this accounts for the slow physical growth of the affected children.

Histological examinations reveal dilated proximal tubules and Bowman's space, mesangial cell hyperplasia, capillary dilation, increased size and number of glomeruli, and fibrosis in the glomeruli (Tryggvason and Kouvalainen, 1975; Autio-Harmainen and Rapola, 1981). The GBM seems to be unaffected with no changes in the expression level of its components, although it appears to be thinner in CNF patients (Autio-Harmainen and Rapola, 1981). Electron microscopy studies show effacement and fusion of the foot processes, a decrease in the number of slits between podocytes, and an absence of the typical zipper-like structure of the junction in the slit diaphragms (Ruotsalainen et al., 2000; Wartiovaara et al., 2004).

Historically NPHS1 patients have had a poor prognosis with most of them dying during the first half a year after birth. At present the only life-saving treatment for these children remains the kidney transplantation (Holmberg et al., 1996).

2.2 Genetic and molecular basis of NPHS1

Even though CNF was first described in the middle of the last century by Hallman and colleagues (Ahvenainen et al., 1956), the molecular basis of the disease remained unknown for a long time. In 1994, a genome wide screen in 17 affected families allowed the identification of a 150 kb candidate region on chromosome 19q13.1 (Kestilä et al., 1994). Analyses of the coding sequences within that region led to the identification of mutations in a novel gene named NPHS1 (nephrotic syndrome 1) (Kestilä et al., 1998).

The NPHS1 gene codes for a transmembrane protein of the Ig super family, called nephrin due to its nephron specific expression. It has 29 exons spanning over 26 kb area on the long arm of chromosome 19. Northern blot, *in situ* hybridization and immuno EM show that it is expressed in kidney podocytes and is located in the slit diaphragm (Kestilä et al., 1998; Ruotsalainen et al., 1999).

Two main nephrin mutations are found in the majority of the affected chromosomes in Finnish patients and carriers (Kestilä et al., 1998). Fin_{major} is a 2 bp deletion in exon 2, causing a frameshift and generation of stop codon in the same exon. It is found in approximately 78% of all Finnish NPHS1 chromosomes. The second most common mutation, Fin_{minor}, is a nonsense mutation in exon 26, found in approximately 16% of the affected chromosomes. Very few CNF patients of Finnish origin have other mutations. This uniform mutation pattern can be explained with the founder effect in the isolated Finnish population (Lenkkeri et al., 1999). Similar enrichment of founder CNF mutations has also been observed in Old Order Mennonites (Bolk et al., 1999) and Maltese families (Koziell et al., 2002).

In non-Finnish patients a large number of different mutations has been described (Kestilä et al., 1998; Bolk et al., 1999; Lenkkeri et al., 1999; Aya et al., 2000; Patrakka et al., 2000; Gigante et al., 2002; Koziell et al., 2002; Lahdenkari et al., 2004; Weber et al., 2004). The list of “non-Finnish” nephrin mutations includes small deletions and insertions causing changes in the reading frame or deletion of single amino acids, nonsense, missense and splice-site mutations. It is very intriguing that missense mutations represent more than half of the CNF causing sequence variants. How single amino-acid substitutions may completely abolish the

function of nephrin in the podocyte, causing renal failure, will be discussed in more detail in another chapter of this thesis.

It is interesting to note that deletions of multiple dinucleotides in a GA repeat region in the putative nephrin promoter have been observed in several patients (Lenkkeri et al., 1999). Similar deletion has been recently reported in a case of two siblings with sporadic nephrotic syndrome (Caridi et al., 2003). DNA analysis of both NPHS1 and NPHS2 revealed that the disease causing mutation is in the podocin gene. The healthy father of the patients is homozygous for the nephrin promoter mutation, which shows that the GA deletion is probably not disease causing.

Another promoter region mutation, G to C substitutions at positions -340, has been described in two independent studies (Gigante et al., 2002; Koziell et al., 2002). The effect of this sequence variant has not been determined so far, because it has only been found in heterozygous state.

Mutations in the nephrin gene have also been identified in patients with other glomerular diseases such as focal segmental glomerulosclerosis (Koziell et al., 2002), diffuse mesangial sclerosis (Schultheiss et al., 2004) and minimal change nephrotic syndrome (Lahdenkari et al., 2004). Surprisingly, a well known NPHS1 polymorphism – G to A substitution at position 349 (E117K on protein level), has been shown to modify the clinical manifestation of Ig A nephropathy (Narita et al., 2003). Moreover, the patients with AG or GG genotype presented with more proteinuria and increased deterioration of the renal filter compared to those with AA genotype. The authors speculate that the presence of GG alleles is a risk factor for deteriorated renal function.

2.3 Prenatal diagnostics

While the pathogenesis of CNF remained unknown, the prenatal diagnosis in affected families had been based solely on measurement of alpha-fetoprotein (AFP) levels in maternal serum and amniotic fluid during the second trimester (Aula et al., 1978). However, elevated AFP levels are not specific for congenital nephrotic syndrome of the Finnish type, which could lead to false positive results. It has now been shown that in some cases, increased AFP levels can be detected in pregnancies with heterozygous fetuses, indicating proteinuria *in utero* (Patrakka et al., 2002). This demonstrates once again the unreliability of AFP measurements as a diagnostic test for CNF.

With the cloning of NPHS1 it became possible to perform prenatal diagnostics based initially on haplotype analyses (Mannikko et al., 1997) and later using mutation screening. At present DNA tests represent much more reliable alternative for the affected families.

3 Determination and maintenance of podocyte phenotype – transcription regulation

Podocytes are highly specialized, terminally differentiated cells, which play a unique role in the filtration process in the kidney. As discussed earlier, podocytes originate from the metanephric mesenchymal cells. Their fate is determined and later maintained through initiation of a specific transcription program.

3.1 Transcription factors

In eukaryotic organisms, transcription of protein coding genes is carried out by polymerase II (PolII). PolII is a large complex formed by nearly 60 polypeptides. In the process of transcription PolII is assisted and regulated by a whole set of transcription factors – general transcription factors (GTFs), gene specific factors and co-regulators.

The assembly of the PolII with the GTFs is required for the initiation of transcription. In yeast, where transcription is best studied, the following GTFs have been identified: TFIIA, TFIIB, TFIID, TFIIF, TFIIE and TFIIH (Orphanides and Reinberg, 2002). Some of these factors are, in turn, formed of more than one subunit. For example, TFIID is formed by association of TBP (TATA binding protein) and TAFs (TBP associated factors). The transcription is initiated by binding of PolII and the GTFs to the core promoter sequence. This complex of factors, which are minimally essential for initiation of transcription from an isolated core promoter *in vitro*, is defined as basal transcription machinery.

The levels of transcription for each individual gene are determined by the binding of gene specific transcription factors. The gene specific transcription factors, which from now on will be referred to as simply transcription factors, are sequence-specific DNA binding proteins. They recognize and bind, directly or indirectly, different sites of the promoter. In some cases they bind in close proximity to the core promoter, but they often recognize sequences located in greater distances up or downstream of the transcription initiation site. The transcription factors have been classified into several groups, depending on common structural motives.

The TRANSFAC® database on eukaryotic transcription offers the following classification based on the nature of the DNA-binding domains: transcription factors with basic domain; zinc-coordinated DNA-binding factors; helix-turn-helix (HTH) transcription factors; β-scaffold factors with minor groove contacts. Few transcription factors fall outside of these categories.

The transcription factors regulate the activity of the transcription initiation complex through co-regulators. The most important co-regulator is the mediator complex. It was initially identified in yeast (Kelleher et al., 1990; Flanagan et al., 1991), but was later discovered in mammals as well (Chao et al., 1996; Fondell et al., 1996). It is a large complex, which has a conserved core of eight proteins found in all eukaryotic cells and different number of other, variable subunits (Bjorklund and Gustafsson, 2004). Unlike PolII, the GTFs and the majority of the transcription factors, the mediator is not able to bind DNA. It acts as a link between the promoter specific transcription factors and the basic transcription machinery. While the core subunits are responsible for binding of the mediator to the PolII and TFIIH, the variable subunits interact with the cell specific regulatory factors. Its modular structure, allows the mediator to integrate both positive and negative regulatory signals. A schematic drawing of the pre-initiation complex in eukaryotic cells is shown on figure 5.

During the recent years several transcription factors have been shown to be involved in podocyte development and differentiation (Quaggin, 2002; Kreidberg, 2003; Ly et al., 2004).

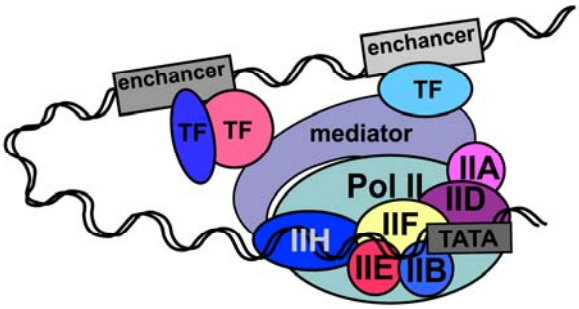

Fig. 5. Transcription pre-initiation complex. Pol II – polymerase II; TF – gene specific transcription factors; IIA, IIB, IID, IIE, IIF, IIH – general transcription factors.

3.1.1 WT1

WT1 is a zinc-finger transcription factor and RNA-binding protein. It was first identified as a tumour suppressor gene in a children kidney tumour – Wilms' tumour (Call et al., 1990; Gessler et al., 1990). WT1 is expressed during mammalian embryonic development in many tissues and is thought to play a role in the mesenchyme to epithelium differentiation, although its expression in tissue with ectodermal origin like brain, indicates other tissue specific roles in development (Armstrong et al., 1993). During kidney development WT1 is expressed in the metanephric mesenchyme, but appears not to be needed for its initial differentiation (Donovan et al., 1999). With the formation of the S-shaped bodies, WT1 expression becomes restricted to the podocytes (Armstrong et al., 1993). WT1 is expressed in podocytes throughout adulthood and is one of the main podocyte-specific molecular markers.

Through alternative splicing, RNA editing, and the use of alternative translation initiation sites, 24 different WT1 isoforms are produced, which seem to have different, though sometimes overlapping functions (Wagner et al., 2003).The four main isoforms are generated by two alternative splicing events (Haber et al., 1991). Alternative splicing of exon 5 inserts 17 amino acids in between the proline rich amino terminal domain and the four zinc-fingers. Mice lacking exon 5 develop without abnormalities, which shows that these isoforms have no major role in development or that the transcriptional changes occurring are not obvious unless a disease condition develops (Natoli et al., 2002b). The other major alternative splicing in WT1 involves exon 9, where two alternative splice sites give rise to isoforms differing by only 3 amino acids – lysine-threonine-serine (KTS) isoform. This change affects the third zinc-finger and alters its DNA binding capability (Drummond et al., 1994)

As mentioned earlier, several human diseases are associated with defects in the WT gene. Denys-Drash syndrome results from dominant negative mutations in the DNA-binding region of WT1 (Little et al., 1993). It is characterised by Wilms' tumour (WT), partial gonadal dysgenesis and nephropathy due to diffuse mesangial scelorosis in the glomeruli. Frasier syndrome is another WT1 associated disease. It is caused by mutations in exon 9 of the WT1 gene, which shift the ratio between the KTS+ and KTS- splice isoforms (Hammes et al., 2001). Frasier patients have focal segmental glomerular sclerosis and male-to-female sex reversal. Some cases of isolated diffuse mesangial sclerosis are also due to mutations in WT1 (Jeanpierre et al., 1998). Another disease caused by WT1 mutations is the WAGR syndrome, which is characterised with Wilms' tumours, aniridia, genitourinary malformations and

mental retardation. It is caused by a loss of one WT1 allele due to a chromosome 11p13 deletion (Riccardi et al., 1978).

Several mouse models exist with different defects in WT1 and diverse phenotypes. Some of the studies done on these animals give contradicting results regarding the involvement of WT1 in different stages of the renal development. This is probably due to lack of sufficient knowledge about the involvement of WT1 isoforms in different biological processes, its downstream targets and its relationship with other regulatory proteins, which makes the interpretation of the data more difficult.

Some of the experimental work supports the hypothesis for the major role of WT1 in podocyte differentiation. One such example is the WT1 knockout mouse. The WT1 null animals die at E13.5 due to heart defects without developing kidneys. Approximately 80% of the aging heterozygous animals develop glomerulosclerosis, effacement of the podocyte foot processes and proteinuria (Menke et al., 2003). A complementation experiment with human derived WT1 YAC shows rescue of the heart defects in the knockouts. However, hypoplasia is observed in kidneys of the rescued animals, demonstrating the importance of WT1 throughout the nephrogenesis and particularly the glomerular formation (Moore et al., 1999).

WT1 also seems to be involved in capillary formation during the development of the glomeruli. In a transgenic mouse model, animals heterozygous for a dominant negative mutation in the transcription factor have abnormally developed glomeruli, with dilated capillaries (Natoli et al., 2002a). Interestingly, nephrin, podocin, CD2-AP, synaptopodin and podocalyxin, are all unaffected in the diseased glomeruli. The authors speculate that WT1 is mainly responsible for regulating the expression of vascular factors important for angiogenesis in the kidney, rather than for regulating the differentiation of the podocytes themselves. This study seems to contradict a previous work, where the inducible expression of WT1 in immortalized rat kidney cell line induces expression of endogenous podocalyxin (Palmer et al., 2001).

In a recent work, mice unable to produce either KTS+ or KTS- isoforms were generated (Hammes et al., 2001). Both mutations result in glomerular abnormalities, although the KTS - null mice have a more severe phenotype, indicating that this isoform may be required earlier or that it has a more important role in the renal development. A decrease in nephrin expression is observed in the KTS - null mice, again contradicting the dominant negative WT1 mutation study of Natoli and co-workers (Wagner et al., 2004).

There have been other studies with different mouse models suggesting that WT1 may be involved in regulation of the nephrin gene expression (Guo et al., 2002; Menke et al., 2003).

Recently several publications on the nephrin promoter support this notion (Guo et al., 2004; Wagner et al., 2004).

3.1.2 Pax2

Pax2 is expressed in the intermediate mesoderm, which gives rise to the metanephros. It plays an important role in kidney development – Pax2 null mice lack kidney, ureters and genital tracts (Torres et al., 1995). During kidney development Pax2 is down-regulated in the podocyte precursors in the S-shape body simultaneously with the marked increase of WT1 expression in these cells (Ryan et al., 1995). Even though Pax2 does not have a direct role in podocyte differentiation, deregulation of its expression leads to development of abnormal and dysfunctional kidney epithelium with renal histology similar to that of congenital nephrotic syndrome (Dressler et al., 1993). Abnormalities of Pax2 expression due to WT1 mutations have been observed in several human diseases. In the majority of Denys-Drash syndrome patients, as well as some patients with isolated diffuse mesangial sclerosis, persistent expression of Pax2 in podocytes has been reported, which is suspected to attribute to the disease phenotype (Yang et al., 1999).

3.1.3 Lmx1b

Lmxb1 is a LIM-homeodomain transcription factor, mutations in which cause nail-patella syndrome. Up to one-half of the patients with this disease suffer from nephropathy, resulting in renal failure (Bennett et al., 1973). In kidney this transcription factor is expressed specifically in podocytes. Lmx1b knockout mice show glomerular abnormalities similar to those in nail-patella syndrome patients, i.e. no branching of the capillaries and less fenestrated endothelial cells due to decreased expression of VEGF, split GBM and cubical podocytes with no foot processes and slit diaphragm (Chen et al., 1998; Rohr et al., 2002). Podocalyxin, synaptopodin, ZO-1, α3-integrin and nephrin, but not podocin and CD2AP, are still expressed in the podocytes of the knockout animals (Miner et al., 2002; Rohr et al., 2002). Using electromobility shift assay (EMSA), the authors showed that Lmx1b binds regions in the podocin and CD2AP promoters and co-transfection assays confirmed the ability of this transcription factor to activate the podocin promoter.

3.1.4 Pod1

Pod1 is a member of the basic domain family of transcription factors. In the kidney, it is expressed early in development, in the condensing metanephric mesenchyme and is required for the epithelial differentiation. In podocytes, Pod1 is expressed first in the podocyte precursors at the S-shape stage and persists in adult kidneys (Quaggin et al., 1998). Knockout mice die at birth from lung and heart defects and show marked kidney abnormalities (Quaggin et al., 1999). The renal phenotype in the Pod1 knockouts includes vascular defects, disruption of the branching morphogenesis and failure of interstitial cell differentiation. The number of glomeruli in the mutant kidneys is reduced due to the limited branching. Glomeruli develop a single capillary and the podocytes remain undifferentiated, with a cubical shape and rudimentary foot processes. These data show that Pod1 plays an important role in the differentiation of the podocyte lineage.

3.1.5 Kreisler

Kreisler, also called MafB or Maf-1, is a basic domain transcription factor. In the kidney, Kreisler is expressed in mature podocytes, after the capillary loop stage. In a mouse model, generated by means of chemical mutagenesis, a single A to G substitution causes severe renal phenotype - the affected homozygous animals die from dehydration within the first 24 hours after birth (Sadl et al., 2002). Podocytes in these animals fail to develop foot processes and slit diaphragm and appear arrested in the capillary loop stage. Nephrin, podocin and CD2AP are still expressed in the mutant glomeruli, as well as the transcription factor Pod1. In contrast, Kreisler expression can be observed in Pod1 knockout animals, indicating that Kreisler is acting downstream of Pod1 at a later developmental stage.

3.1.6 RAR

The family of retinoic acid receptors belongs to the zinc-finger transcription factors. It has been shown that all-*trans*-retinoic acid (ATRA) has the ability to improve the glomerular function in puromycin aminonucleoside-induced nephrosis (PAN induced nephrosis) in rats. Moreover, the marked decrease in nephrin expression in the PAN treated animals is reversed after one week of daily administration of ATRA (Suzuki et al., 2003). The nephrin promoter was shown to contain three putative retinoic acid response elements and to be activated by

ATRA in a dose-dependant manner. It has been proposed that ATRA acts on the nephrin promoter via the retinoic acid receptors.

3.1.7 Sp1

Sp1 is a zinc-finger transcription factor. Although it is ubiquitously expressed, studies in mouse embryos have shown 100 fold differences in the levels of Sp1 expression between different cell types (Saffer et al., 1991). A role for Sp1 in nephrogenesis has been proposed based on data for its spatial and temporal expression in developing glomeruli and its ability to regulate WT1 expression (Cohen et al., 1997).

3.2 Regulatory sequences

The promoter region of each gene is the sequence, which determines its temporal and spatial expression. This is the sequence targeted by the PolII complex and the cell- specific transcription factors. Each promoter contains several elements with different functions during the transcription.

3.2.1 Core promoter

The core promoters recognized by the general transcription machinery, show considerable structural and functional diversity (Fig. 6). The most common elements of the core promoter are TATA box, initiator element, (Inr) downstream promoter element (DPE), TFIIB recognition element (BRE) and CpG islands (Smale and Kadonaga, 2003).

The TATA box is the first discovered core promoter element. It is located 25-30 bp upstream of the transcription initiation site and contains the sequence TATAAA. Single base pair differences from the consensus sequence have been found in certain promoters. The TATA box is found in 30-40% of the PolII promoters. It is recognized and bound by the TBP general transcription factor.

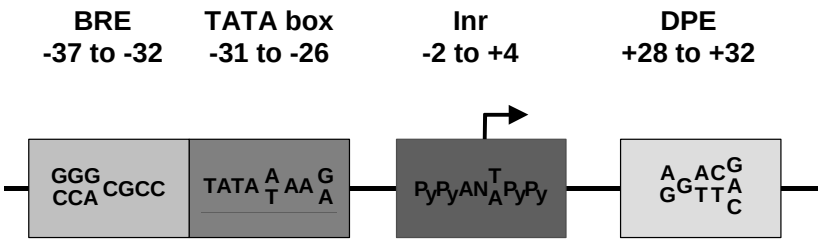

Fig. 6. Common core promoter elements. The name and localization of each element is shown on the top. The boxes contain the consensus sequences of the respective elements.

The initiator is an element of the core promoter, functionally similar but independent of the TATA box. Database analyses of Inr sequences in mammals reveal the following consensus sequence PyPyA(+1)NT/APyPy. Mutation analyses indicate that the presence of this Inr sequence between –3 and +5 around the transcription initiation site is necessary and sufficient for accurate transcription initiation *in vivo* and *in vitro*. The Inr element alone is able to initiate similar levels of transcription as the TATA box by itself. It is recognized by the TAFs.

The DPE is another promoter element needed for the binding of the TFIID complex. It is typically found in promoters lacking TATA box. This core promoter sequence is conserved from Drosophila to human, acts together with the Inr and is located at position +28; +32 bp from the A(+1) nucleotide of the Inr. A decrease in the distance between Inr and DPE by even one nucleotide, results in marked decrease of the transcription activity.

BRE is a TFIIB recognition sequence found initially in *Archaea*. The human TFIIB binding sequence, contrary to the archaeal, appears to be capable of not only activating, but also suppressing the transcription.

It has been estimated that in mammals approximately half of the promoters are associated with CpG islands. They appear to be protected from *de novo* methylation in all tissues and through different developmental stages. Even though many promoters have CpG islands, these core elements are not well understood. Promoters with CpG islands usually lack TATA box, DPE and Inr elements and have multiple transcription start sites. The CpG islands are commonly associated with multiple Sp1 binding sites.

3.2.2 Enhancers

Enhancer sequences are recognized by the gene specific transcription factors, which convey external and internal signals and thus regulate the expression in agreement with the cell's needs and functions. Unlike the core promoter sequences, the enhancers are located at varying distances from the transcription initiation site. Sometimes they can be found tens or hundreds of thousands of base pairs up or downstream of the gene they regulate. Enhancer elements are very diverse, which together with their unpredictable localization explains why regulatory sequences have been found in only few podocyte specific genes.

In recent years there have been several studies of nephrin promoter sequences in man and mice (Moeller et al., 2000; Wong et al., 2000; Eremina et al., 2002; Moeller et al., 2002) The identification of RAR and WT1 binding sites in human and mouse nephrin promoters has been reported (Suzuki et al., 2003; Guo et al., 2004; Wagner et al., 2004).

The podocin promoter containing all necessary elements for podocyte specific expression has recently been shown to reside within 2.5 kb upstream of the transcription start site (Moeller et al., 2002). One enhancer element identified in this sequence is the Lmx1b binding site (Miner et al., 2002; Rohr et al., 2002). Lmx1b recognizes short AT rich motifs, known as FLAT-E (5' TAATTA 3') and FLAT-F (5' TTAATAAT 3') sites (German et al., 1992). Both types of motives have been identified in the podocin promoter at positions -832; -809 and -1079; -1072, respectively. Using EMSA, it has been shown that both sites can be recognized and specifically bound by human and hamster Lmx1b protein. Co-transfection of an Lmx1b expression plasmid and luciferase reporter plasmid under the 4.4 kb podocin promoter in COS-7 cells or an immortalized podocyte cell line fail to show activation of the podocin promoter (Rohr et al., 2002). The authors explain that lack of trans-activation with possible absence of additional transcription factor(s) needed for the proper regulation. In another study, the FLAT element located at position -825 in the podocin promoter was cloned together with its flanking sequence in multiple copies upstream of a minimal Col2a1 promoter and co-transfected with Lmx1b expression plasmid in NIH 3T3 cells (Miner et al., 2002). In this system Lmx1b could up-regulate the luciferase expression through the podocin enhancer.

As discussed earlier, the Lmx1b transcription factor is also needed for the expression of CD2AP in podocytes. FLAT sites are present at positions -2855, -1817 and -1170 in the promoter of that gene and are specifically recognized by full length human Lmx1b in EMSA (Miner et al., 2002).

A recent study reveals new information about the VEGF-A regulation in podocytes. VEGF-A is a multifunctional cytokine with an important role in vasculogenesis and

angiogenesis. In glomeruli it is expressed by developing and mature podocytes and is known to play a role in proliferation, differentiation and survival of the capillary endothelial cells. Several mouse models and some human disease conditions indicate that the precise regulation of the VEGF-A expression levels is essential for maintenance of the glomerular function. Patients treated with VEGF-A inhibitors often develop high blood pressure and proteinuria. Heterozygous animals of the podocyte specific VEGF-A knockout mouse model show endotheliosis, “bloodless glomeruli” and develop nephrotic syndrome. The homozygotes die shortly after birth from kidney failure (Eremina et al., 2003). On the other hand, overexpression of this cytokine may also cause kidney failure(Eremina et al., 2003). It has been shown that VEGF-A expression is controlled by binding of hypoxia-inducible factor (HIF) to enhancer element in the promoter of the gene (Liu et al., 1995). In podocytes, which are not situated in hypoxic region in the kidney, there is a specific pathway for the activation and binding of HIF to the VEGF-A promoter. It has been shown that the extracellular matrix plays a major role in this process. Binding of the GBM laminins to the $\alpha 3\beta 1$ integrin on the podocyte membrane triggers a cascade involving PKC, which stimulates the binding of HIF to its adaptor protein p300 and respectively to the VEGF-A enhancer element (Datta et al., 2004).

Protein tyrosine phosphatase RQ (PTPRQ) is a member of the type II receptor-like tyrosine phosphatase family, which is expressed in podocytes in human kidney. The PTPRQ gene was found to be upregulated in a rat model of glomerular injury (Wright et al., 1998). Its expression is regulated by alternative promoters and alternative slicing (Seifert et al., 2003). One promoter drives expression of a transcript encoding a transmembrane protein in the basal membrane of human podocytes. The second promoter drives expression of another transcript encoding a cytoplasmic protein in rat mesangial cells and human testis. The differential regulation of the transmembrane and cytosolic forms appears to be cell dependant.

Aims of the present study

The work presented in this thesis was initiated after the discovery of nephrin. Mutations in its gene were found to cause Congenital nephrotic syndrome of the Finnish type – a severe kidney disease, associated with destruction of the renal ultrafilter and massive proteinuria. The clinical manifestations of patients lacking nephrin were the first indications for the essential role of this protein in the kidney filtration barrier. These findings raised numerous questions regarding the particular role of nephrin in development of CNF and the mechanisms controlling its cell- and tissue-specific expression. Thus, the specific aims of the present study were to:

1. Further characterise the mutation pattern in the nephrin gene in congenital nephrotic syndrome of the Finnish type.

2. Search for possible mutation hot spots in the nephrin gene, which may prove useful for the purposes of DNA testing in CNF families.

3. Search for and characterise the mouse nephrin promoter which drives its tissue specific expression.

4. Attempt to identify specific transcription factors involved in the nephrin gene regulation.

Materials and methods

This chapter contains a brief overview of the materials and methods used in the presents study. More detailed descriptions of the experimental procedures can be found in the respective original articles included in this thesis and designated here with Roman numerals.

Patient samples (I) DNA samples from CNF children and their families were sent to us for analyses by the respective physicians in charge of the patients. The samples were accompanied by detailed information about the family history, diagnosis and treatment. The origin of the subjects was diverse - samples were sent to us from France, Sweden, Germany, Turkey, the Netherlands, USA and the UK.

Nephrin gene mutation screening (I) The entire nephrin gene was sequenced for each CNF patient and the available family members. All 29 exons were amplified by PCR and sequenced using previously described primers (Lenkkeri et al., 1999). Automated sequencing was performed using ABI310 and ABI377 sequencers. The results were analysed with FASTA (Genetics Computer Group) and BLAST (GenBank).

Generation and analysis of transgenic mice (II) The nephrin gene regulation study presented here was carried out using transgenic mice. Five different regions of the putative nephrin promoter sequence were cloned in front of *LacZ* as a marker gene and the resulting constructs were injected in fertilized mouse oocytes. Embryos were dissected on days E12.5, E14.5 or E17.5 and genotyped by PCR of amnionic DNA. Tissue expression of the transgene was studied on paraffin sections after *LacZ* staining.

RNA isolation, 5'RACE and RT-PCR (II) For the transcription start site identification a 5'RACE assay was performed, using total RNA from mouse kidney and cerebellum. Primers from exons 2 and 4 were used for the reverse transcriptase reactions and the resulting PCR products were cloned and sequenced.

Analyses of cDNA and amino acid sequences (II) Homology searches were performed using FASTA (Genetics Computer Group), BLAST (GenBank™) and mVISTA (Dubchak et al., 2000; Mayor et al., 2000). For signal peptide predictions SPScan program (Genetics Computer Group) and SIGFIND (Nielsen et al., 1997) were used.

Immunohistochemistry (II) The distribution of nephrin in brains of knockout and wild type new-born mice was studied by immunohistochemistry with a polyclonal rabbit anti-mouse antiserum raised against the intracellular part of nephrin (Putaala et al., 2001). Immunoreactivity in the brain sections was analysed using confocal laser scanning microscope. The specificity of the antibodies was tested by preabsorbtion tests with an excess of nephrin protein.

Cell culture (III) The following cell lines were used for this study: HEK293 cells, immortalized mouse podocyte cell line (Schiwek et al., 2004) and macrophage-like cells (MLC-6) (Sakiyama et al., 2001). The podocyte cells were a kind gift from K. Endlich.

Nuclear extracts preparation (III) Nuclear extracts from an immortalized mouse podocyte cell line were used for the identification of transcription factors involved in the regulation of the nephrin gene. Cells grown in both permissive and non-permissive conditions were used as a source of nuclear proteins. Extracts of HEK293 and MLC-6 cells were used in control reactions.

Electromobility shift assay (EMSA) (III) EMSA was performed using labeled probes generated by PCR with biotinylated primers or by annealing of biotinylated oligonucleotides. Fragments were resolved with PAGE and transferred to a positively charged nylon membrane. Visualization of the results was done using chemiluminescence detection kits. As a zinc-finger inhibitor 1,10-phenanthroline was used in different concentrations. Antibody directed against the N-terminus of WT1 was added to some of the binding reactions to induce super-shift of the bands.

Transcription factor binding sites predictions (III) Analysis for putative transcription factor recognition sites was performed using rVISTA (Loots et al., 2002), Match (BioBase), AliBaba2 (Biobase) and MOTIF (GenomeNet) prediction programmes.

Results

1 Nephrin mutations in non-Finnish CNF patients (I)

In the course of the present study more than 30 patients with congenital nephrotic syndrome and their families were referred to us for NPHS1 screening from medical centres in France, Sweden, Germany, Turkey, the Netherlands, USA and the UK. Sequencing of all 29 exons of the nephrin gene for each subject resulted in the identification of 20 novel disease causing nucleotide changes. From these, five generated a premature stop codon, nine caused amino acid substitutions, one was a small non-frameshift deletion, one a frameshift insertion, one a combined deletion/insertion resulting in change of the reading frame and three affected the splicing of the RNA. These mutations are listed in Table 1. The missense mutation P264R was initially classified as "sequence variant with unknown phenotype effect" (Table 3, Article I) but was later shown to cause CNF (Koziell et al., 2002). The phenotype of three other novel amino acid substitutions, H617R, E725D and A851V, is still unclear.

Six polymorphisms were identified along with the disease causing mutations (Table 1). Five of them did not cause any change on protein level. In one case a threonine residue was replaced with isoleucine at position 295. This mutation was found in a mother, healthy CNF carrier, heterozygous for a known disease causing mutation. Since both mutations were on different chromosomes we could safely conclude that T295I is a neutral polymorphism.

2 Characterization of the nephrin promoter sequence (II)

For the purposes of our nephrin gene regulation study several transgenic mouse lines were generated using different promoter constructs. A genomic clone containing 6 kb of the sequence upstream of the mouse nephrin ATG codon was sequenced and used for the promoter study. Five different segments of the 5' upstream region of nephrin were cloned in front of β-galactosidase as a reporter gene and the constructs were then used to generate transgenic mice. Tissue expression of *LacZ* in the different mouse lines was studied by histochemistry. The results were confirmed in at least two independent lines for each of the

constructs. Schematic presentation of all five promoter constructs used in the present study, the number of independent lines analysed and the results obtained for each of them can be seen in Article II, Fig. 1.

Table 1. Sequence variants in NPHS1 identified in the present study

Number	Exon	Sequence variant	Effect on protein	Phenotype
1.	4	468 C→G	Y156X	CNF
2.	4	513-5 delCAC	del 172T	CNF
3.	5	532 C→T	Q178X	CNF
4.	6	692 C→A	S231X	CNF
5.	7	791 C→G	P264R	CNF
6.	8	881 C→T	T295I	polymorphism
7.	9	1100 G→A	R367H	CNF
8.	9	1103 C→T	P368L	CNF
9.	9	1135 C→T	R379W	CNF
10.	10	1250 G→T	C417F	CNF
11.	11	1379 G→A	R460Q	CNF
12.	13	1672 C→T	R558C	CNF
13.	13	1701 C→A	C567X	CNF
14.	15	2071+2 T→C	splice	CNF
15.	17	2216 C→T	A739V	CNF
16.	17	2289 C→A	V763V	polymorphism
17.	18	2500 G→T	V834F	CNF
18.	19	2617 delTCA ins CC	frameshift	CNF
19.	23	3165 A→T	S1055S	polymorphism
20.	26	3315 G→A	S1105S	polymorphism
21.	26	3357 ins GG	frameshift	CNF
22.	27	3388+2 A→G	splice	CNF
23.	27	3424 C→T	L1142L	polymorphism
24.	27	3426 G→T	L1142L	polymorphism
25.	27	3442 C→T	Q1148X	CNF
26.	27	3481+1 G→T	splice	CNF

Constructs nephA and nephB contained 6,242 and 4,013 bp, respectively, of the 5' UTR and the upstream region (with the exception of bases -1 to -3). Mouse lines generated with these two longest constructs had *LacZ* expression in podocytes, central nervous system – brain and spinal cord, and pancreas (Fig. 2B, Article II). This expression pattern was identical to that described for the endogenous nephrin (Putaala et al., 2001).

A third construct, nephC, was generated by deletion in the 5' end of nephB. It contained 2,148 bp of the upstream region and the 5' UTR. Analysis of the nephC animals showed complete lack of *LacZ* expression in 4 independent mouse lines.

NephB-Δ700, the fourth promoter construct used for generation of transgenic mice, was based on nephB but a 700 bp deletion was introduced between -1,249 and -1,908 bp. Expression of this constructs was observed only in podocytes and brain but not in spinal cord and pancreas (Fig. 2C, Article II).

3 Identification of a zinc-finger transcription factor binding site (III)

Together, the results from the four different promoter constructs indicated that the sequence between -4 and -2.1 kb upstream of the ATG codon of exon 1 was important for directing podocyte-specific expression. Homology search using VISTA scan revealed three regions within this sequence, conserved between mouse and human (Fig. 6, Article II; Fig. 1, Article III). Regions I and II correspond to the first two exons of the newly identified gene – filtrin or kirrel2 (Ihalmo et al., 2003; Sun et al., 2003), so the search for regulatory sequences focused on the third homologous region (reg3).

Electromobility shift assay (EMSA) was used to determine if putative cis-regulatory sequences were present in reg3. For that purpose, a biotin labeled reg3 fragment was generated with PCR and after incubation with podocyte nuclear proteins from immortalized podocyte cell line (Schiwek et al., 2004) it was subjected to PAGE. Figure 2 in Article III shows the prominent shift observed in this assay. It could be competed out with as little as 100 times molar excess of unlabeled probe – an indication for specificity of the binding (Fig. 2, Article III). Specific binding was also observed using nuclear extracts from human embryonic kidney cells (HEK293) (Fig. 2, Article III). Nuclear proteins from macrophage-like cells, however, were not able to bind reg3 (Fig. 2, Article III).

In order to determine the precise region recognized by the putative transcription factor(s) we used several overlapping unlabeled competitors in an EMSA assay and managed to show

that a 20 bp sequence, reg3.7, was involved in the binding (Fig. 3A, B, Article III). We could further narrow down the analysed region using four reg3.7 mutant non-biotinilated competitors in a competition assay – a stretch of six guanines was shown to be essential for the binding (Fig. 3C, Article III). Using two different chelating agents – EDTA and 1, 10 phenanthroline, we could abolish the binding of the putative transcription factor to reg3.7 (Fig. 3D, Article III), which indicated that the unknown protein belongs to the family of zinc-finger factors.

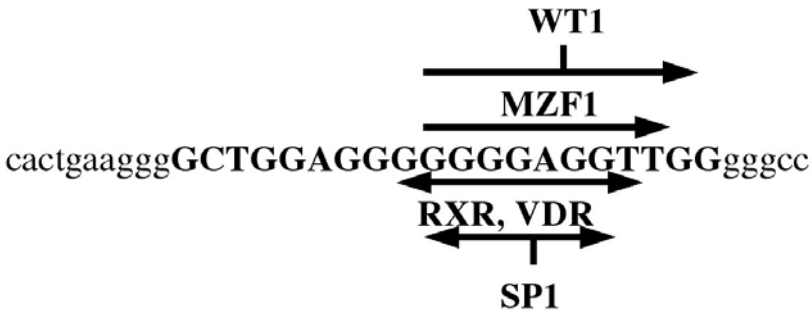

Fig. 7. Predicted transcription factors binding sites reg3.7. The sequence of reg3.7 is shown in bold capital letters. Arrows indicate the orientation of the respective *cis*-elements.

The identification of the putative recognition site allowed the use of different prediction programs to search for candidate transcription factors. As shown in figure 7, among the hits were several Zn-finger proteins – SP1, WT1, retinoid X receptor α, vitamin D receptor, MZF1. Based on the data in the literature we considered WT1 and MZF1 promising candidates and tried to determine whether one of them was responsible for the shift observed in the EMSA assays. A WT1 antibody, known to bind the factor without disturbing its association with DNA, thus generating a supershifted band, was used in EMSA (Heckman et al., 1997). As figure 3D in Article III shows, no supershift was observed with this antibody, so we concluded that WT1 most likely was not the factor binding to reg3.7. Similarly, we tried to determine the possible involvement of MZF1 in the EMSA binding using antibodies (data not shown). Again, we could neither obtain supershifted band nor abolish the binding – indication that MZF1 is also not likely to bind reg3.7.

4 Alternative splicing. Expression of nephrin in extra-renal tissues (II)

Analyses of the transgenic animals generated with construct nephA-Δ500 led to the identification of alternative splicing in the 5' end of the nephrin gene. This construct lacked the 5' UTR, including the transcription initiation site and part of the immediate promoter region. It was generated as a negative control and was not expected to be able to drive *LacZ* expression. Surprisingly, the transgenic embryos showed strong *LacZ* staining in the central nervous system (Fig. 2D, Article II) similar to that shown for the native nephrin promoter (Putaala et al., 2001). No staining was observed in kidney or pancreas of the nephA-Δ500 mice.

The ability of nephA-Δ500 to drive nephrin-specific CNS expression, led us to investigate the existence of alternative transcription start site(s), and novel exon(s) upstream from the main transcription initiation site. For that purpose, both kidney and cerebellar mouse mRNA were examined for the presence of alternative nephrin 5' ends using 5' RACE. For kidney mRNA, the transcription start site was found to be located at position -381 bp, slightly upstream of the previously published one (Putaala et al., 2000). When cerebellar mRNA was used for the 5' RACE different results were obtained - an alternative 5' end and respectively a novel exon were located at position -1,871. The new exon was designated exon 1B and the previously described first exon was named exon 1A. Using RT-PCR we could see that exon 1A is expressed in both kidney and brain, while exon 1B is specific for brain. Exon1B was found to be spliced together with exons 2 through 30 (Fig. 3 and Fig. 4, Article II). It is 271 bp long and has a typical GT-donor site at its 3' end. For both kidney and cerebellar nephrin isoforms no TATA box was identified. In both cases the nucleotides around the transcription start site were in agreement with the requirements for Inr element in mammalian cells (Javahery et al., 1994). The ATG codon of exon 1B was found to agree with the Kozak consensus sequence (RNNatgG) (Kozak, 1986).

The two alternative first exons give rise to different sequences in the amino-terminus of the nephrin protein (Fig. 4, Article II). Exon 1A codes for a typical signal peptide sequence, directing nephrin to its location on the cell membrane (Putaala et al., 2000). Analysis of the amino-terminus encoded by exon 1B, performed with two different prediction programs, however, did not reveal a signal peptide cleavage site.

The existence of a brain specific nephrin was confirmed using the knockout mice created in our laboratory (Putaala et al., 2001). As described by Putaala and co-authors, the nephrin knockout was generated by the insertion of a *LacZ-neo* cassette in exon 1A and disruption of

the reading frame. It was expected that knocking out exon 1A would not affect the expression of exon 1B nephrin and we wanted to confirm that that was indeed the case. Using a polyclonal antibody raised against the intracellular part of the mouse nephrin we analysed tissue sections of -/- mice. As expected, nephrin could not be found in the kidneys of knockout animals. In brain sections, however, we could see quite similar staining in knockout mice compared to their wild – type or heterozygous littermates (Fig. 5, Article II).

In order to improve the understanding of the role of nephrin in extra renal tissues, we performed a detailed study of its expression in brain of rodents. The exact locations of nephrin in mouse brain can be seen in Fig. 5 in Article II. We detected a nephrin signal in the cerebral cortex, choroid plexus, pia and arachnoid maters, hypothalamus and axons in the posterior pirtuitary. Similar results were obtained with rat samples (unpublished data).

Discussion

1 Mutation screening in non-Finnish CNF patients (I)

In the Finnish population, due to the founder effect, many inherited diseases are caused by a limited number of mutations in the respective genes. Such is also the case with CNF, where two mutations are found in the majority of the patients of Finnish origin. In Finland 65% of the NPHS1 patients are homozygous for Fin_{major}, 8% homozygous for Fin_{minor} and 16% Fin_{major} / Fin_{minor} compound heterozygous (Patrakka et al., 2000). Other mutations account for only limited number of cases. We were interested in analyzing the mutation pattern in patients of non-Finnish origin, because we believed that the identification of a broader spectrum of mutations will give new insights into the function of nephrin and the roles of its different domains.

As a part of the present study we performed screening of the nephrin gene in more than 30 foreign CNF families from different countries. The results from our work, described in details in the previous section and in the original publication included in this thesis, were very interesting in several respects. First of all the majority of newly identified mutations resulted in "simple", single amino acid substitutions, the effects of which on protein level was hard to predict. Second, we identified very large number of novel sequence variants, with almost every family having their private disease causing change.

Missense mutations represent approximately half of the disease-causing nucleotide changes in CNF patients described to-date. While it is easy to understand the effect of deletions and nonsense mutations on protein level, it is more difficult to explain the consequence of a single amino acid substitution for the function of nephrin. It has been proposed that some of the missense mutations occur in highly conserved regions, remove cysteine residues or change the charge in certain domains, thus disturbing the proper folding and function of nephrin (Koziell et al., 2002). Having in mind that the missense mutations are distributed all over the protein it is not very likely that this hypothesis is valid for all cases. Indeed, it has been shown that many of the single amino acid substitutions in fact affect the trafficking of nephrin within the cell (Liu et al., 2001b). When 21 different nephrin mutants were expressed in HEK293 cells the protein failed to reach the plasma membrane and were

retained in the endoplasmic reticulum (ER) in 75% of the cases. Based on this finding, in a follow-up study the authors show the ability of chemical chaperons to rescue some of the mutants from ER (Liu et al., 2004). These results show that at least some of the missense mutations affect only the proper localization of nephrin but not its function and allow the authors of the study to speculate on the prospects of using such compounds for treatment of CNF patients (Liu et al., 2004).

The high percentage of missense mutations involved in the pathogenesis of CNF can also present a diagnostic challenge. In cases when such a mutation has not been previously identified in patients or healthy controls it would be difficult to prove whether it is disease causing or a neutral polymorphism since the effect on protein level will be hard to predict. The situation becomes even more complicated by the fact that recently other genes have been implicated in nephrotic syndromes (Kaplan et al., 2000; Koziell et al., 2002; Schultheiss et al., 2004). In such cases, it would be essential for the family to have correct diagnosis of the disease in order to decrease the risk for false positive results.

Another difficulty in performing DNA tests for non-Finnish CNF families arises from the variability of the disease causing mutations in the nephrin gene. This is of particular significance when fast genetic tests for the purpose of prenatal diagnostics are needed. In Finland, where the majority of patients have the Fin_{major} and/or Fin_{minor} mutations it is easy to analyse the two potentially affected exons in the family, identify the disease causing change and prepare for genotyping of the fetus. In a non-Finnish family, however, sequencing of the whole gene is required, which is more time consuming. This will especially affect families waiting for results from prenatal diagnostic tests.

For all these diagnostic considerations, it was deemed useful to identify particular mutation hot-spots in the nephrin gene. Such types of mutation-prone sequences have been described for other genes (Beggs et al., 1990; Caron de Fromentel and Soussi, 1992; Bonneau and Longy, 2000). The phenomenon is explained by the nature of the affected DNA stretch, which triggers errors in the replication and/or repair machinery. The knowledge of such hot-spots would direct genetic consultants performing DNA tests in their search for disease causing variants in CNF families and would decrease the time needed for genotyping and prenatal diagnostics in affected individuals.

A review of all available nephrin mutations at the time this study was performed revealed that the distribution of the 50 known mutations was not uniform along the gene (Fig.1, Article I). Three exons, namely 4, 9 and 18, contained 37% of the mutations, even though they represent only 12% of the coding sequence. Despite the large number of mutations identified

it was obvious that some exons remained essentially mutation free – exons 1, 3, 8, 20-23, 28 and 29. This distribution may be explained with the small number of mutations identified so far, or with greater functional importance of the protein domains coded for by the mutation free exons. With the addition of the novel mutations identified since the publication of our work, however, the distribution along the gene does not look much different (Patrakka et al., 2000; Gigante et al., 2002; Koziell et al., 2002; Schultheiss et al., 2004; Weber et al., 2004; Sako et al., 2005). Approximately one third of the 62 mutations shown to-date to cause CNF are located in exons 4, 9 and 18 despite their small size. The identification of two novel mutations in exons 3 and 23 (Koziell et al., 2002; Weber et al., 2004), leaves only eight unaffected by disease causing nucleotide changes i.e. 1, 8, 20-22, 28 and 29. Three novel mutations in the relatively short exon 14 place it among the mutation rich regions of NPHS1 (Koziell et al., 2002; Schultheiss et al., 2004).

Even though these observations may indicate greater functional importance of some nephrin domains, and despite some other publications depicting Ig - like motives 2, 4 and 7 as nephrin mutation hot-spots (Koziell et al., 2002), it is not possible to assert that there are actual hot spots in the NPHS1 gene. The performance of reliable DNA diagnostics for the CNF patients and their families still requires the sequencing of all 29 exons.

The variability of the mutations identified in this study did not allow us to draw any conclusions about possible phenotype/genotype relationship. There are, however, several reports in the literature of CNF patients with milder symptoms, which respond to treatment with ACE-inhibitors (Patrakka et al., 2000; Koziell et al., 2002). They have attracted a lot of attention and attempts have been made to reveal the genetic traits determining the milder disease course. In one case, a female patient, with very mild proteinuria, was found to be compound heterozygous for Fin_{major} and a missense mutation R743C. Electron microscopy study of renal biopsy sample showed that nephrin was still expressed in her kidneys and the slit diaphragm appeared intact (Patrakka et al., 2000). Another example of milder phenotype was described for Maltese and Indian patients homozygous for the nonsense mutation R1109X (Koziell et al., 2002). Interestingly, the milder phenotype appeared to be gender related and was predominantly seen in female patients.

2 Regulation of the nephrin gene expression (II, III)

In man nephrin is expressed only in podocytes in kidney glomeruli (Kestilä et al., 1998; Kuusniemi et al., 2004). Surprisingly, in mouse nephrin was also found in the central nervous system and pancreatic β-cells (Palmen et al., 2001; Putaala et al., 2001). The aim of the present work was to try to identify the regulatory sequences and transcription factors responsible for this expression pattern in mice. Studies of the mechanisms directing the tissue-specific expression will not only help us to understand the regulation of the nephrin gene, but may also give us insights into podocyte differentiation and biology.

In order to study the gene regulation we used transgenic mice, an experimental model which was considered to give the most reliable picture of the regulatory mechanisms in the nephrin expressing cells. Cloning different fragments of the nephrin promoter in front of the *LacZ* gene and consecutive analyses of the expression pattern of the reporter in the transgenic animals allowed us to identify sequences essential for the tissue specific expression of the endogenous gene.

The marker gene expression observed with the two longest constructs, nephA (6.2 kb) and nephB (4 kb), was basically the same as the described for nephrin in mice (Putaala et al., 2000). Glomerular podocytes, spinal cord, brain and pancreas in these transgenic animals stained positive for *LacZ*. These results were in agreement with previously published works, reporting -5.4 kb and -4.1 kb mouse promoter regions (Moeller et al., 2000; Eremina et al., 2002), and indicated that elements necessary and sufficient for ensuring podocyte specific expression reside within the first 4 kb upstream of the ATG codon of exon 1A.

The lack of *LacZ* expression observed with the third construct, nephC (2.1 kb), was, however, in contradiction with results reported by other groups. Much shorter fragments spanning only up to -1.25 kb, have been shown to be sufficient for driving podocyte specific expression for both mouse and human promoters (Wong et al., 2000; Moeller et al., 2002). One possible explanation for this contradiction might be that the 1.25 kb region has a weaker kidney enhancer, inhibited by suppressor elements immediately upstream, -2.1 ; -1.25 kb, while other podocyte specific regulatory elements reside in the region -4 kb ; -2.1 kb. It can be speculated that collaborative action of the two regions would ensure stable and specific podocyte expression.

This hypothesis could explain some of our unpublished transgenic mouse data. In one out of two founder lines generated with a 1,214 bp promoter construct we observed podocyte

staining. However, the mice from both lines also had clearly ectopic, extra-renal *LacZ* staining in tissues such as brain, cartilage and bone, possibly due to an integration site dependent influence over the weak podocyte specific elements contained in this fragment.

Whether or not suppressor elements exist in the region -2.1; -1.25 has not yet been determined. However, based on the results of the analysis of the nephB-Δ700 transgenic mice, it could be concluded that this region was not important for podocyte expression of the transgene. The introduction of 700 bp deletion between -1.25 and -1.9 kb did not affect the expression of *LacZ* in podocytes and brain (Fig. 2C, Article II). Spinal cord and pancreas, on the other hand, lacked the expression of the transgene (Fig. 2C, Article II). Comparison of the results for nephB, nephC and nephB-Δ700, showed that the region between -1.25 and -1.9 kb was required for expression in pancreas and spinal cord, while the sequence -4; -2.1 contained podocyte specific elements.

Very recently two independent studies showed that WT1 binding elements exist in close proximity to the basic promoter of the nephrin gene in both human and mouse (Guo et al., 2004; Wagner et al., 2004). Moreover, a 186 bp fragment of the human nephrin promoter, located 743 bp upstream of the ATG codon and later shown to contain WT1 binding sites, is capable of directing podocyte specific expression of *LacZ* marker gene when cloned in front of a minimal promoter (Guo et al., 2004). This short fragment is active when cloned in single or multiple copies and no neuronal expression is seen in any of the transgenic lines generated with it. It is worth mentioning that one of the two transgenic lines carrying a single copy of the 186 bp fragment showed mosaic *LacZ* expression - not all glomeruli were positively stained. In one of the two mouse lines generated using multiple copies of the fragment extra-renal staining in whisker follicles and epicardium was observed. This unspecific expression of the transgene probably results from integration site-dependent influence. The weaker enhancer activity in the first case and the extra-renal expression in the second case, are in agreement with our hypothesis that more than one element is needed for directing endogenous, nephrin-like expression. It is possible that the 186 bp WT1 responsive element needs to work in co-operation with the -4; -2.1 kb enhancer region to ensure the proper tissue specific expression of nephrin.

In an extensive, EMSA based screening, we managed to identify a short region (reg3.7) within the -4; -2.1 kb enhancer, which was recognized and bound by, as yet undetermined, protein factor(s) in nuclear extracts from a podocyte cell line. Similar results were observed for nuclear extracts from human embryonic kidney cells (HEK293), but not with nuclear proteins from macrophage-like cells. This indicated that HEK293 cells probably had part of

the transcription factors needed for initiation of transcription from the nephrin gene. The inability of the macrophage-like nuclear extracts to induce an EMSA shift indicated that the transcription factor in question was not widely expressed, or required additional cell specific factors.

The 20 bp reg3.7 was a part of a highly conserved stretch of DNA, located between exon 1B of nephrin and exon 1 of the newly identified gene – filtrin or kirrel2. A mutations screening of the region revealed the importance of six successive guanine nucleotides for the binding. An inhibition assay using chelating agents abolished the binding of the nuclear protein(s) to reg3.7, which indicated that the putative transcription factor(s) belong to the family of the zinc-coordinated DNA binding proteins. The zinc-finger family is the most abundant class of transcription factors - almost half of the predicted 2,000 human transcription factors are dependant on zinc ions for binding DNA (Urnov, 2002). As discussed earlier, many members of this family have been implicated in kidney development - WT1, RAR, Krueppel-like transcription factors, SP1, etc. Using different TRANSFAC® based prediction programs we tried to identify candidate factors that could be binding within or close by the six G stretch in reg3.7. As shown in Figure 7, among those were several zinc-finger proteins – SP1, WT1, retinoid X receptor α, vitamin D receptor, MZF1. Based on the data in the literature we considered two of these factors, namely WT1 and MZF1, good candidates. WT1 has been shown to play important role in podocyte development in general and in nephrin gene regulation in particular (Armstrong et al., 1993; Guo et al., 2002; Natoli et al., 2002a; Menke et al., 2003; Wagner et al., 2003; Guo et al., 2004; Wagner et al., 2004). MZF1 is a zinc-finger protein previously shown to be involved in regulation of gene expression through alternative splicing (Nomoto et al., 1999). Using antibodies against the two candidate factors we showed that neither of them was likely to be binding reg3.7.

Since the prediction programs could not help us in identifying the putative transcription factor and because it is always possible that we were dealing with an unknown protein we tried to purify it by DNA affinity chromatography. However, our efforts have failed, so far. The lack of success in these experiments is probably due to the low amounts of the transcription factor in nuclear extracts, which, together with the high background, makee the visualization of the protein on Coomassie stained gels very difficult.

It is interesting to note that reg3.7, which contained a podocyte specific transcription factor binding site, was not well conserved in the human sequence (Fig.1, Article III). This observation came as a surprise, since the work on region III was based on the homology between the two species. However, there are data showing that this is not always the case.

Recently Dermitzakis *et al*. have shown that between 32% and 40% of the human transcription binding sites are not functional in rodents due to the turnover of the promoter sequences in evolution (Dermitzakis and Clark, 2002). It is quite possible that the reg3.7 transcription factor binding site is located in another region in the human promoter. This human sequence remains to be identified.

An important question about the nature of the reg3.7 enhancer rises in connection with the recent discovery of kirrel2/filtrin. This novel gene is located upstream of nephrin and transcribed in opposite direction from a transcription start site located in close proximity to exon 1B. It codes for an immunoglobuline–like protein, with several splice isoforms and is expressed in kidney podocytes, pancreatic islet cells and nervous system (Ihalmo et al., 2003; Sun et al., 2003). The similarity in the expression pattern of the two genes is striking. It is tempting to speculate that they both share some regulatory elements as it has been shown for other genes (Heikkila et al., 1993). It would be interesting to test if the reg3.7 enhancer can work in both directions and whether it is involved in the regulation of both genes.

3 Identification of a brain-specific nephrin isoform (II)

In the present study we report the discovery of a novel nephrin isoform, which in rodents is specifically expressed in brain. The identification of alternative splicing in the 5' end of the nephrin gene came as unexpected result from our transgenic mouse work. The novel nephrin variant was shown to be specific for the central nervous system of mouse and rat, where it possibly co-localizes with the original kidney isoform.

Alternative splicing is a common event in the genome of the higher organisms, which is believed to contribute to the increase of the protein diversity and in this way to facilitate evolution (Maniatis and Tasic, 2002). A typical example of a protein with several variants generated as a result of alternative splicing of the mRNA is WT1 with its 24 isoforms (Wagner et al., 2003). In recent years alternative splicing has been described also for nephrin (Holthofer et al., 1999; Luimula et al., 2000). Four different splice variants, α-, β-, γ- and δ- involving exons 24 through 27 have been described in rats.

So far, nothing is known about the function of the nephrin isoforms. In an attempt to elucidate the function of the brain-specific nephrin a detailed study of the expression of the protein in mouse and rat brain was performed. We studied both nephrin knockout mice

lacking the kidney nephrin and their wild type and heterozygous littermates in order to distinguish between the two isoforms.

Using immunohistochemistry it was found that nephrin is expressed in several regions of the brain. Detailed description of these observations is included in the "Results" section of this thesis as well as in the original publication (Article II). We found the expression of nephrin in the choroid plexus especially intriguing. The choroid plexus is involved in blood filtration and in establishing the blood/brain barrier, function similar to that of the podocytes in kidney glomeruli. Since nephrin has been shown to be essential for the filtration in kidney it was tempting to speculate on a similar role for it in brain.

No apparent difference in the distribution of exon 1A and 1B variants were detected in sections of rodent brains. The localization of the immunoreactivity in both knockout mice and their wild type control was identical.

It is interesting that while nephrin can be found in CNS in mouse and rat, an extensive search for it in extra renal tissues in human and pig has been unsuccessful (Kuusniemi et al., 2004). It is probable still that expression of nephrin in brains of rodents is not accidental and has biological meaning, but more studies need to be carried out in order to reveal its role there.

4 General conclusions

The kidney filter has for a long time been a centre of attention of both scientists and medical specialists. The discovery of nephrin, the first podocyte specific protein shown to be part of the slit diaphragm and the consecutive identification of other slit proteins helped for unravelling the molecular structure of the renal ultrafilter.

In man nephrin is expressed only in kidney podocytes, where it is located in the slit diaphragm between adjacent foot processes (Kestilä et al., 1998; Kuusniemi et al., 2004). The identification of mutations in the NPHS1 gene led to better understanding of the pathogenesis behind the congenital nephrotic syndrome of the Finnish type. Recently, new studies report on involvement of nephrin in other common diseases of the renal ultrafilter such as focal segmental glomerulosclerosis and minimal change nephrotic syndrome (Koziell et al., 2002; Lahdenkari et al., 2004). Even some polymorphisms in nephrin have been shown to affect certain disease phenotypes, as it is in the case of IgA nephropathy patients (Narita et al., 2003). This thesis work reports on the identification of 20 novel CNF causing mutations and 6

neutral polymorphisms. These findings have not only had a diagnostic meaning, but have also provided valuable information regarding the molecular filter of the kidney in health and disease.

The studies on nephrin gene regulation described in this thesis together with those performed by several other groups during the last years (Moeller et al., 2000; Wong et al., 2000; Moeller et al., 2002; Guo et al., 2004; Wagner et al., 2004), have revealed a lot of information regarding the *cis* and *trans* elements involved. Regulatory sequences for both human and mouse nephrin have been reported (Wong et al., 2000; Moeller et al., 2002). It has also been shown that two transcription factors, namely RAR and WT1, are involved in regulating nephrin expression (Guo et al., 2004; Wagner et al., 2004). These discoveries have already found practical application. The generation of a glomerular-specific Cre-recombinase transgenic mice using nephrin promoter will be a valuable tool in studying different podocyte specific genes and proteins (Eremina et al., 2002). The present study has shown that the mouse nephrin expression is regulated by both alternative splicing in the 5' end of the gene and presence of enhancer elements in two regions of the promoter -4; -2.1 kb and -1.9; -1.2kb. A transcription factor binding site, consisting of a stretch of six Gs was identified and shown to be recognized by a zinc-finger protein present in podocyte nuclear extracts. Hopefully further studies will lead to the purification and identification of this putative zinc-coordinated factor. Further investigations would also be required for understanding the role of nephrin in extra-renal tissues in rodents.

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References:

Ahola H, Heikkila E, Astrom E, Inagaki M, Izawa I, Pavenstadt H, Kerjaschki D, and Holthofer H (2003). A novel protein, densin, expressed by glomerular podocytes. J Am Soc Nephrol *14*, 1731-1737.

Ahvenainen EK, Hallman N, and Hjelt L (1956). Nephrotic syndrome in newborn and young infants. Ann Paediatr Fenn *2*, 227-241.

Antikainen M, Holmberg C, and Taskinen MR (1992). Growth, serum lipoproteins and apoproteins in infants with congenital nephrosis. Clin Nephrol *38*, 254-263.

Antikainen M, Sariola H, Rapola J, Taskinen MR, Holthofer H, and Holmberg C (1994). Pathology of renal arteries of dyslipidemic children with congenital nephrosis. Apmis *102*, 129-134.

Armstrong JF, Pritchard-Jones K, Bickmore WA, Hastie ND, and Bard JB (1993). The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. Mech Dev *40*, 85-97.

Aula P, Rapola J, Karjalainen O, Lindgren J, Hartikainen AL, and Seppala M (1978). Prenatal diagnosis of congenital nephrosis in 23 high-risk families. Am J Dis Child *132*, 984-987.

Autio-Harmainen H, and Rapola J (1981). Renal pathology of fetuses with congenital nephrotic syndrome of the Finnish type. A qualitative and quantitative light microscopic study. Nephron *29*, 158-163.

Aya K, Tanaka H, and Seino Y (2000). Novel mutation in the nephrin gene of a Japanese patient with congenital nephrotic syndrome of the Finnish type. Kidney Int *57*, 401-404.

Barletta GM, Kovari IA, Verma RK, Kerjaschki D, and Holzman LB (2003). Nephrin and Neph1 co-localize at the podocyte foot process intercellular junction and form cis hetero-oligomers. J Biol Chem *278*, 19266-19271.

Beggs AH, Koenig M, Boyce FM, and Kunkel LM (1990). Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction. Hum Genet *86*, 45-48.

Beltran PJ, Bixby JL, and Masters BA (2003). Expression of PTPRO during mouse development suggests involvement in axonogenesis and differentiation of NT-3 and NGF-dependent neurons. J Comp Neurol *456*, 384-395.

Bennett WM, Musgrave JE, Campbell RA, Elliot D, Cox R, Brooks RE, Lovrien EW, Beals RK, and Porter GA (1973). The nephropathy of the nail-patella syndrome. Clinicopathologic analysis of 11 kindred. Am J Med *54*, 304-319.

Benzing T (2004). Signaling at the slit diaphragm. J Am Soc Nephrol *15*, 1382-1391.

Bjorklund S, and Gustafsson CM (2004). The mediator complex. Adv Protein Chem *67*, 43-65.

Bolk S, Puffenberger EG, Hudson J, Morton DH, and Chakravarti A (1999). Elevated frequency and allelic heterogeneity of congenital nephrotic syndrome, Finnish type, in the old order Mennonites. Am J Hum Genet *65*, 1785-1790.

Bonneau D, and Longy M (2000). Mutations of the human PTEN gene. Hum Mutat *16*, 109-122.

Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, Dahan K, Gubler MC, Niaudet P, and Antignac C (2000). NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. Nat Genet *24*, 349-354.

Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis WH, and et al. (1990). Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell *60*, 509-520.

Caridi G, Bertelli R, Di Duca M, Dagnino M, Emma F, Onetti Muda A, Scolari F, Miglietti N, Mazzucco G, Murer L, *et al.* (2003). Broadening the spectrum of diseases related to podocin mutations. J Am Soc Nephrol *14*, 1278-1286.

Caron de Fromentel C, and Soussi T (1992). TP53 tumor suppressor gene: a model for investigating human mutagenesis. Genes Chromosomes Cancer *4*, 1-15.

Chao DM, Gadbois EL, Murray PJ, Anderson SF, Sonu MS, Parvin JD, and Young RA (1996). A mammalian SRB protein associated with an RNA polymerase II holoenzyme. Nature *380*, 82-85.

Chen H, Lun Y, Ovchinnikov D, Kokubo H, Oberg KC, Pepicelli CV, Gan L, Lee B, and Johnson RL (1998). Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat Genet *19*, 51-55.

Ciani L, Patel A, Allen ND, and ffrench-Constant C (2003). Mice lacking the giant protocadherin mFAT1 exhibit renal slit junction abnormalities and a partially penetrant cyclopia and anophthalmia phenotype. Mol Cell Biol *23*, 3575-3582.

Cohen HT, Bossone SA, Zhu G, McDonald GA, and Sukhatme VP (1997). Sp1 is a critical regulator of the Wilms' tumor-1 gene. J Biol Chem *272*, 2901-2913.

Cybulsky AV, Carbonetto S, Huang Q, McTavish AJ, and Cyr MD (1992). Adhesion of rat glomerular epithelial cells to extracellular matrices: role of beta 1 integrins. Kidney Int *42*, 1099-1106.

Datta K, Li J, Karumanchi SA, Wang E, Rondeau E, and Mukhopadhyay D (2004). Regulation of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF-A) expression in podocytes. Kidney Int *66*, 1471-1478.

Dermitzakis ET, and Clark AG (2002). Evolution of transcription factor binding sites in Mammalian gene regulatory regions: conservation and turnover. Mol Biol Evol *19*, 1114-1121.

Donovan MJ, Natoli TA, Sainio K, Amstutz A, Jaenisch R, Sariola H, and Kreidberg JA (1999). Initial differentiation of the metanephric mesenchyme is independent of WT1 and the ureteric bud. Dev Genet *24*, 252-262.

Donoviel DB, Freed DD, Vogel H, Potter DG, Hawkins E, Barrish JP, Mathur BN, Turner CA, Geske R, Montgomery CA, *et al.* (2001). Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. Mol Cell Biol *21*, 4829-4836.

Dressler GR, Wilkinson JE, Rothenpieler UW, Patterson LT, Williams-Simons L, and Westphal H (1993). Deregulation of Pax-2 expression in transgenic mice generates severe kidney abnormalities. Nature *362*, 65-67.

Drummond IA, Rupprecht HD, Rohwer-Nutter P, Lopez-Guisa JM, Madden SL, Rauscher FJ, 3rd, and Sukhatme VP (1994). DNA recognition by splicing variants of the Wilms' tumor suppressor, WT1. Mol Cell Biol *14*, 3800-3809.

Dubchak I, Brudno M, Loots GG, Pachter L, Mayor C, Rubin EM, and Frazer KA (2000). Active conservation of noncoding sequences revealed by three-way species comparisons. Genome Res *10*, 1304-1306.

Dunne J, Hanby AM, Poulsom R, Jones TA, Sheer D, Chin WG, Da SM, Zhao Q, Beverley PC, and Owen MJ (1995). Molecular cloning and tissue expression of FAT, the human homologue of the Drosophila fat gene that is located on chromosome 4q34-q35 and encodes a putative adhesion molecule. Genomics *30*, 207-223.

Dustin ML, Olszowy MW, Holdorf AD, Li J, Bromley S, Desai N, Widder P, Rosenberger F, van der Merwe PA, Allen PM, and Shaw AS (1998). A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. Cell *94*, 667-677.

Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH, and Quaggin SE (2003). Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest *111*, 707-716.

Eremina V, Wong MA, Cui S, Schwartz L, and Quaggin SE (2002). Glomerular-specific gene excision in vivo. J Am Soc Nephrol *13*, 788-793.

Flanagan PM, Kelleher RJ, 3rd, Sayre MH, Tschochner H, and Kornberg RD (1991). A mediator required for activation of RNA polymerase II transcription in vitro. Nature *350*, 436-438.

Fondell JD, Ge H, and Roeder RG (1996). Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc Natl Acad Sci U S A *93*, 8329-8333.

Gavin LA, McMahon FA, Castle JN, and Cavalieri RR (1978). Alterations in serum thyroid hormones and thyroxine-binding globulin in patients with nephrosis. J Clin Endocrinol Metab *46*, 125-130.

Gerke P, Huber TB, Sellin L, Benzing T, and Walz G (2003). Homodimerization and heterodimerization of the glomerular podocyte proteins nephrin and NEPH1. J Am Soc Nephrol *14*, 918-926.

German MS, Wang J, Chadwick RB, and Rutter WJ (1992). Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. Genes Dev *6*, 2165-2176.

Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, and Bruns GA (1990). Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature *343*, 774-778.

Gigante M, Monno F, Roberto R, Laforgia N, Assael MB, Livolti S, Caringella A, La Manna A, Masella L, and Iolascon A (2002). Congenital nephrotic syndrome of the Finnish type in Italy: a molecular approach. J Nephrol *15*, 696-702.

Gloy J, Reitinger S, Fischer KG, Schreiber R, Boucherot A, Kunzelmann K, Mundel P, and Pavenstadt H (2000). Amino acid transport in podocytes. Am J Physiol Renal Physiol *278*, F999-F1005.

Gonzalez-Mariscal L, Betanzos A, and Avila-Flores A (2000). MAGUK proteins: structure and role in the tight junction. Semin Cell Dev Biol *11*, 315-324.

Groffen AJ, Veerkamp JH, Monnens LA, and van den Heuvel LP (1999). Recent insights into the structure and functions of heparan sulfate proteoglycans in the human glomerular basement membrane. Nephrol Dial Transplant *14*, 2119-2129.

Guo G, Morrison DJ, Licht JD, and Quaggin SE (2004). WT1 Activates a Glomerular-Specific Enhancer Identified from the Human Nephrin Gene. J Am Soc Nephrol *15*, 2851-2856.

Guo JK, Menke AL, Gubler MC, Clarke AR, Harrison D, Hammes A, Hastie ND, and Schedl A (2002). WT1 is a key regulator of podocyte function: reduced expression levels cause crescentic glomerulonephritis and mesangial sclerosis. Hum Mol Genet *11*, 651-659.

Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, and Housman DE (1991). Alternative splicing and genomic structure of the Wilms tumor gene WT1. Proc Natl Acad Sci U S A *88*, 9618-9622.

Hammes A, Guo JK, Lutsch G, Leheste JR, Landrock D, Ziegler U, Gubler MC, and Schedl A (2001). Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. Cell *106*, 319-329.

Harris HW, Jr., Umetsu D, Geha R, and Harmon WE (1986). Altered immunoglobulin status in congenital nephrotic syndrome. Clin Nephrol *25*, 308-313.

Heckman C, Mochon E, Arcinas M, and Boxer LM (1997). The WT1 protein is a negative regulator of the normal bcl-2 allele in t(14;18) lymphomas. J Biol Chem *272*, 19609-19614.

Heikkila P, Soininen R, and Tryggvason K (1993). Directional regulatory activity of cis-acting elements in the bidirectional alpha 1(IV) and alpha 2(IV) collagen gene promoter. J Biol Chem *268*, 24677-24682.

Holmberg C, Laine J, Ronnholm K, Ala-Houhala M, and Jalanko H (1996). Congenital nephrotic syndrome. Kidney Int Suppl *53*, S51-56.

Holthofer H, Ahola H, Solin ML, Wang S, Palmen T, Luimula P, Miettinen A, and Kerjaschki D (1999). Nephrin localizes at the podocyte filtration slit area and is characteristically spliced in the human kidney. Am J Pathol *155*, 1681-1687.

Honda K, Yamada T, Endo R, Ino Y, Gotoh M, Tsuda H, Yamada Y, Chiba H, and Hirohashi S (1998). Actinin-4, a novel actin-bundling protein associated with cell motility and cancer invasion. J Cell Biol *140*, 1383-1393.

Huber TB, Hartleben B, Kim J, Schmidts M, Schermer B, Keil A, Egger L, Lecha RL, Borner C, Pavenstadt H*, et al.* (2003a). Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. Mol Cell Biol *23*, 4917-4928.

Huber TB, Kottgen M, Schilling B, Walz G, and Benzing T (2001). Interaction with podocin facilitates nephrin signaling. J Biol Chem *276*, 41543-41546.

Huber TB, Schmidts M, Gerke P, Schermer B, Zahn A, Hartleben B, Sellin L, Walz G, and Benzing T (2003b). The carboxyl terminus of Neph family members binds to the PDZ domain protein zonula occludens-1. J Biol Chem *278*, 13417-13421.

Hudson BG, Reeders ST, and Tryggvason K (1993). Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis. J Biol Chem *268*, 26033-26036.

Ihalmo P, Palmen T, Ahola H, Valtonen E, and Holthofer H (2003). Filtrin is a novel member of nephrin-like proteins. Biochem Biophys Res Commun *300*, 364-370.

Inoue T, Yaoita E, Kurihara H, Shimizu F, Sakai T, Kobayashi T, Ohshiro K, Kawachi H, Okada H, Suzuki H*, et al.* (2001). FAT is a component of glomerular slit diaphragms. Kidney Int *59*, 1003-1012.

Javahery R, Khachi A, Lo K, Zenzie-Gregory B, and Smale ST (1994). DNA sequence requirements for transcriptional initiator activity in mammalian cells. Mol Cell Biol *14*, 116-127.

Jeanpierre C, Denamur E, Henry I, Cabanis MO, Luce S, Cecille A, Elion J, Peuchmaur M, Loirat C, Niaudet P*, et al.* (1998). Identification of constitutional WT1 mutations, in patients with isolated diffuse mesangial sclerosis, and analysis of genotype/phenotype correlations by use of a computerized mutation database. Am J Hum Genet *62*, 824-833.

Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, Mathis BJ, Rodriguez-Perez JC, Allen PG, Beggs AH, and Pollak MR (2000). Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. Nat Genet *24*, 251-256.

Kelleher RJ, 3rd, Flanagan PM, and Kornberg RD (1990). A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. Cell *61*, 1209-1215.

Kestilä M, Lenkkeri U, Mannikko M, Lamerdin J, McCready P, Putaala H, Ruotsalainen V, Morita T, Nissinen M, Herva R*, et al.* (1998). Positionally cloned gene for a novel glomerular protein-nephrin-is mutated in congenital nephrotic syndrome. Mol Cell *1*, 575-582.

Kestilä M, Mannikko M, Holmberg C, Gyapay G, Weissenbach J, Savolainen ER, Peltonen L, and Tryggvason K (1994). Congenital nephrotic syndrome of the Finnish type maps to the long arm of chromosome 19. Am J Hum Genet *54*, 757-764.

Khoshnoodi J, Sigmundsson K, Ofverstedt LG, Skoglund U, Obrink B, Wartiovaara J, and Tryggvason K (2003). Nephrin promotes cell-cell adhesion through homophilic interactions. Am J Pathol *163*, 2337-2346.

Khoshnoodi J, and Tryggvason K (2001a). Congenital nephrotic syndromes. Curr Opin Genet Dev *11*, 322-327.

Khoshnoodi J, and Tryggvason K (2001b). Unraveling the molecular make-up of the glomerular podocyte slit diaphragm. Exp Nephrol *9*, 355-359.

Kim JM, Wu H, Green G, Winkler CA, Kopp JB, Miner JH, Unanue ER, and Shaw AS (2003). CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. Science *300*, 1298-1300.

Kobayashi N, Gao SY, Chen J, Saito K, Miyawaki K, Li CY, Pan L, Saito S, Terashita T, and Matsuda S (2004). Process formation of the renal glomerular podocyte: is there common molecular machinery for processes of podocytes and neurons? Anat Sci Int *79*, 1-10.

Kozak M (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell *44*, 283-292.

Koziell A, Grech V, Hussain S, Lee G, Lenkkeri U, Tryggvason K, and Scambler P (2002). Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration. Hum Mol Genet *11*, 379-388.

Kreidberg JA (2003). Podocyte differentiation and glomerulogenesis. J Am Soc Nephrol *14*, 806-814.

Kuure S, Vuolteenaho R, and Vainio S (2000). Kidney morphogenesis: cellular and molecular regulation. Mech Dev *92*, 31-45.

Kuusniemi AM, Kestilä M, Patrakka J, Lahdenkari AT, Ruotsalainen V, Holmberg C, Karikoski R, Salonen R, Tryggvason K, and Jalanko H (2004). Tissue expression of nephrin in human and pig. Pediatr Res *55*, 774-781.

Kwiatkowski AV, Gertler FB, and Loureiro JJ (2003). Function and regulation of Ena/VASP proteins. Trends Cell Biol *13*, 386-392.

Lahdenkari AT, Kestilä M, Holmberg C, Koskimies O, and Jalanko H (2004). Nephrin gene (NPHS1) in patients with minimal change nephrotic syndrome (MCNS). Kidney Int *65*, 1856-1863.

Lahdenperä J, Kilpelainen P, Liu XL, Pikkarainen T, Reponen P, Ruotsalainen V, and Tryggvason K (2003). Clustering-induced tyrosine phosphorylation of nephrin by Src family kinases. Kidney Int *64*, 404-413.

Lehtonen S, Lehtonen E, Kudlicka K, Holthofer H, and Farquhar MG (2004). Nephrin forms a complex with adherens junction proteins and CASK in podocytes and in Madin-Darby canine kidney cells expressing nephrin. Am J Pathol *165*, 923-936.

Lehtonen S, Zhao F, and Lehtonen E (2002). CD2-associated protein directly interacts with the actin cytoskeleton. Am J Physiol Renal Physiol *283*, F734-743.

Lenkkeri U, Mannikko M, McCready P, Lamerdin J, Gribouval O, Niaudet PM, Antignac CK, Kashtan CE, Homberg C, Olsen A, *et al.* (1999). Structure of the gene for congenital nephrotic syndrome of the finnish type (NPHS1) and characterization of mutations. Am J Hum Genet *64*, 51-61.

Li C, Ruotsalainen V, Tryggvason K, Shaw AS, and Miner JH (2000). CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere. Am J Physiol Renal Physiol *279*, F785-792.

Li H, Lemay S, Aoudjit L, Kawachi H, and Takano T (2004). SRC-family kinase Fyn phosphorylates the cytoplasmic domain of nephrin and modulates its interaction with podocin. J Am Soc Nephrol *15*, 3006-3015.

Little MH, Williamson KA, Mannens M, Kelsey A, Gosden C, Hastie ND, and van Heyningen V (1993). Evidence that WT1 mutations in Denys-Drash syndrome patients may act in a dominant-negative fashion. Hum Mol Genet *2*, 259-264.

Liu G, Kaw B, Kurfis J, Rahmanuddin S, Kanwar YS, and Chugh SS (2003). Neph1 and nephrin interaction in the slit diaphragm is an important determinant of glomerular permeability. J Clin Invest *112*, 209-221.

Liu L, Aya K, Tanaka H, Shimizu J, Ito S, and Seino Y (2001a). Nephrin is an important component of the barrier system in the testis. Acta Med Okayama *55*, 161-165.

Liu L, Done SC, Khoshnoodi J, Bertorello A, Wartiovaara J, Berggren PO, and Tryggvason K (2001b). Defective nephrin trafficking caused by missense mutations in the NPHS1 gene: insight into the mechanisms of congenital nephrotic syndrome. Hum Mol Genet *10*, 2637-2644.

Liu XL, Done SC, Yan K, Kilpeläinen P, Pikkarainen T, and Tryggvason K (2004). Defective trafficking of nephrin missense mutants rescued by a chemical chaperone. J Am Soc Nephrol *15*, 1731-1738.

Liu XL, Kilpeläinen P, Hellman U, Sun Y, Wartiovaara J, Morgunova E, Pikkarainen T, Yan K, Jonsson AP, and Tryggvason K (2005). Characterization of the interactions of the nephrin intracellular domain. Febs J *272*, 228-243.

Liu Y, Cox SR, Morita T, and Kourembanas S (1995). Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. Circ Res *77*, 638-643.

Loots GG, Ovcharenko I, Pachter L, Dubchak I, and Rubin EM (2002). For comparative sequence-based discovery of functional transcription factor binding sites. Genome Res *12*, 832-839.

Luimula P, Aaltonen P, Ahola H, Palmen T, and Holthofer H (2000). Alternatively spliced nephrin in experimental glomerular disease of the rat. Pediatr Res *48*, 759-762.

Ly J, Alexander M, and Quaggin SE (2004). A podocentric view of nephrology. Curr Opin Nephrol Hypertens *13*, 299-305.

Maniatis T, and Tasic B (2002). Alternative pre-mRNA splicing and proteome expansion in metazoans. Nature *418*, 236-243.

Mannikko M, Kestilä M, Lenkkeri U, Alakurtti H, Holmberg C, Leisti J, Salonen R, Aula P, Mustonen A, Peltonen L, and Tryggvason K (1997). Improved prenatal diagnosis of the congenital nephrotic syndrome of the Finnish type based on DNA analysis. Kidney Int *51*, 868-872.

Mayor C, Brudno M, Schwartz JR, Poliakov A, Rubin EM, Frazer KA, Pachter LS, and Dubchak I (2000). VISTA : visualizing global DNA sequence alignments of arbitrary length. Bioinformatics *16*, 1046-1047.

Menke AL, A IJ, Fleming S, Ross A, Medine CN, Patek CE, Spraggon L, Hughes J, Clarke AR, and Hastie ND (2003). The wt1-heterozygous mouse; a model to study the development of glomerular sclerosis. J Pathol *200*, 667-674.

Miner JH, Morello R, Andrews KL, Li C, Antignac C, Shaw AS, and Lee B (2002). Transcriptional induction of slit diaphragm genes by Lmx1b is required in podocyte differentiation. J Clin Invest *109*, 1065-1072.

Miner JH, Patton BL, Lentz SI, Gilbert DJ, Snider WD, Jenkins NA, Copeland NG, and Sanes JR (1997). The laminin alpha chains: expression, developmental transitions, and chromosomal locations of alpha1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel alpha3 isoform. J Cell Biol *137*, 685-701.

Moeller MJ, Kovari IA, and Holzman LB (2000). Evaluation of a new tool for exploring podocyte biology: mouse Nphs1 5' flanking region drives LacZ expression in podocytes. J Am Soc Nephrol *11*, 2306-2314.

Moeller MJ, Sanden SK, Soofi A, Wiggins RC, and Holzman LB (2002). Two Gene Fragments that Direct Podocyte-Specific Expression in Transgenic Mice. J Am Soc Nephrol *13*, 1561-1567.

Moeller MJ, Soofi A, Braun GS, Li X, Watzl C, Kriz W, and Holzman LB (2004). Protocadherin FAT1 binds Ena/VASP proteins and is necessary for actin dynamics and cell polarization. Embo J *23*, 3769-3779.

Moore AW, McInnes L, Kreidberg J, Hastie ND, and Schedl A (1999). YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. Development *126*, 1845-1857.

Mundel P, Heid HW, Mundel TM, Kruger M, Reiser J, and Kriz W (1997). Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes. J Cell Biol *139*, 193-204.

Narita I, Goto S, Saito N, Song J, Kondo D, Omori K, Kawachi H, Shimizu F, Sakatsume M, Ueno M, and Gejyo F (2003). Genetic polymorphism of NPHS1 modifies the clinical manifestations of Ig A nephropathy. Lab Invest *83*, 1193-1200.

Natoli TA, Liu J, Eremina V, Hodgens K, Li C, Hamano Y, Mundel P, Kalluri R, Miner JH, Quaggin SE, and Kreidberg JA (2002a). A mutant form of the Wilms' tumor suppressor gene WT1 observed in Denys-Drash syndrome interferes with glomerular capillary development. J Am Soc Nephrol *13*, 2058-2067.

Natoli TA, McDonald A, Alberta JA, Taglienti ME, Housman DE, and Kreidberg JA (2002b). A mammal-specific exon of WT1 is not required for development or fertility. Mol Cell Biol *22*, 4433-4438.

Nielsen H, Engelbrecht J, Brunak S, and von Heijne G (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng *10*, 1-6.

Nishibori Y, Liu L, Hosoyamada M, Endou H, Kudo A, Takenaka H, Higashihara E, Bessho F, Takahashi S, Kershaw D*, et al.* (2004). Disease-causing missense mutations in NPHS2 gene alter normal nephrin trafficking to the plasma membrane. Kidney Int *66*, 1755-1765.

Noakes PG, Miner JH, Gautam M, Cunningham JM, Sanes JR, and Merlie JP (1995). The renal glomerulus of mice lacking s-laminin/laminin beta 2: nephrosis despite molecular compensation by laminin beta 1. Nat Genet *10*, 400-406.

Nomoto S, Tatematsu Y, Takahashi T, and Osada H (1999). Cloning and characterization of the alternative promoter regions of the human LIMK2 gene responsible for alternative transcripts with tissue-specific expression. Gene *236*, 259-271.

Ohtsuka A, Nakatani S, Horiuchi K, Taguchi T, and Murakami T (2001). Repulsive charge mechanism serve to maintain lumens and cavities. An histochemical study of rat serosa and kidney. Ital J Anat Embryol *106*, 379-384.

Orphanides G, and Reinberg D (2002). A unified theory of gene expression. Cell *108*, 439-451.

Palmen T, Ahola H, Palgi J, Aaltonen P, Luimula P, Wang S, Jaakkola I, Knip M, Otonkoski T, and Holthofer H (2001). Nephrin is expressed in the pancreatic beta cells. Diabetologia *44*, 1274-1280.

Palmer RE, Kotsianti A, Cadman B, Boyd T, Gerald W, and Haber DA (2001). WT1 regulates the expression of the major glomerular podocyte membrane protein Podocalyxin. Curr Biol *11*, 1805-1809.

Panicucci F, Sagripanti A, Vispi M, Pinori E, Lecchini L, Barsotti G, and Giovannetti S (1983). Comprehensive study of haemostasis in nephrotic syndrome. Nephron *33*, 9-13.

Patrakka J, Kestilä M, Wartiovaara J, Ruotsalainen V, Tissari P, Lenkkeri U, Mannikko M, Visapaa I, Holmberg C, Rapola J*, et al.* (2000). Congenital nephrotic syndrome (NPHS1): features resulting from different mutations in Finnish patients. Kidney Int *58*, 972-980.

Patrakka J, Martin P, Salonen R, Kestilä M, Ruotsalainen V, Mannikko M, Ryynanen M, Rapola J, Holmberg C, Tryggvason K, and Jalanko H (2002). Proteinuria and prenatal diagnosis of congenital nephrosis in fetal carriers of nephrin gene mutations. Lancet *359*, 1575-1577.

Pavenstadt H, Kriz W, and Kretzler M (2003). Cell biology of the glomerular podocyte. Physiol Rev *83*, 253-307.

Putaala H, Sainio K, Sariola H, and Tryggvason K (2000). Primary structure of mouse and rat nephrin cDNA and structure and expression of the mouse gene. J Am Soc Nephrol *11*, 991-1001.

Putaala H, Soininen R, Kilpeläinen P, Wartiovaara J, and Tryggvason K (2001). The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. Hum Mol Genet *10*, 1-8.

Quaggin SE (2002). Transcriptional regulation of podocyte specification and differentiation. Microsc Res Tech *57*, 208-211.

Quaggin SE, Schwartz L, Cui S, Igarashi P, Deimling J, Post M, and Rossant J (1999). The basic-helix-loop-helix protein pod1 is critically important for kidney and lung organogenesis. Development *126*, 5771-5783.

Quaggin SE, Vanden Heuvel GB, and Igarashi P (1998). Pod-1, a mesoderm-specific basic-helix-loop-helix protein expressed in mesenchymal and glomerular epithelial cells in the developing kidney. Mech Dev *71*, 37-48.

Radice GL, Ferreira-Cornwell MC, Robinson SD, Rayburn H, Chodosh LA, Takeichi M, and Hynes RO (1997). Precocious mammary gland development in P-cadherin-deficient mice. J Cell Biol *139*, 1025-1032.

Rastaldi MP, Armelloni S, Berra S, Li M, Pesaresi M, Poczewski H, Langer B, Kerjaschki D, Henger A, Blattner SM*, et al.* (2003). Glomerular podocytes possess the synaptic vesicle molecule Rab3A and its specific effector rabphilin-3a. Am J Pathol *163*, 889-899.

Reiser J, Kriz W, Kretzler M, and Mundel P (2000). The glomerular slit diaphragm is a modified adherens junction. J Am Soc Nephrol *11*, 1-8.

Riccardi VM, Sujansky E, Smith AC, and Francke U (1978). Chromosomal imbalance in the Aniridia-Wilms' tumor association: 11p interstitial deletion. Pediatrics *61*, 604-610.

Rodewald R, and Karnovsky MJ (1974). Porous substructure of the glomerular slit diaphragm in the rat and mouse. J Cell Biol *60*, 423-433.

Rohr C, Prestel J, Heidet L, Hosser H, Kriz W, Johnson RL, Antignac C, and Witzgall R (2002). The LIM-homeodomain transcription factor Lmx1b plays a crucial role in podocytes. J Clin Invest *109*, 1073-1082.

Roselli S, Gribouval O, Boute N, Sich M, Benessy F, Attie T, Gubler MC, and Antignac C (2002). Podocin localizes in the kidney to the slit diaphragm area. Am J Pathol *160*, 131-139.

Roselli S, Heidet L, Sich M, Henger A, Kretzler M, Gubler MC, and Antignac C (2004). Early glomerular filtration defect and severe renal disease in podocin-deficient mice. Mol Cell Biol *24*, 550-560.

Rossi M, Morita H, Sormunen R, Airenne S, Kreivi M, Wang L, Fukai N, Olsen BR, Tryggvason K, and Soininen R (2003). Heparan sulfate chains of perlecan are indispensable in the lens capsule but not in the kidney. Embo J *22*, 236-245.

Ruotsalainen V, Ljungberg P, Wartiovaara J, Lenkkeri U, Kestilä M, Jalanko H, Holmberg C, and Tryggvason K (1999). Nephrin is specifically located at the slit diaphragm of glomerular podocytes. Proc Natl Acad Sci U S A *96*, 7962-7967.

Ruotsalainen V, Patrakka J, Tissari P, Reponen P, Hess M, Kestilä M, Holmberg C, Salonen R, Heikinheimo M, Wartiovaara J*, et al.* (2000). Role of nephrin in cell junction formation in human nephrogenesis. Am J Pathol *157*, 1905-1916.

Ryan G, Steele-Perkins V, Morris JF, Rauscher FJ, 3rd, and Dressler GR (1995). Repression of Pax-2 by WT1 during normal kidney development. Development *121*, 867-875.

Sadl V, Jin F, Yu J, Cui S, Holmyard D, Quaggin S, Barsh G, and Cordes S (2002). The mouse Kreisler (Krml1/MafB) segmentation gene is required for differentiation of glomerular visceral epithelial cells. Dev Biol *249*, 16-29.

Saffer JD, Jackson SP, and Annarella MB (1991). Developmental expression of Sp1 in the mouse. Mol Cell Biol *11*, 2189-2199.

Sakiyama H, Masuda R, Inoue N, Yamamoto K, Kuriiwa K, Nakagawa K, and Yoshida K (2001). Establishment and characterization of macrophage-like cell lines expressing osteoclast-specific markers. J Bone Miner Metab *19*, 220-227.

Sako M, Nakanishi K, Obana M, Yata N, Hoshii S, Takahashi S, Wada N, Takahashi Y, Kaku Y, Satomura K*, et al.* (2005). Analysis of NPHS1, NPHS2, ACTN4, and WT1 in Japanese patients with congenital nephrotic syndrome. Kidney Int *67*, 1248-1255.

Saleem MA, Ni L, Witherden I, Tryggvason K, Ruotsalainen V, Mundel P, and Mathieson PW (2002). Co-localization of nephrin, podocin, and the actin cytoskeleton: evidence for a role in podocyte foot process formation. Am J Pathol *161*, 1459-1466.

Schiwek D, Endlich N, Holzman L, Holthofer H, Kriz W, and Endlich K (2004). Stable expression of nephrin and localization to cell-cell contacts in novel murine podocyte cell lines. Kidney Int *66*, 91-101.

Schmieder S, Nagai M, Orlando RA, Takeda T, and Farquhar MG (2004). Podocalyxin activates RhoA and induces actin reorganization through NHERF1 and Ezrin in MDCK cells. J Am Soc Nephrol *15*, 2289-2298.

Schultheiss M, Ruf RG, Mucha BE, Wiggins R, Fuchshuber A, Lichtenberger A, and Hildebrandt F (2004). No evidence for genotype/phenotype correlation in NPHS1 and NPHS2 mutations. Pediatr Nephrol.

Schwarz K, Simons M, Reiser J, Saleem MA, Faul C, Kriz W, Shaw AS, Holzman LB, and Mundel P (2001). Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. J Clin Invest *108*, 1621-1629.

Seifert RA, Coats SA, Oganesian A, Wright MB, Dishmon M, Booth CJ, Johnson RJ, Alpers CE, and Bowen-Pope DF (2003). PTPRQ is a novel phosphatidylinositol phosphatase that can be expressed as a cytoplasmic protein or as a subcellularly localized receptor-like protein. Exp Cell Res *287*, 374-386.

Sellin L, Huber TB, Gerke P, Quack I, Pavenstadt H, and Walz G (2003). NEPH1 defines a novel family of podocin interacting proteins. Faseb J *17*, 115-117.

Shih NY, Li J, Cotran R, Mundel P, Miner JH, and Shaw AS (2001). CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. Am J Pathol *159*, 2303-2308.

Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, Miner JH, and Shaw AS (1999). Congenital nephrotic syndrome in mice lacking CD2-associated protein. Science *286*, 312-315.

Smale ST, and Kadonaga JT (2003). The RNA polymerase II core promoter. Annu Rev Biochem *72*, 449-479.

Sprecher E, Bergman R, Richard G, Lurie R, Shalev S, Petronius D, Shalata A, Anbinder Y, Leibu R, Perlman I*, et al.* (2001). Hypotrichosis with juvenile macular dystrophy is caused by a mutation in CDH3, encoding P-cadherin. Nat Genet *29*, 134-136.

Srivastava T, Garola RE, Whiting JM, and Alon US (2001). Synaptopodin expression in idiopathic nephrotic syndrome of childhood. Kidney Int *59*, 118-125.

Sun C, Kilburn D, Lukashin A, Crowell T, Gardner H, Brundiers R, Diefenbach B, and Carulli JP (2003). Kirrel2, a novel immunoglobulin superfamily gene expressed primarily in beta cells of the pancreatic islets. Genomics *82*, 130-142.

Suzuki A, Ito T, Imai E, Yamato M, Iwatani H, Kawachi H, and Hori M (2003). Retinoids regulate the repairing process of the podocytes in puromycin aminonucleoside-induced nephrotic rats. J Am Soc Nephrol *14*, 981-991.

Tanoue T, and Takeichi M (2004). Mammalian Fat1 cadherin regulates actin dynamics and cell-cell contact. J Cell Biol *165*, 517-528.

Thomas PE, Wharram BL, Goyal M, Wiggins JE, Holzman LB, and Wiggins RC (1994). GLEPP1, a renal glomerular epithelial cell (podocyte) membrane protein-tyrosine phosphatase. Identification, molecular cloning, and characterization in rabbit. J Biol Chem *269*, 19953-19962.

Timpl R, and Brown JC (1996). Supramolecular assembly of basement membranes. Bioessays *18*, 123-132.

Torres M, Gomez-Pardo E, Dressler GR, and Gruss P (1995). Pax-2 controls multiple steps of urogenital development. Development *121*, 4057-4065.

Tryggvason K (2001). Nephrin: role in normal kidney and in disease. Adv Nephrol Necker Hosp *31*, 221-234.

Tryggvason K, and Kouvalainen K (1975). Number of nephrons in normal human kidneys and kidneys of patients with the congenital nephrotic syndrome. A study using a sieving method for counting of glomeruli. Nephron *15*, 62-68.

Tryggvason K, and Wartiovaara J (2005). How does the kidney filter plasma? Physiology (Bethesda) *20*, 96-101.

Urnov FD (2002). A feel for the template: zinc finger protein transcription factors and chromatin. Biochem Cell Biol *80*, 321-333.

Verma R, Wharram B, Kovari I, Kunkel R, Nihalani D, Wary KK, Wiggins RC, Killen P, and Holzman LB (2003). Fyn binds to and phosphorylates the kidney slit diaphragm component Nephrin. J Biol Chem *278*, 20716-20723.

Vize PD, Seufert DW, Carroll TJ, and Wallingford JB (1997). Model systems for the study of kidney development: use of the pronephros in the analysis of organ induction and patterning. Dev Biol *188*, 189-204.

Wagner KD, Wagner N, and Schedl A (2003). The complex life of WT1. J Cell Sci *116*, 1653-1658.

Wagner N, Wagner KD, Xing Y, Scholz H, and Schedl A (2004). The major podocyte protein nephrin is transcriptionally activated by the Wilms' tumor suppressor WT1. J Am Soc Nephrol *15*, 3044-3051.

Wartiovaara J, Ofverstedt LG, Khoshnoodi J, Zhang J, Makela E, Sandin S, Ruotsalainen V, Cheng RH, Jalanko H, Skoglund U, and Tryggvason K (2004). Nephrin strands contribute to a porous slit diaphragm scaffold as revealed by electron tomography. J Clin Invest *114*, 1475-1483.

Weber S, Gribouval O, Esquivel EL, Moriniere V, Tete MJ, Legendre C, Niaudet P, and Antignac C (2004). NPHS2 mutation analysis shows genetic heterogeneity of steroid-resistant nephrotic syndrome and low post-transplant recurrence. Kidney Int *66*, 571-579.

Welsch T, Endlich N, Kriz W, and Endlich K (2001). CD2AP and p130Cas localize to different F-actin structures in podocytes. Am J Physiol Renal Physiol *281*, F769-777.

Wong MA, Cui S, and Quaggin SE (2000). Identification and characterization of a glomerular-specific promoter from the human nephrin gene. Am J Physiol Renal Physiol *279*, F1027-1032.

Wright MB, Hugo C, Seifert R, Disteche CM, and Bowen-Pope DF (1998). Proliferating and migrating mesangial cells responding to injury express a novel receptor protein-tyrosine phosphatase in experimental mesangial proliferative glomerulonephritis. J Biol Chem *273*, 23929-23937.

Yan K, Khoshnoodi J, Ruotsalainen V, and Tryggvason K (2002). N-linked glycosylation is critical for the plasma membrane localization of nephrin. J Am Soc Nephrol *13*, 1385-1389.

Yang Y, Jeanpierre C, Dressler GR, Lacoste M, Niaudet P, and Gubler MC (1999). WT1 and PAX-2 podocyte expression in Denys-Drash syndrome and isolated diffuse mesangial sclerosis. Am J Pathol *154*, 181-192.

Yu CC, Yen TS, Lowell CA, and DeFranco AL (2001). Lupus-like kidney disease in mice deficient in the Src family tyrosine kinases Lyn and Fyn. Curr Biol *11*, 34-38.

Yuan H, Takeuchi E, and Salant DJ (2002). Podocyte slit-diaphragm protein nephrin is linked to the actin cytoskeleton. Am J Physiol Renal Physiol *282*, F585-591.