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Novel Conditional Mouse Models for Targeted Kidney Research

Emphasis on the Analysis of the Biological Function of Nephrin

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ACADEMIC DISSERTATION

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List of original publications

This thesis is based on three original publications, which are referred to in the text by their Roman numerals. In addition, some unpublished work is also included.

I J Juhila, R Roozendaal, M Lassila, JS Verbeek and H Holthöfer: Podocyte cell specific expression of doxycycline inducible cre recombinase in mice. Journal of American Society of Nephrology, 52:2969-74, 2006.

II J Juhila, M Lassila, R Roozendaal, E Lehtonen, M Messing, JS Verbeek and H Holthöfer: Inducible nephrin transgene expression in podocytes rescues nephrin deficient mice from perinatal death. Submitted.

III M Lassila, J Juhila, E Heikkilä and H Holthöfer: Densin is a novel cell membrane protein of Sertoli cells in the testis. Molecular reproduction and development, 74:641-645, 2007.

Abbreviations

ACTN4	alpha-actinin-4 gene
AKT	v-akt murine thymoma viral oncogene homolog 1
AP	alkaline phosphatase
BAD	BCL2 antagonist of cell death
CD2AP	CD2 associated protein
cDNA	complementary DNA
CMV	cytomegalovirus
CNF	congenital nephrotic syndrome of the Finnish type
CNS	central nervous system
dsRNA	double stranded RNA
ER	estrogen receptor
ES	embryonic stem
ESRD	end-stage renal disease
FRT	FLP recombination target
FSGS	focal and segmental glomerulosclerosis
FSH	follicle-stimulating hormone
GBM	glomerular basement membrane
Grb4	Nck2 gene
Ig	immunoglobulin
KO	knock-out
LH	luteinicing hormone
loxP	locus of X-over of P1
LRRC7	densin gene
LSD	least significant difference
MAGUK	membrane-associated guanylate kinase
mRNA	messenger RNA
NEPH	nephrin-like protein
Nck	non-catalytic region of tyrosine kinase
NPHS1	nephrin gene
NPHS1as	nephrin antisense

NPHS2	podocin gene
NS	nephrotic syndrome
N-WASP	Wiskott Aldrich syndrome like gene
pA	polyA tail
PCR	polymerase chain reaction
PDZ	PSD-95/Dlg-A/ZO-1 domain
PI3K	phosphoinositide 3 kinase
PSD	postsynaptic density
RISC	RNA-inducing silencing complex
RLU	relative luciferase unit
RNAi	RNA interference
RT-PCR	reverse transcription PCR
rtTA	reverse transcriptional activator
SD	slit diaphragm
SH2 or 3	Src homology 2 or 3
shRNA	short hairpin RNA
siRNA	small interfering RNA
Tet-O	tetracycline operator sequence
TJP1	ZO-1 gene
TRPC6	member 6 of the transient receptor potential cation channel subfamily C
tTA	transcriptional activator
tTS	transcriptional silencer
VZ	ventricular zone
WT	wild-type
WT1	Wilm's tumour 1 gene or protein
Z/AP	lacZ/human placental alkaline phoshatase reporter mouse-line
ZO-1,2	zonula occludens 1 or 2 protein

Abstract

Nephrin is a transmembrane protein belonging to the immunoglobulin superfamily and express in the podocytes, which are highly differentiated epithelial cells needed for primary urine filtration in the kidney. Mutations leading to nephrin loss abrogate podocyte morphology, and result in massive protein loss through urine and early death in humans and mice. The purpose of this thesis was to generate novel inducible mouse-lines that allow targeted gene deletion in a time and tissue specific manner. A gene therapy proof of principle model that allowed podocyte specific transgene replacement to rescue gene deficient mice from perinatal lethality was studied. Furthermore, the phenotypic consequences of nephrin restoration in the kidney and nephrin deficiency in the testis, brain and pancreas in rescued mice were investigated.

A novel podocyte-specific construct was achieved by using standard cloning techniques to provide an inducible tool for *in vitro* and *in vivo* gene targeting. Using modified constructs and standard micro-injection procedures two novel transgenic mouse-lines were generated. First, a mouse-line with doxycycline inducible expression of Cre recombinase that allows podocyte-specific gene deletion was generated. Second, a mouse-line with doxycycline inducible expression of rat nephrin, which allows podocyte-specific nephrin over-expression was made. Furthermore, it was possible to rescue nephrin deficient mice from perinatal lethality by cross-breeding them with a mouse-line with inducible rat nephrin expression that restored the missing endogenous nephrin only in the kidney after doxycycline treatment. The rescued mice were smaller, infertile, showed genital malformations and developed distinct histological abnormalities in the kidney with an altered molecular composition of the podocytes. Histological changes were also found in the testis, cerebellum and pancreas. The expression of another molecule with limited tissue expression, densin, was localized to the plasma membranes of Sertoli cells in the testis by immunofluorescence staining. Densin may be an essential adherens junction protein between Sertoli cells and developing germ cells and these junctions share similar protein assembly with kidney podocytes.

This single, binary conditional construct appears to serve as a cost- and time-efficient tool to increase the basic understanding of podocyte-specific key proteins in health and disease. These results showed a tightly controlled inducible podocyte-specific transgene expression *in vitro* and *in vivo* as expected. These novel mouse-lines with doxycycline inducible Cre recombinase and with rat nephrin expression will be useful for conditional deletion of essential podocyte proteins and to study in detail their functions after over-expression in adult mice. This is important for future diagnostic and pharmacologic development platforms.

This thesis also proves that inducible podocyte-specific expression of a rat nephrin transgene rescues nephrin deficient mice from perinatal death, but in a complex fashion that should be considered while designing gene replacement therapies. Findings from kidneys of rescued animals indicate that nephrin plays a major role in the podocyte, both in the formation of functional filtration slits during development and in the formation and maintenance of foot process integrity during adulthood, via specific intracellular signaling. Furthermore, the phenotype of rescued animals has also revealed the importance of nephrin in male reproductive track development. Additional work on the functional role of slit diaphragm components in the testis is needed to reveal whether these molecules have a direct role in spermatogenesis.

Introduction

The nephron is the functional unit for filtration of the kidney consisting of a glomerulus, proximal tubule, Henle's loop, distal tubule, and collecting duct [1]. The glomerulus maintains filtration by forming a three-layered barrier that is composed of a layer of endothelial cells, the glomerular basement membrane (GBM) and podocytes that form a special extracellular structure called the slit diaphragm (SD) [1, 2]. Leaking of proteins into the urine, proteinuria, is often due to dysfunction of the SD with functional or structural changes in the corresponding molecules.

Children born with congenital nephrotic syndrome of the Finnish type (CNF) suffer from severely impaired podocyte function. Before the development of current transplantation therapy these children died within a few months after birth due to massive proteinuria [3]. After a meticulous search for the causative gene of CNF, the nephrin (*NPHS1*) gene encoding the slit diaphragm specific protein nephrin was identified [4]. Consistent with the human CNF disease phenotype, nephrin knock-out (KO) mice die within a few days after birth and the podocytes show morphological abnormalities closely similar to those in the glomeruli of CNF patients [5, 6].

In the kidney, nephrin generates junctions between two podocyte cells and forms the molecular sieve in the SD [5, 7]. Nephrin also has a role in signal transduction and maintenance of the architecture of the podocytes by regulating the actin cytoskeleton [8, 9]. However, expression of nephrin is not limited to the podocytes, but is also present in the testis [10], the central nervous system (CNS) and the pancreas [5, 11]. The most striking kidney manifestation in nephrin KO mice is a loss of SD structures and a fusion of podocyte foot processes [5, 6]. These mice die as a consequence of massive proteinuria soon after birth and the precise glomerular functions of nephrin especially in adulthood remain unidentified. Furthermore, whether the absence of nephrin in extra-renal sites in nephrin KO mice, also contributes to the lethality is not known and cannot be studied with present conventional gene silencing techniques. Importantly, the conventional silencing of many other podocyte-specific genes in mice is also

associated with early postnatal lethality. Therefore, it is of great importance to generate mouse-lines, which allow the study of podocyte-specific gene functions in a tissue- and time-specific manner.

To achieve this goal, a novel tetracycline inducible (Tet-on) podocyte-specific construct for *in vitro* and *in vivo* gene targeting was generated. By modifying this construct, two transgenic mouse-lines were developed, (A) an inducible podocyte-specific expression of Cre recombinase and (B) a rat nephrin protein. These mouse-lines are suitable for conditional gene deletion and further characterization of nephrin function in adult mice, respectively.

Review of the literature

1. The kidney: Structure and main functions

The kidneys are paired organs located retroperitoneally. The kidneys are responsible for the ultrafiltration of plasma, excretion of metabolic waste products, maintaining water and pH balance and secretion of hormones. Thus, the filtration regulates the body fluid balance by affecting the electrolyte contents, osmolarity and acidity of body fluids. The nephron is the functional unit for filtration of the kidney. Each of the approximately one million nephrons in the human kidney consists of a renal corpuscle (glomerulus), proximal tubule, Henle's loop, distal tubule, and collecting duct [1].

The glomerulus is responsible for the formation of the primary urine. The afferent arteriole, which imports blood to the glomerulus, divides further into five to seven smaller capillary branches forming a tight tuft structure of the glomerulus surrounded by the urinary space and an epithelial cell-lined Bowman's capsule. The glomerulus maintains filtration by forming a three-layered barrier between the blood circulation and the tubular system, which is responsible for the reabsorbation of substances like excessive water, smaller proteins and glucose. Even though approximately 180 l of primary urine is produced every day, the final urine volume is only 1-1.5 l/day [1]. The three-layered glomerular filter filtrates primary urine in a size, charge and shape-selective manner [12-15]. Structurally, the glomerular filter is composed of a layer of endothelial cells, the glomerular basement membrane (GBM) and visceral epithelial cells, called podocytes (Figure 1A).

The first layer of the filtration barrier is the endothelial cell layer of the capillary lumen wall. The endothelium has large fenestrations and openings up to ~100 nm, which are spanned by negatively charged glycoproteins and lipids [1]. This layer maintains blood cells in circulation, whereas water and many macromolecules, e.g. albumin, flow without major restraint through the pores [16, 17].

The second layer, the negatively charged GBM, seems to be the principal charge-selective barrier, and probably also the main size-selective barrier [18, 19]. The GBM forms a size-selective barrier for particles with a size similar to or larger than that of albumin, which has a molecular weight of 68 kDa. The GBM is composed mainly of Type IV collagen and laminin, but contains also heparan sulphate proteoglycans, fibronectin and entactin/nidogen [20, 21]. These molecules form a negative charge on the GBM and favour filtration of cationic proteins (Figure 1A).

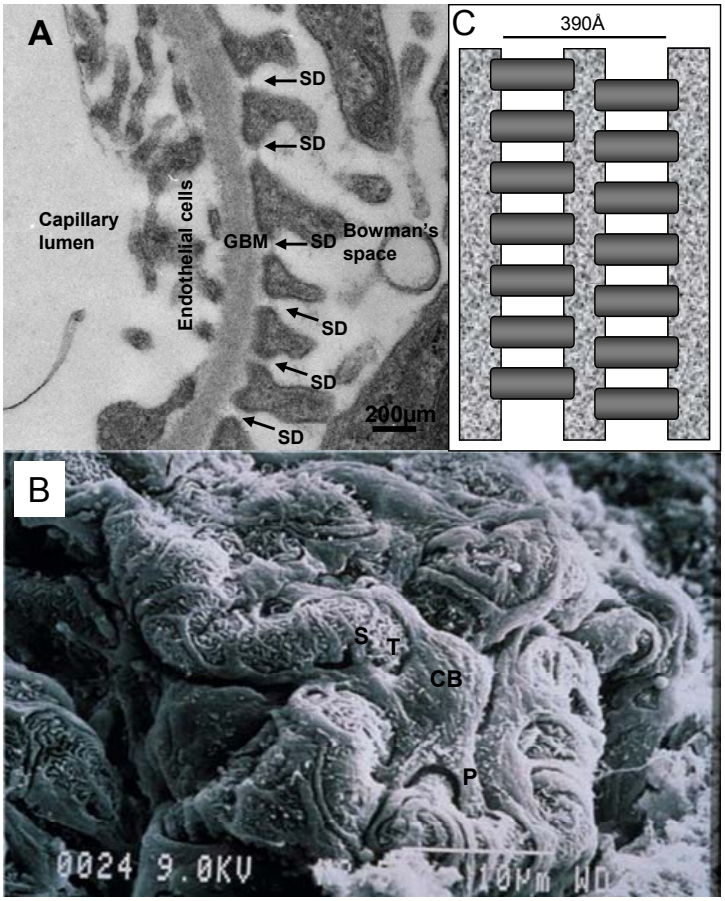

Figure 1. *The podocyte and the slit diaphragm.* ***A****) Transmission electron micrograph of glomeruli. Filtrate passes from the glomerular capillary lumen through fenestrated endothelial cells, across the glomerular basement membrane (GBM), and through the slit diaphragm (SD) between the podocytes into the Bowman's space.* ***B****) Scanning electron micrograph of podocytes. Podocytes have long primary processes (P) that extend towards the capillaries to which they attach by foot secondary (S) and tertiary (T) processes. In contrast to the cell body (CB), the foot processes contain only few organelles.* ***C****) A modified slit diaphragm model by Karnowsky et al. 1974. Podocyte foot processes (left and right) with a central filament in parallel. Perpendicular cross strands form a reticular lattice, with rectangular pores between the strands*.

1.1. Podocytes and slit diaphragm

The last layer of the filtration barrier is composed of podocytes, which are polarized and highly differentiated epithelial cells. They have a voluminous cell body, which bulges into the urinary space. The cell body contains a prominent nucleus, a well developed Golgi system, rough and smooth endoplasmic reticulum, lysosomes, and many mitochondria [2]. Podocytes have long primary processes that extend towards capillaries to which they attach by secondary and tertiary foot processes (Figure 1B). In contrast to the cell body, the foot processes contain only few organelles. The foot processes between neighbouring podocytes regularly interdigitate and a special extracellular structure called the slit diaphragm (SD) is formed between them [1, 2].

The filtration slit has a constant width of ~30-40 nm and is bridged by the SD [22-24]. Electron microscopy studies on the structure of the SD reveal a zipper-like structure with rod-like units connected to the central filament running parallel to the foot processes. This structure forms a molecular sieve with pores of the approximate size of an albumin molecule [24, 25]. The SD appears to be a crucial structure regulating the glomerular filtration in a size and shape selective manner (Figure 1C).

1.2. Filtration barrier injury leads to proteinuria

Small and neutral proteins pass the filtration barrier freely and active reabsorbtion in the downstream tubular system returns the majority of the proteins back into circulation. A filtration barrier injury results in a proteinuric condition, in which proteins that are normally kept in the blood circulation leak into the urine. This is due to an increased amount of total protein passing through the filtration barrier and this subsequently overloads the tubular reabsorption capacity, whereafter proteins in the tubular lumen pass freely into the collecting duct and further into urine. Leaking of proteins into the urine is due to dysfunction of the glomerular filter with functional or structural changes in the corresponding molecules in the filter. However, the molecular events that occur in proteinuria are still partly unsolved. Glomerular proteinuria is accompanied by podocyte foot processes effacement where the foot processes are fused together along the GBM. In proteinuric disorders, the effacement can be irreversible leading to progressive

proteinuria and in certain cases, development of end-stage renal disease (ESRD). The filtration function damage may be reversible with subsequent restoration of the foot processes and the SD. There are several known causes of proteinuric conditions including autoimmune diseases, local inflammation, toxic effects and genetic mutations [2, 19]. Glomerular anomalities and clinical features in some familial nephrotic syndromes (NS) caused by genetic mutations are summarized in Table 1.

Table 1. *Some familial nephrotic syndromes and their clinical features.*

Glomerular Anomalities	Mutated Protein	Nephrotic Syndrome	Clinical Features	Refe-rences
I. Changes in the SD.	a. Nephrin	a. CNF	a. Develop refractory NS within 3 months of life and progress ESRD in 2-3 years of age.	[4]
	b. Podocin	b. Steroid resistant NS	b. Early onset of NS and progression to ESRD in childhood.	[26]
II. Altered podocyte homeostasis due to changes in Ca influx.	TRPC 6	Focal and segmental sclerosis (FSGS)	Onset of proteinuria in adolescence and early adulthood. ERSD after the fourth decade.	[27]
III. Unstable cytoskeleton of the podocyte foot processes.	Alpha-actinin-4	FSGS	Onset of proteinuria in adolescence and early adulthood. ERSD after the fifth decade.	[28]
IV. Changes in the GBM.	Laminin	Pierson Syndrome	Early onset of NS soon after birth and progression to ESRD in childhood.	[29]
V. Alterations in early glomerular development.	a. Wilm's tumour protein (WT1)	a. Denys-Drash Syndrome	a. Diffuse mesangial sclerosis or FSGS occur in combination with Wilms tumor and urogenital abnormalities.	[30]

2. Congenital nephrotic syndrome of the Finnish type

CNF is an autosomal recessive renal disease [31-33] and its approximate incidence in Finland is 1/10000 live births [34]. Children born with CNF suffer from severely impaired podocyte function and, before current transplantation therapy, used to die within a few months after birth due to massive proteinuria [3]. The understanding of the glomerular filter function has improved, with the finding that mutations in *NPHS1* gene causes CNF disease [4].

At present, more than 70 mutations in *NPHS1* gene have been identified [35, 36]. In the Finnish population, two main mutations of the *NPHS1 gene*, Fin_{major} and Fin_{minor} have been reported [4]. The most common mutation, Fin_{major}, is a frameshift deletion in exon 2 that forms a stop codon within the same exon resulting in complete loss of the nephrin protein [35]. The other Finnish mutation, Fin_{minor}, is a nonsense mutation in exon 26 that leads to a large deletion of the intracellular domain of nephrin [35]. While most prevalent mutations are found in Finland, a variety of different mutations leading to various clinical manifestations have also been described in other countries [35, 36]. The absence of SD structures and nephrin protein expression with clinical manifestations typical to CNF disease, has also been reported from patients with either the Fin_{major} or Fin_{minor} mutation [37].

The clinical features of CNF include massive proteinuria *in utero*, oedema, enlarged placenta and low birth weight [3, 31]. The kidneys of CNF patients are bigger compared to healthy controls, and the number and size of glomeruli are significantly increased [38, 39]. The number of podocytes is decreased and the podocytes show effacement of the foot processes and absence of SDs [40]. Other histological findings include dilated proximal tubules, mesangial hypercellularity with expansion of the mesangial area, inflammation, thickened GBM and thickened parietal epithelial cell layer of Bowman's capsule [32, 40-43].

Current treatment of CNF consists of early nephrectomy and dialysis followed by transplantation [44]. This treatment removes all symptoms with no apparent manifestations in other organs [45]. After transplantation 20% of CNF patients develop a recurrence of the nephrotic syndrome, which has been shown to result from the presence of nephrin-specific circulating auto-antibodies [46, 47]. This recurrence has only been reported in the patients with the Fin_{major} mutation. These patients have an early stop codon in exon 2 of the nephrin gene and lack nephrin immunological tolerance [47]. Interestingly, patients with Fin_{minor} mutation do not show a recurrence of proteinuria after the kidney graft [46, 47]. This may be due to immunological tolerance against the partly normal nephrin protein, because of a nonsense mutation that occurs relatively late in exon 26.

3. Molecular composition of the SD

During development, the SD originates from the apically located tight junctions of the prepodocytes. During formation of the glomerulus, these apical junction complexes migrate toward the basal surface and prior to reaching it, nephrin appears in the complex [4]. The expression of nephrin is associated with the development of foot processes and the emergence of other nephrin-associated SD proteins [5]. The molecular composition of the SD is partly similar to tight junctions [48, 49] and partly to adherens junctions [49-52]. Therefore the SD can be defined as a modified adherens junction. A multitude of podocyte proteins relevant to the filtration slit function have recently been characterized. The outgrowth of foot processes from the primary process requires polymerization of actin microfilaments and their connection to nephrin and other SD proteins [8, 9]. The proteins that are crucial for this process remain partly unidentified. Nephrin and its associated proteins will be further discussed in this section.

3.1. Nephrin

3.1.1. Nephrin gene

The *NPHS1* gene is located on human chromosome 19q13.1. The human gene consist of 29 exons and is approximately 26 kb in size [35] and produces a 4.3 kb long messenger RNA (mRNA), which is translated into the nephrin protein.

3.1.2. Structure of nephrin protein

Human nephrin is a transmembrane protein that consists of 1241 amino acids and belongs to the immunoglobulin (Ig) superfamily and cell-cell adhesion molecules [4]. Nephrin has different functions in the podocytes. It generates junctions between two podocyte cells and forms the molecular sieve in the SD [5, 7]. In addition, nephrin also has been suggested to have a role in signal transduction and maintenance of the architecture of the podocytes by regulating the actin cytoskeleton [8, 9]. Different domains of the nephrin protein are involved in these multiple roles (Figure 2A).

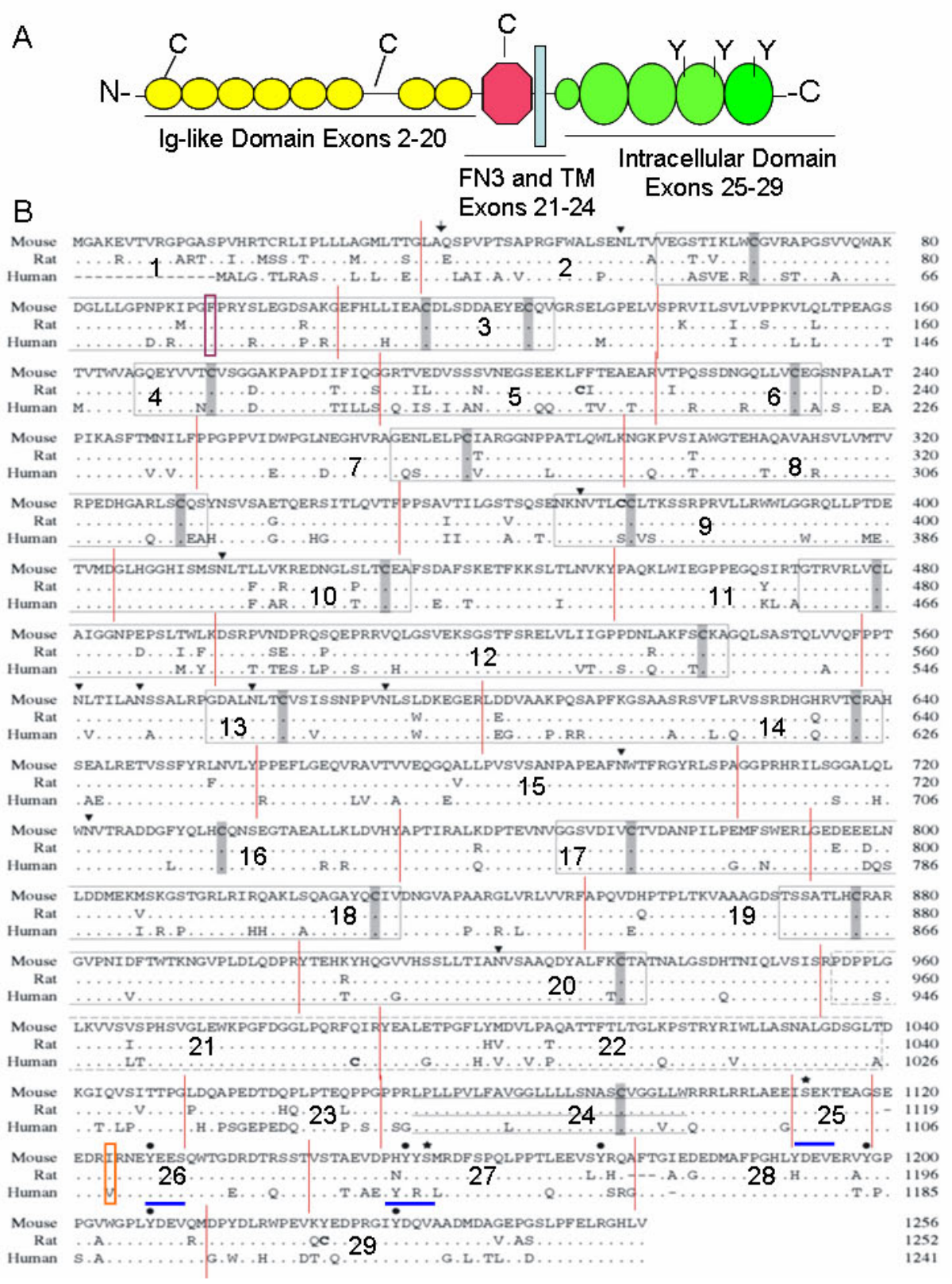

Figure 2. *Nephrin protein structure and phylogenetic protein sequence.* ***A****) Nephrin's extra-cellular domain contains eight Ig-like domains and a fibronectin type III-like domain (FN3). After transmembrane domain (TM) is an intracellular domain with multiple tyrosine phosphorylation sites (Y). In addition, the extracellular domain of nephrin also contains free cysteine residues (C) that*

*may serve as linkers between binding partner(s). **B**) Phylogenetic comparison of the nephrin amino acid sequences of mouse, rat and human. Modified from the original article by Putaala et al. 2000. Exon borders are marked with a vertical solid red line. Ig motifs are boxed and the fibronectin type III-like domain is boxed with a hatched line. The transmembrane domain is underlined with a solid line. The signal peptide cleavage site is marked by an arrow (↓).Conserved cysteine residues are shaded, unconserved residues are shown in bold, and conserved N-glycosylation sites are indicated with a triangle (▼). Conserved tyrosine residues in the intracellular domain are indicated with a dot (•), and two possible protein kinase C phosphorylation sites with the consensus sequence Ser/Tyr-X-Arg/Lys are marked with a star (*).Tyrosine residues shown to be important to Nck adaptor protein binding are overlined with a blue solid line. Places for Fin_{major} and Fin_{minor} mutations are boxed with a black line.*

Like other cell adhesion molecules, nephrin binds itself and related proteins by its extracellular domain [53, 54]. The extracellular domain of nephrin consists of eight Ig motifs with cysteine residues, which take part in protein-protein interactions [4]. All eight Ig motifs in nephrin have two cysteines containing thiol-groups that form disulfide bonds within the motif, and thus stabilize the folded protein structure. The extracellular domain of nephrin also contains free cysteine residues that may serve as linkers between binding partner(s) [4].

Fibronectin type III-like domain encoded by exons 21 and 22, is found upstream of the transmembrane domain that is encoded by exon 24. The fibronectin domain includes a 90-residue structure with conserved tryptophan and tyrosine residues. Its secondary structure resembles an Ig-like fold, thus indicating its role in protein-protein interactions [55].

The intracellular domain of nephrin is encoded by exons 25-29 resulting in a 155-amino acid domain. The presence of nine tyrosine residues, which are tentative phosphorylation sites for intracellular signaling, suggest a role for nephrin in signal transduction [8, 9, 56-58].

3.1.3. Phylogenetic comparison of mouse, rat and human nephrin sequence

The mouse nephrin amino acid sequence shows 93% and 83% identity with the rat and the human sequence, respectively (Figure 2B). The most notable difference is at the amino terminus, where the mouse and rat proteins are 14 amino acid residues longer than the human protein. Mouse nephrin has 1256 and

rat nephrin has 1252 amino acid residues both predicting a molecular weight of 136 kD without post-translational modifications [59]. Human nephrin contains 1241 residues, with a molecular weight of 135 kD [4].

The signal peptide cleavage site in human nephrin is located between residues 22 and 23, whereas it is predicted to be between residues 36 and 37 in mouse and rat. All 10 potential N-glycosylation sites are conserved between the three species. Glycosylation has been postulated to occur based on the finding of a difference between the calculated (135 kDa) and the identified (185 kDa) molecular weight of nephrin by Western blot analysis [4]. Of the seven serine-glycine doublets that are potential heparan sulfate attachment sites, only two are conserved between the three species [59].

All eight Ig motifs contain two cysteines that have been conserved in all three species. In addition, the mouse and rat nephrin contain three cysteine residues that are conserved between the species, and one or two unconserved residues depending on the species. The conserved residues are; one in the first Ig- motif, one in the spacer domain, and one in the transmembrane domain. The unconserved residues include; one cysteine in Ig motif 4 in the mouse, one each in Ig motifs 2 and 4 in the rat, and one residue in the fibronectin III domain in human nephrin. Furthermore, both the mouse signal peptide and the rat intracellular part have one cysteine residue each [4].

The intracellular domain in human nephrin has nine tyrosine residues that are tentative phosphorylation sites for intracellular signaling, while the corresponding domains of mouse and rat contain ten and eight residues, respectively. Six of those tyrosine residues are conserved between the three species. However, the sequences around the tyrosines are completely conserved between the three species only in the case of three of the residues. In human nephrin, these are tyrosines 1113, 1193, and 1217. Recently, the importance of three tyrosine residues (1176, 1193, 1217) in the cytoplasmic tail of human nephrin has been shown to convey actin polymerization, via the non-catalytic region of tyrosine kinase (Nck) adaptor proteins after tyrosine kinase Fyn phosphorylation [8, 9, 58]. Furthermore, two potential conserved protein kinase C

phosphorylation sites with the consensus sequence Ser/Tyr-X-Arg/Lys have also been reported [59].

3.1.4. Complex fashion of nephrin expression

In the kidney, nephrin is expressed only in the podocytes and is mainly localized in the SD area [7, 60, 61]. In disagreement with the initial reports, expression of nephrin is not limited to the kidney, but can also be found in the testis [10], CNS [5], pancreas [5, 11, 62], placenta [63] and lymphoid tissues [64]. The role of nephrin in each of these tissues remains elusive, but most likely reflects some shared functions. One study in human states that nephrin expression is restricted only to the kidney and that CNF patients do not have complications in extra-renal organs [65]. However, early nephrectomy and dialysis treatment is clinically used to prevent early lethality in CNF patients [44]. This treatment comes along with a massive life-long anti-rejection therapy which is needed to modulate the immunoreaction against the transplant. Thus, even if initially removing the symptoms [45] it is still premature to conclude that no later onset symptoms could occur, as other tissue sites could not express nephrin. As described elsewhere in this thesis, many of the patients show signs of neurological symptoms [66], which interestingly is not a common site of an immunological reaction. Thus, symptoms from extra-CNS expression sites of nephrin may be controlled by strong immunomodulatory medications.

3.1.4.1. Alternative nephrin promoter

An alternative nephrin promoter directs tissue-specific expression of nephrin [67] and different transcription factors most likely have tissue- and function specific roles in the modulation of *NPHS1* gene expression [68-72]. An upstream segment of mouse nephrin promoter, between 4 kb and 4 bp, was shown to be sufficient for driving expression in the kidney, CNS and pancreas [67]. Surprisingly, these studies showed that a 5.7 kb construct lacking the transcription initiation site and the immediate upstream region of the gene still drove expression in the CNS. This lead to the identification of a novel, additional exon 1B in the mouse *NPHS1* gene located 1.8 kb upstream from the ATG translation initiation codon of the previously known first exon, now termed exon 1A. The existence and functionality

of exon 1B was verified in nephrin KO mice in which exon 1A is deleted. Neither nephrin mRNA or nephrin protein was observed in the kidneys of the KO mice. However, immunohistochemical staining of brain sections from newborn KO mice using antibody against the intracellular part of the mouse nephrin, showed similar distribution of nephrin protein in the brains of the KO mice compared to wild-type controls (WT). These results indicate that KO mice still express nephrin in the brain through exon 1B [67]. Deletion of exon 1B and its immediate surrounding sequence abolished the expression of the transgene in the pancreas and the spinal cord, but not in the kidney and brain in transgenic mice [67]. The function of exon 1A is to form the aminoterminal signal sequence, which is cleaved by a peptidase in the lumen of the endoplasmic reticulum [4]. In the variant form of nephrin with exon 1B this signal sequence does not exist [67]. The biological role of the two nephrin isoforms in the brain and kidney remains to be clarified.

3.1.4.2. Bidirectional nephrin promoter and alternative splicing

Nephrin also shares a bidirectional promoter area with another immunoglobulin superfamily protein, nephrin-like protein 3 (Neph3, Kirrel2 or filtrin), and these two genes are transcribed in opposite directions [73]. Nephrin is also regulated by a post-transcriptional mechanism. One alternative splicing variant for nephrin protein has been reported in humans [60] and four variants in rats [74]. According to sequence analysis of two rat variants, nephrin α/β, could be translated into correct open reading frames [60]. When compared to the human sequence, α-nephrin lacks the transmembrane domain encoding for exon 24 and the intracellular exons 25, 26 and 27, and β-nephrin lacks part of exon 24 (bp 3166 – 3205) [35]. The α-nephrin protein, was also found to be expressed in humans, but in contrast to rat variant, it lacks only the transmembrane-encoding exon 24 [60]. The role of a bidirectional promoter and the alternative splicing variants remains to be elucidated.

3.1.4.3. Endogenous nephrin antisense transcript

An endogenous antisense transcript originating from the nephrin-encoding locus was found when studying the nephrin-deficient mouse-line generated by the gene-trapping technique [6]. Further evidence of the antisense transcript

(*NPHS1*as) was obtained by searching for *NPHS1*-like expressed sequence tags [75]. Surprisingly, one clone showed exact sequence similarity compared to the *NPHS1* transcript, but spanning from exons 7 to 12 in the reverse orientation. *NPHS1*as is expressed in the mouse brain, thymus, peripheral lymph nodes and embryonic stem (ES) cells. However, the mesenteric lymph nodes and the main sites of nephrin expression, the kidney and pancreas, were negative. The relative amounts of sense and antisense mRNAs as well as nephrin protein were determined by semiquantitative RT-PCR and immunoblotting in various mouse tissues. These results suggest that *NPHS1*as may be important for the regulation of the tissue- and cell-type-specific expression of nephrin.

3.1.5. Nephrin KO mice die perinatally and show kidney abnormalities

Two different nephrin KO mouse-lines have been generated by using different cloning techniques (See Chapter 5). Consistent with the human CNF disease phenotype, nephrin KO mice die within a few days after birth and the podocytes show morphological abnormalities closely similar to the glomeruli of CNF patients [5, 6].

3.1.5.1. Nephrin KO mouse-line generated by homologous recombination

The first published nephrin KO mouse-line was generated by inactivating the *NPHS1* gene in ES cells by homologous recombination using a targeting vector. In this case exon 1 was fused in-frame to the *Escherichia coli lacZ* gene located in a cassette with, the phosphoglycerine kinase promoter driving the neo selection gene, which is transcribed in the opposite direction. Mice homozygous for inactivated *NPHS1* were born at an expected Mendelian frequency of 25%. Although seemingly normal at birth, they immediately developed massive proteinuria and oedema and died within 24 h. The kidneys of KO mice exhibited enlarged Bowman's spaces, dilated tubules, effacement of podocyte foot processes and absence of the SD, essentially as found in human CNF patients [5].

3.1.5.2. Nephrin KO mouse-line generated by random mutagenesis

The second nephrin KO mouse-line was generated by the gene-trapping technique using random insertional mutagenesis [6]. In this method, mouse ES

cells are electroporated with the promoterless splice-acceptor type vector, PT1βgeo [76], and selected by gentamycin treatment. Resistant clones are isolated, and the site of integration is determined by 5'RACE [77]. The sequence obtained from 5'RACE revealed integration of the trap vector in an inverted orientation in exon 8 of the *NPHS1* gene corresponding to the third Ig-motif of the extracellular domain of nephrin [6]. Chimeric mice were generated by blastocyst injection of the trapped nephrin clone. Southern blot analysis using a lacZ probe showed a single integration of the gene-trap vector within the *NPHS1* gene [6].

Nephrin deficient pups died instantly after birth. One homozygote survived for 7 d, but it was significantly smaller than the other pups and showed severe proteinuria of > 20 mg/ml. Kidneys of nephrin KO mice showed characteristic features of proteinuric disease including fibrotic and hypercellular glomeruli as well as excessive extracellular matrix deposition. The Bowman's capsules and tubules were dilated, and tubules had severe cystic lesions. Despite the observed morphological changes in kidneys of nephrin KO newborn littermates, no prominent changes in the branching morphogenesis of the developing collecting ducts could be found compared to WT mice. Immunohistochemical staining showed that the expression and localization of other SD-associated proteins i.e. CD2-associated protein (CD2AP) and zonula occludens-1 (ZO-1) protein were unchanged. Electron microscopy revealed that over 90% of the podocyte foot processes were fused. The remaining inter-podocyte junctions lacked SDs but showed tight adhering areas. Interestingly, in the heterozygote phenotype approximately one third of the foot processes are fused, in addition down-regulation of nephrin-specific mRNA levels occurs [6].

3.2. Nephrin associated proteins

Following the identification of nephrin and the characterization of nephrin's role as a key molecule in the filtration barrier, it has become important to identify the molecular complexes associated with nephrin and the physiological function of this molecular machinery. These proteins can be subdivided into scaffold proteins or signaling proteins.

3.2.1. Scaffold proteins

Scaffold proteins are structural proteins forming adherens junction and participating in the SD formation between two foot processes. A hypothetical representation of selected key-protein components of the SD is shown in Figure 3 according to the current literature.

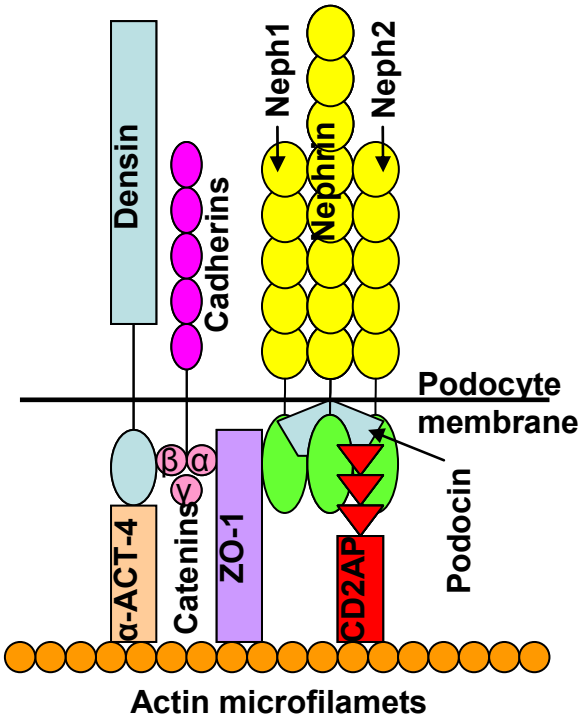

Figure 3. *A hypothetical representation of the protein components of the SD according to the current literature.*

3.2.1.1. Densin

The densin protein was first reported from a postsynaptic density (PSD) fraction of rat forebrain [78]. Recently, densin has been reported also in podocytes of the rat kidney [79] and in beta-cells of the islets of Langerhans in the human pancreas [80]. The densin gene (*LRRC7*) is located on human chromosome 1p31.1.

The protein sequence of densin contains 17 leucine-rich repeats, a sialomucin domain, an apparent transmembrane domain, and a PSD-95/Dlg-A/ZO-1 (PDZ) domain. PDZ domains were first identified in the postsynaptic density protein 95 (PSD-95), the *Drosophila melanogaster* discs large protein (Dlg) and the zonula occludens 1 protein (ZO-1) contract to form the name PDZ according to the first letters of these proteins. PDZ domain consists of

approximately 90 residues and has conserved structural elements, that are present as single or repeated copies in a variety of proteins [81]. In addition, the PDZ domain functions as a protein-protein interaction module, and is known as a central organizer of protein complexes at the plasma membrane [81]. The arrangement of densin protein domains is similar to several other adhesion molecules including erbin, scribble and lano [82-84]. The molecular weight of densin detected in rat brain was 180 kDa [78] and in rat kidney lysate 210 kDa [79]. This difference suggests post-translational modification or alternative splicing of densin in different tissues [79, 85, 86].

In rat brain, densin participates in specific adhesion between presynaptic and postsynaptic membranes at glutamatergic synapses and has interactions with alpha-actinin-4 [78, 87]. Furthermore, densin was co-immunoprecipitated with delta-catenin/neural plakophilin-related armadillo repeat protein and N-cadherin in the rat brain [88]. In rat kidney, densin localizes particularly in the SD and appears to interact with nephrin [79], however a direct interaction has not yet been proven. In addition to cell-cell contact, densin plays roles in signal transduction [89] and maintaining cell polarity [90]. Furthermore recent data from the kidney, show that there is an interaction between densin and ß-catenin in the podocytes [91].

No densin deficient mouse-lines or clinical data concerning mutations in the densin gene have been published to date. However, increased densin mRNA and protein expression, but reduced glomerular immunostaining has been detected in CNF patient's glomeruli compared to healthy subjects [79]. This apparent controversy may reflect an abnormal distribution and localization of densin, in the severely distorted and flattened podocytes, in CNF glomeruli missing the nephrin backbone for proper anchoring. With missing feedback control, this leads to an overproduction and dispersed localization of densin within the podocyte, in addition to the functional association with nephrin [79].

3.2.1.2. Neph-proteins

Neph-proteins are expressed in multiple tissues including kidney, testis, CNS, pancreas and lymph nodes [73, 92, 93]. Genes expressing Neph1, Neph2 and filtrin are *Kirrel1* (1q21-25), *Kirrel3* (11q24) and *Kirrel2* (19q13.12), respectively. Interestingly, filtrin and nephrin genes are located in an adjacent genetic locus

and they share a partially over lapped promoter sequence, but are expressed in opposite directions [73]. Neph1, Neph2 and filtrin are all transmembrane proteins that belong to the Ig superfamily with different numbers of extracellular Ig-like domains and intracellular parts with signaling capabilities [73, 92, 93].

The extracellular domain of nephrin has been shown to interact with Neph1 [53, 54, 93]. Neph1 interacts also with other SD proteins like podocin [93] and ZO-1 [94]. ZO-1 binding was associated with a strong increase in tyrosine phosphorylation of the cytoplasmic tail of Neph1 and dramatically accelerated the ability of Neph1 to induce signal transduction [94]. All three Neph proteins share a conserved podocin-binding motif and a mutation of a centrally located tyrosine residue dramatically lowers the affinity of Neph1 for podocin [93]. Neph2 forms homodimers and specifically interacts with the extracellular domain of nephrin *in vitro* and *in vivo* [95]. Neph1 however fails to interact with Neph2. Immunoreactive Neph2 has been detected in the urine of healthy subjects, suggesting that the extracellular domain is cleaved under physiological conditions [95]. These findings were confirmed *in vitro* in podocyte cell culture. Cleaving was increased by tyrosine phosphatase inhibitors and diminished by GM6001, an inhibitor of metalloproteinases. These findings suggested a role for Neph2 in the organization and/or maintenance of the glomerular SD that may differ from the functions of Neph1 and nephrin [95]. Interaction partners for filtrin are not yet known.

Neph1 KO mice generated by the gene-trapping approach were represented at the expected normal Mendelian ratios at one to three days of age but at only 10% of the expected frequency at 10 to 12 days after birth, suggesting an early postnatal lethality [92]. The Neph1 KO mice that survived beyond the first week of life were sick and small but without oedema, and all died between three and eight weeks of age. All Neph1 KO mice showed severe proteinuria and podocyte foot processes effacement. Three-weeks-old Neph1 KO kidneys exhibited dilated juxtamedullary glomeruli, diffuse mesangial hypercellularity, and increased mesangial matrix and occasional mesangiolysis. Kidneys from newborn Neph1 KO mice showed occasional cystic glomeruli, dilated proximal tubules lined by epithelial cells that in some cases contained vacuoles and/or protein droplets, and protein-filled tubules. These findings suggest that Neph1, like nephrin, may

play an important role in maintaining the structure of the filtration barrier, which prevents proteins from freely entering the glomerular urinary space [92].

Recently, the relevance of glomerular expression of filtrin in human glomerular diseases was demonstrated in patients with focal segmental FSGS [96]. These patients filtrin mRNA level was only one-tenth compared to samples from the kidneys of healthy controls. In addition, changes in the expression pattern of filtrin in renal biopsies were found by immunostaining. However, no correlation between the expression of filtrin and the levels of serum creatinine and proteinuria was observed. These results indicate a potential role of down-regulation of the filtrin gene and protein expression in the pathogenesis of proteinuric diseases.

3.2.1.3. Podocin

Podocin protein has been shown to be expressed only in the podocytes [26] and has been localized in the SD [97]. A gene encoding podocin, *NPHS2*, is mapped to chromosal locus 1q25-31 [26]. *NPHS2* gene is mutated in autosomal recessive steroid-resistant nephrotic syndrome [26] and in some patients with a phenotype of CNF but without mutations in nephrin [98].

The podocin protein presents similarities with proteins of the stomatin family and is predicted to be an integral membrane protein of 383 amino acids, with a single membrane domain forming a hairpin-like structure and with both N- and C-terminal domains in the cytosol [26]. The structure and the subcellular localization of the podocin indicates it's role as a linker between the components of the SD and the actin cytoskeleton [97].

Podocin interacts with the intracellular domain of nephrin [99]. Podocin has been shown to interact also with CD2AP [99] and Neph1 [93]. Podocin is anchored in lipid rafts, which are microdomains rich in sphingolipids and cholesterol in the outer leaflet of the plasma membrane [100]. Nephrin and Neph1 are also found in these lipid rafts, where they associate with podocin and form a transmembrane receptor complex [53, 99, 100]. Interestingly, interaction with podocin affects the signaling properties of nephrin and nephrin-induced signaling is greatly enhanced by podocin binding [101]. Furthermore, when the tyrosine

kinase Fyn phosphorylates tyrosine residues in the cytoplasmic domain of nephrin, it leads to enhanced association with podocin and downstream signaling of nephrin [102].

In the recent study, the RNA interference (RNAi) expression vector against podocin mRNA was generated and transfected into a mouse podocyte cell-line [103]. The results showed decreased podocin and nephrin mRNA and protein levels. The distribution and the mRNA and protein level of alpha-actinin-4 showed no appreciable change. Alpha-actinin-4 localized mainly in the cytoplasm and also extended to the processes. These results suggested that podocin may interact directly with nephrin, but not with alpha-actinin-4.

A podocin KO mouse-line was generated by inactivating the *NPHS2* gene in ES cells by homologous recombination [104]. These mice developed proteinuria during the early antenatal period and died in the first five weeks of life due to renal failure caused by massive mesangial sclerosis. Electron microscopy revealed extensive fusion of podocyte foot processes and lack of a SD in the remaining foot process junctions. Also, the expression of the nephrin gene was downregulated, whereas that of the ZO-1 and CD2AP genes appeared to be upregulated. These findings demonstrate the crucial role of podocin in the establishment of the glomerular filtration barrier.

3.2.1.4. CD2AP

CD2AP was first found in mouse T cells interacting with the cell adhesion protein CD2, but is broadly expressed and found in all tissues except CNS [105]. The *CD2AP* gene is located on human chromosome 6p12. Two human patients with focal segmental glomerulosclerosis had a mutation predicted to ablate expression of one *CD2AP* allele, implicating CD2AP as a determinant of human susceptibility to glomerular disease [106].

The CD2AP protein contains three amino-terminal Src homology 3 (SH3) domains followed by a proline-rich region and a coiled-coil domain at the carboxyl terminus [105]. The SH3 domain has been described on several cytoskeletal and signaling proteins, where it mediates protein–protein binding to proline-rich regions [107].

CD2AP was the first identified protein interacting with the intracellular domain of nephrin [108-111]. Other studies however, have presented controversial data that does not support the interaction between nephrin and CD2AP [101, 112]. CD2AP has been suggested to act as an adaptor protein between other proteins and the actin cytoskeleton [52, 111, 113-115].

Mice lacking CD2AP were generated by homologous recombination [108]. Homozygous CD2AP KO mice were born with a normal Mendelian frequency of 25%. At three weeks of age, CD2AP KO animals began to exhibit substantial growth retardation, and most KO mice died at six to seven weeks of age. Postmortem examination of these mice revealed cardiac hypertrophy, splenic and thymic atrophy, and ascites. Histological examination revealed evidence of severe kidney pathology including defects in podocyte foot processes, accompanied by mesangial cell hyperplasia and extracellular matrix deposition. This pathology correlated with proteinuria, elevated blood urea nitrogen and creatinine concentrations, and reduced serum albumin concentrations, all signs of kidney dysfunction. Proteinuria was first detected at approximately two weeks of age [108]. Interestingly, mice heterozygote for CD2AP deficiency, also developed glomerular changes at nine months of age and had increased susceptibility to glomerular injury by nephrotoxic antibodies or immune complexes [106]. In these mice, defects in the formation of multivesicular bodies in podocytes were found by electron microscopic analysis, suggesting an impairment of the intracellular degradation pathway.

Although CD2AP protein is widely expressed, the severity of renal failure precludes a clear determination of the role of CD2AP in other tissues. Recently, transgenic mouse-line expressing CD2AP under the control of the nephrin promoter [116] was generated and cross-bred with a CD2AP KO mouse-line [108]. Podocyte-specific expression of CD2AP prevented the development of proteinuria, demonstrating that the renal failure is solely due to loss of CD2AP in podocytes and not in other renal or immune cells [117]. Rescued CD2AP KO mice were long-lived and phenotypically normal. Interestingly, rescued CD2AP KO male animals were infertile and histological analysis demonstrated testicular abnormalities that were age-related. This suggests that CD2AP also plays an important role in the development of sperm in the testes. CIN85, one of paralogs

of CD2AP, is poorly expressed in both the podocyte and the basal seminiferous tubule, suggesting that loss of CD2AP protein expression in specific tissues may be compensated for by CIN85 [117].

Furthermore, combinations in mice with CD2AP heterozygosity and heterozygosity of either synaptopodin or Fyn but not Neph1 resulted in spontaneous proteinuria and in FSGS-like damage [118]. This result was explained by protein interactions, where CD2AP associates with Fyn and synaptopodin but not with Neph1. It also demonstrates that bigenic heterozygosity can lead to FSGS and suggests that combined mutations in podocyte genes may be a common etiology for glomerular disease.

3.2.1.5. ZO-1

The tight junction protein ZO-1 was the first protein found in the cytoplasmic base of the SD area [48]. ZO-1 is expressed in multiple tissues including testis, CNS and pancreas [119-121]. The gene encoding ZO-1, *TJP1*, is located on human chromosome 15q13. The ZO-1 protein is a member of the membrane-associated guanylate kinase homologue protein family (MAGUK) [122]. MAGUK proteins are composed of modular units including a PDZ domain, a SH3 domain, a guanylate kinase domain and a proline-rich moiety [122]. These domains have been shown to function in binding integral membrane proteins such as ion channels at synapses [123, 124]. In addition, the guanylate kinase domain has been shown to bind a novel synaptic protein [125], and the SH3 domain of ZO-1 binds a serine protein kinase that phosphorylates a region at the COOH-terminal end of the protein [126].

ZO-1 interacts with many different cell-junction components including occludin, alpha-catenin and ZO-2 as well as with the cytoskeletal molecules spectrin and F-actin [127]. The role of ZO-1 in the SD remained unclear until the interaction between ZO-1 and Neph1 was reported [94]. These results show that ZO-1 may serve as a cytoplasmic organizer by coupling the nephrin-Neph1 complex to the actin cytoskeleton and recruiting appropriate signal transduction components to the SD area.

Although no ZO-1 KO mouse-line has been published some *in vivo* data are available. Alterations in ZO-1 localization and expression have been observed in puromycin aminonucleoside and in protamine sulphate induced nephropathy in rats [128]. These changes were inhibited by cyclosporine A administration [129]. However, in CNF patient kidneys ZO-1 expression remains unaffected [130]. In podocin KO mice, the expression of the nephrin gene was downregulated, whereas that of the ZO-1 and CD2AP genes appeared to be upregulated [104]. Recently, decreased expression of ZO-1 in glomeruli and redistribution of ZO-1 from the podocyte membrane to the cytoplasm has been shown in diabetic animals, and in glomerular epithelial cells after high glucose treatment [131]. In the same study, it was shown that serine and tyrosine phosphorylation of ZO-1 was decreased in cells exposed to high glucose. These findings suggest that alterations in the content and localization of ZO-1 may be relevant to the pathogenesis of proteinuria in diabetes [131].

3.2.1.6. Alpha-actinin-4

Alpha-actinin-4 is ubiquitously expressed and also reported to be present in podocytes [28]. The *ACTN4* gene is closely located in the same human chromosome 19q13 as the *NPHS1* gene. Mutations in *ACTN4* lead to an inheritable form of glomerulosclerosis [28].

The alpha-actinin-4 protein is an actin-bundling protein [28]. It has also been shown to interact with densin in rat brain [78, 87]. Recently, interaction of alpha-actinin-4 with ZO-1 was confirmed by co-immunoprecipitation in various tissues including brain, liver and heart [132]. In RNAi studies *in vitro*, nephrin, podocin, or CD2AP silencing did not change the expression of alpha-actinin-4, whereas silenced alpha-actinin-4 downregulated nephrin and upregulated podocin and CD2AP [112].

A transgenic mouse-line, which expressed murine alpha-actinin-4 containing a mutation analogous to that affecting a human FSGS family in a podocyte-specific manner was previously generated [133]. Consistent with the association of human alpha-actinin-4 in FSGS, a proportion of the transgenic mice exhibited significant albuminuria. Only proteinuric mice exhibited histological features consistent with human alpha-actinin-4-associated FSGS, including

segmental sclerosis and tuft adhesion of some glomeruli, tubular dilatation, mesangial matrix expansion, as well as regions of podocyte vacuolization and foot process fusion. Consistent with such podocyte damage, proteinuric alpha-actinin-4 mutant mouse kidneys exhibited significantly reduced nephrin mRNA and protein. These results suggest a relationship between dysregulation of actin cytoskeleton in the mutant alpha-actinin-4 mouse and consequent deterioration of the nephrin-supported SD complex [133].

An alpha-actinin-4 KO mouse-line was generated by homologous recombination [134]. The number of mice homozygous at birth was lower than expected under Mendelian inheritance indicating embryonic lethality. Surviving homozygous KO mice showed progressive proteinuria, glomerular disease, and pups typically died at several months of age. Light microscopic analysis revealed extensive glomerular disease and proteinaceous casts in the tubules. Electron microscopic examination showed foot-process effacement in young mice and diffuse effacement with globally disrupted podocyte morphology in older mice. Interestingly, despite the widespread distribution of alpha-actinin-4, histological examination of mice showed abnormalities only in the kidneys [134]. However, the mechanism by which alpha-actinin-4 deficiency leads to glomerular disease has not been clarified.

Recently, the effect of alpha-actinin-4 deficiency on the adhesive properties of podocytes was examined further [135]. A comparison with alpha-actinin-4-KO mice to WT controls, showed a decrease in the number of podocytes/glomerulus as well as the presence of podocyte markers in the urine. Podocyte cell-lines generated from alpha-actinin-4 KO mice were less adherent to GBM components collagen IV and laminin 10 and 11 than cells from WT mice. Furthermore, the strength of the integrin receptor-mediated linkages to the cytoskeleton was tested by applying force to microbeads bound to integrin using magnetic pulling cytometry. Beads bound to alpha-actinin-4 KO podocytes showed greater displacement in response to the applied force than those bound to WT cells. Consistent with integrin-dependent alpha-actinin-4-mediated adhesion, phosphorylation of beta1-integrins on alpha-actinin-4 KO podocytes was reduced. These results suggest that alpha-actinin-4 interacts with integrins and strengthens

the podocyte-GBM interaction thereby stabilizing glomerular architecture and preventing disease [135].

3.2.2. Signaling proteins

Nephrin has been suggested to have a role in intracellular signaling and in regulating the actin cytoskeleton of podocytes. The hypothetical nephrin-associated signaling molecules and pathways are presented in Figure 4 according to the current literature.

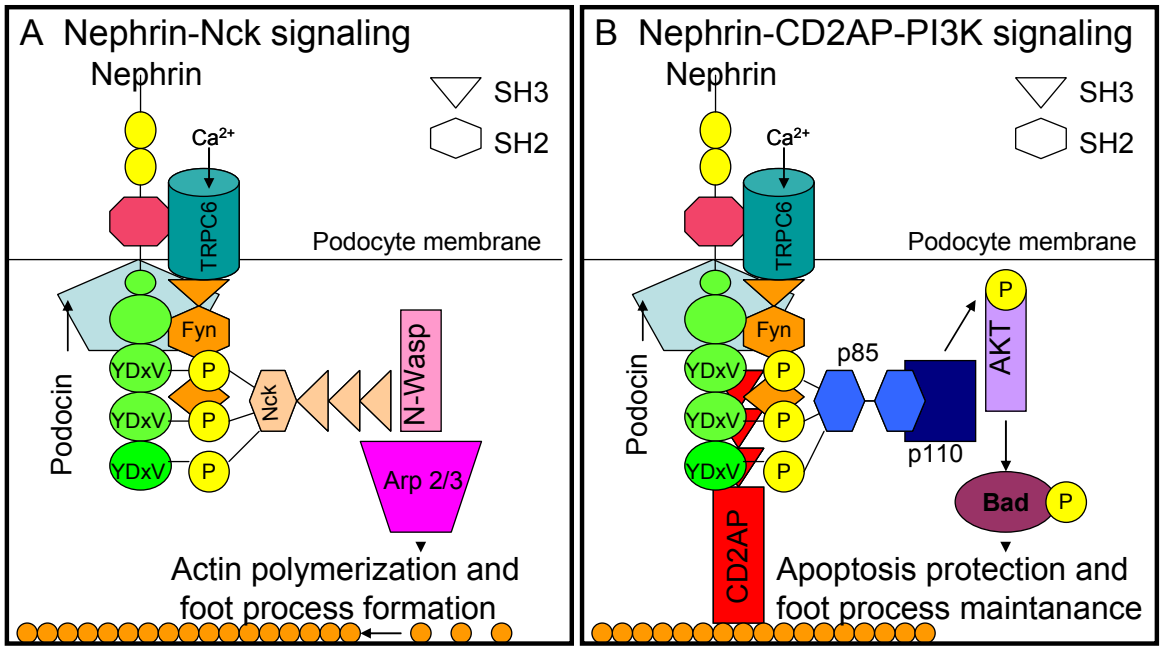

Figure 4. *A hypothetical representation of the nephrin-associated signaling molecules and pathways according to the current literature.* ***A****) The nephrin-Nck signaling pathway participates in actin polymerization and foot process formation.* ***B****) The nephrin-CD2AP-PI3K signaling pathway participates in the protection against apoptosis and in foot process maintenance.*

3.2.2.1. TRPC6

The member 6 of the transient receptor potential cation channel subfamily C (TRPC6) has recently been localized in the podocyte cell membrane [136], but is also expressed in many other tissues. The *TRPC6* gene is located on human chromosome 11q21-22 and a mutation in *TRPC6* was identified as a causative factor for a familial form of FSGS [27]. The TRP family of calcium channels contains over 50 members that serve a large number of cellular functions [137].

For channels containing TRPC6 subunits, the stimulus for calcium influx seems to be the second messenger diacylglycerol [138].

In podocytes, TRPC6 associates with nephrin and podocin, but not with CD2AP [136]. TRPC6 shares similarities to the nephrin cytoplasmic domain as it appears to be regulated by Fyn tyrosine phosphorylation [139]. Recently, it has been shown that TRPC6 is functionally connected to the podocyte actin cytoskeleton that is rearranged upon over-expression of TRPC6 [140]. Furthermore the induced expression of TRPC6 is also a common feature of human proteinuric kidney diseases, with the highest induction observed in membranous nephropathy [140]. In this study, cultured podocytes were exposed to complement and upregulation of TRPC6 protein was observed. Moreover, stimulation of receptor-operated channels by 1-oleoyl-2-acetyl-sn-glycerol in puromycin aminonucleoside-treated podocytes led to an increased calcium influx in a time- and dosage-dependent manner. In addition, a transient *in vivo* gene delivery of *TRPC6* into mice led to expression of TRPC6 protein at the SD and caused proteinuria. These studies suggest the involvement of TRPC6 in the pathology of non-genetic forms of proteinuric disease, but its function and mechanism of action remains unresolved [140].

The TRPC6 KO mouse-line has been generated by homologous recombination [141]. TRPC6 KO mice were viable and showed normal phenotype. Fertility and litter sizes were similar compared to WT mice. Based on the previous data, the loss of TRPC6 function was predicted to lead to a loss of vascular smooth muscle tone and attendant hypotension. Unexpectedly, a higher agonist-induced contractility in isolated aortic rings and an elevated systemic blood pressure was observed in these mice. These effects were explained by *in vivo* replacement of TRPC6 by channels of the TRPC3 type, a closely related but constitutively active member of the diacylglyserol-activated TRPC3/6/7 family [141]. A kidney phenotype of the TRPC6 KO mouse-line has not been reported.

3.2.2.2. Fyn

The Src family kinase Fyn has also been localized in the podocytes [58]. The *Fyn* gene is located on human chromosome 6q21. The Fyn protein contains SH2 and SH3 domains, both of which are capable of binding to nephrin. Moreover, Fyn

contains a tyrosine kinase domain that is capable of phosphorylation of tyrosine residues in target proteins [58].

Nephrin has several intracellular tyrosine residues, some of which are modified by phosphorylation in response to nephrin clustering [142]. These tyrosine residues can be phosphorylated by Fyn, which binds to nephrin most likely via its SH3 domain [58, 102]. Phosphorylation is a biologically important modification of target proteins. It allows the binding of different adaptor proteins to receptor complexes, which then conveys downstream signal transduction to other proteins [143].

Fyn KO mice excreted normal levels of albumin at all ages tested and had a median life span of more than 60 weeks [144]. Histologically Fyn KO mice kidneys were not different from WT mice at any age studied. Further studies with this same mouse-line showed that neither Fyn KO or Yes KO mice (another member of the tyrosine kinase family) developed proteinuria as followed up to 11 months post-gestation [58]. In contrast, double KO mice for both Fyn and Yes developed significantly increased albuminuria at the age of four weeks and their kidneys showed a severe phenotype compared to single KO mouse-lines [58]. The renal histology of Fyn KO and Yes KO mouse-lines was indistinguishable from that of age-matched WT controls. In contrast, kidneys of double KO mice exhibited mesangial expansion and hypercellularity that was readily detectable. In electron microscopy studies, the podocyte foot processes of Fyn KO mice were structurally distorted or coarsened but in some cases retained apparent SDs. Many Fyn KO podocytes appeared entirely "effaced" forming a confluent epithelial mass with the absence of structurally typical SDs. Fyn KO glomeruli also demonstrated mesangial expansion with mild mesangial hypercellularity and a modestly increased mesangial matrix. Yes KO mice displayed a glomerular histology indistinguishable from that of WT age-matched littermates [58]. Glomeruli of FynYes double KO mice demonstrated a distinct and more severe phenotype [58, 144]. In addition, glomerular capillary endothelial swelling and disruption were evident in double KO mice[58].

Furthermore, glomeruli in Fyn, Yes, or double KOs were characterized to explore the relationship between these kinases and nephrin [58]. Fyn deletion

resulted in coarsening of podocyte foot processes and marked attenuation of nephrin phosphorylation in isolated glomerular detergent-resistant membrane fractions. Yes deletion had no identifiable effect on podocyte morphology but dramatically increased nephrin phosphorylating activity. Similar to Fyn deletion, simultaneous deletion of Fyn and Yes reduced nephrin phosphorylation activity. These results demonstrate that endogenous Fyn catalyzes nephrin phosphorylation in podocytes. Although Yes appears to affect the regulation of nephrin phosphorylation, the mechanism by which this occurs requires further investigation [58].

Also combinations of mice with CD2AP heterozygosity and Fyn heterozygosity resulted in spontaneous proteinuria and in FSGS-like glomerular damage. This result was explained by protein interactions and demonstrates that combined mutations in podocyte genes may be a common etiology for glomerular disease [118].

3.2.2.3. Nck adaptor proteins

Mammals have two Nck adaptor family members (Nck1/α and Nck2/ß, also termed Grb4). The genes *Nck1* and *Grb4* are mapped to chromosal locus 3q21 and 2q12, respectively [145]. The two murine Nck proteins share 68% amino acid identity with most of the sequence variation being located in the linker regions between the SH3 and SH2 domains, and are 96% identical to their human counterparts [146]. Nck adaptor proteins consist of three SH3 domains, followed by a C-terminal SH2 domain that binds optimally to pYDEP/D/V motifs in target proteins [147]. Both Nck1 and Nck2 have been shown to be expressed in podocytes [9].

Recently, the importance of nephrin in regulating the actin cytoskeleton in podocytes via Nck adaptor proteins has been shown [8, 9, 148, 149]. The cytoplasmic tail of nephrin has conserved tyrosine-contained motifs (YDxV) that are capable of binding to the SH2 domain of the Nck1 adaptor protein after phosphorylation by Src-family tyrosine kinases, e.g. Fyn [8, 9]. It has been shown that the tyrosine amino acids numbers 1776-Y and 1193-Y are crucial for the interaction of nephrin to Nck1 in humans [8]. Interestingly, one of the two tyrosine motifs needed for Nck1 binding in humans (1176-YDEV) and mice (1191-YDEV)

has not been conserved in rats (1187-HDEV) [8, 9, 59] (Figure 2B). The Nck1 SH3 domains has been shown to bind selectively to proteins that control cytoskeletal organization including Wiskott Aldrich syndrome gene like (N-WASP) [150], WASP-interacting protein [151], and the WAVE1 complex [152]. N-WASP and WAVE1 interact through their C termini with the Arp2/3 complex in a fashion that promotes actin branching and polymerization [153]. Nck1 can bind directly through each of its SH3 domains to N-WASP, and this co-operative binding can stimulate N-WASP-Arp2/3-mediated actin polymerization *in vitro* [154]. Less is known about the binding properties and biological function of Nck2, although these are supposed to be rather similar to Nck1 [155]. Nck2 selectively interacts through its third SH3 domain with the LIM4 domain of PINCH, a protein involved in integrin signaling [156]. In addition, the Nck2 SH2 domain specifically binds to tyrosine phosphorylated B-type ephrins and links B-ephrin reverse signaling to cytoskeletal regulatory proteins [157]. Nck2 has also been proposed to have a unique role in linking the ß-platelet-derived growth factor-receptor to actin polymerization in mouse NIH 3T3 fibroblast [158].

In the rat model of podocyte injury and proteinuria, nephrin tyrosine phosphorylation and nephrin–Nck1 interaction were both reduced significantly [148]. Mice lacking either Nck1 or Nck2 are viable and show no apparent renal defects, suggesting compensation between the subclasses [159]. Double KO mice for both Nck1 and Nck2 died at embryonic day 9.5, precluding analysis of Nck function in podocytes of double-null animals [159]. A mouse-line where Nck2 expression was selectively deleted in podocytes of Nck1 KO animals by expressing Cre recombinase under the control of the podocyte-specific podocin promoter has been developed [9]. These mice at four days of age were smaller than control littermates, and this growth retardation was more apparent at three weeks of age [9]. These mice showed proteinuria and proteinaceous material within the tubules. Most individual glomeruli remained relatively normal in the first few weeks of life, but at three and half weeks of age a range of glomerular defects were detected that include focal sclerosis. Furthermore the expression of molecular markers of glomerular development (Wilm's tumour-1 (WT1) and nephrin) in the glomeruli of Nck KO mice was comparable to littermate controls at one week of age but was reduced by three and half weeks of age, indicating a

loss of podocytes. Electron microscopy examination of kidneys from four-days-old pups showed complete fusion of foot processes of fully differentiated glomeruli in Nck KO animals. Interestingly in glomeruli from both mutant and control embryos (16.5 d), endothelial cell fenestrations were present and podocytes spread normally around the capillary loops. However differentiated foot processes were notably absent at this stage of development in Nck KO embryos compared to controls, consistent with the hypothesis that foot processes fail to form in Nck KO mice [9]. Developmental foot process effacement due to loss of Nck in podocytes resembles that observed in mice deficient in nephrin [5, 6], as well as other proteins of the SD such as Neph1 [92], FAT1 [50] and α_3 integrin [160].

In addition to interacting with Nck, nephrin associates with several cytoplasmic proteins at the SD [161], which may modulate the cellular response to Nck signaling and foot process effacement formation. The importance of actin remodelling in podocyte function is supported by genetic evidence in patients with adult-onset FSGS, who carry mutations in alpha-actinin-4 [28]. Through interaction with alpha-actinin-4 and the Arp2/3 complex, the cytoplasmic adaptor CD2AP has also been proposed to anchor nephrin to the actin cytoskeleton [162]. Although mice lacking CD2AP or alpha-actinin-4 develop nephrotic syndrome, foot process formation is initiated in these mice [108, 134] in contrast to Nck-deficient animals, suggesting that this complex might primarily be required for the maintenance of podocyte foot processes. The roles of Nck and CD2AP in signaling to the podocyte cytoskeleton are therefore likely to be distinct, and it will be of interest to explore whether Nck expression is also required in the mature glomerular filtration barrier maintenance (Figure 4A).

3.2.2.4. P13/AKT kinases

Previously, it has been shown that both nephrin and CD2AP interact with the p85 regulatory subunit of phosphoinositide 3 kinase (PI3K) *in vivo* [163]. This binding recruit PI3K to the plasma membrane, and together with podocin stimulates PI3K-dependent v-akt murine thymoma viral oncogene homolog 1 (AKT) signaling in podocytes [163]. In other systems, binding of the p85 subunit to the catalytic subunit p110 of PI3K further phosphorylates the serine-threonine kinase AKT and enhances cell survival by blocking apoptosis [164]. PI3K binding to the

cytoplasmic domain of nephrin has been shown to be dependent upon phosphorylation by Fyn kinase [163]. PI3K is a heterodimer comprising a regulatory subunit p85 and a catalytic subunit p110. Subunit p85 recognizes phosphorylated nephrin and allows p110 to phosphorylate phospholipids at the inner leaflet of the lipid bilayer stimulating the serine-threonine kinase AKT. Further downstream phosphorylation occurs, one such phosphorylated protein is the BCL2 antagonist of cell death (BAD), which is a proapoptotic factor. Phosphorylation of BAD protects the podocytes from apoptosis induced by detachment [163]. This finding has led to the suggestion that inhibition of nephrin signaling makes podocytes more susceptible to apoptosis. These findings reveal a novel role for the SD proteins nephrin, CD2AP, and podocin suggesting that the nephrin-CD2AP-mediated AKT activity can regulate complex biological programs like apoptosis and foot process maintenance. However, more studies are needed to verify these findings (Figure 4B).

4. SD protein expression in extra-renal tissues

Contrary to early assumptions [4], expression of nephrin is not limited to the podocytes, but is also present in the testis [10], CNS [5], pancreas [5, 11, 62], placenta [63] and lymphoid tissues [64]. Furthermore, many other nephrin-associated SD proteins have been found in extra-renal tissues.

4.1. Testis

It is apparent that protein complexes vital for cell-cell interactions in many organs, such as kidney, testis, CNS and pancreas, are composed of the same junction protein families that resemble or have modified functions in each organ. In mammals, the urinary and reproductive organs also develop from the same intermediate mesoderm during the embryogenesis.

4.1.1. Early organogenesis of the kidney and testis

Three types of renal organs are found during the organogenesis of mammalian species. These structures derived from intermediate mesoderm formed after gastrulation, and are called the pronephros, the mesonephros and metanephros. In mammals, only the metanephros remain through adulthood whereas the

pronephros and mesonephros partly transformed to the developing genital areas during embryogenesis [165, 166]. The pronephros consists of a series of transverse tubules that are suggested to be important in pronephric-mesonephric duct formation. These structures develop into the nephric or Wolffian duct [165, 166].

In humans, the mesonephros has already characteristic kidney structure similar to that is found in the metanephritic kidney. It consists of glomeruli with a well-developed vasculature, proximal and distal tubules, and collecting ducts [165, 166]. In murines, the mesonephros is relatively poorly developed compared to humans and starts to regress at the same time as the development of the metanephros [165, 166]. In male mice, most of the cranial tubules of the mesonephros forms the epidymal ducts of the adult male, while the Wolffian duct serves as the vas deferens [165].

The mesonephros and presumptive gonadal area, called the genital ridge, are developed next to each other [165]. During the development, there is extensive cell migration between the mesonephros and developing testis [167]. These cells participate in shaping of seminiferous cords [168, 169] and Sertoli cell differentiation [170]. Furthermore, some studies indicate that Sertoli cells [171] and Leydig cells [172] might be of mesonephric origin.

Development of the metanephros includes an outgrowth of the primary nephric duct, called the ureteric bud, which extends into the surrounding metanephric mesenchyme. Kidney organogenesis includes further branching and cell-cell interactions, which finally form adult functional kidneys with highly specialized filtration barrier and nephrin protein expression [173].

Interestingly, the mesonephros also shares the same key regulatory molecules as found in the metanephros and adult kidney including paired box gene Pax-2 [174, 175] and WT1 [176, 177]. The mutation in *WT1* gene in humans causes abnormalities of the urinary tract and genitalia formation [176]. Targeted deletion of *WT1* gene in mice resulted in embryonic lethality in homozygotes. In addition these embryos lacked gonads and most of the mesonehric tubules and the metanephros [177]. Inducible PAX2 activation in adult mice decreased WT1 expression and consequently dramatically reduced expression of nephrin [178].

Nephrin protein expression and nephrin promoter activity is detectable in the mesonephros stage and subsequently in the metanephros in late S-shaped bodies at embryonic day 13 [4, 5, 59, 61]. Despite the well characterized role of nephrin in mature podocytes, its function in the mesonephrogenesis remains unknown.

4.1.2. Sertoli cells show morphological and molecular similarities with podocytes

In the testis the arrangement of Sertoli cells closely resembles the podocyte structure in the kidney. Sertoli cells act as feeder cells for the developing germ cells, and constitute the blood-testis barrier that selectively regulates the amount of proteins passing through [179]. Sertoli cells shared molecules with podocytes including extracellular proteins, such as cadherins, and intracellular scaffolding proteins, such as catenins and ZO-1 and ZO-2 [180-185]. Also nephrin has been detected in the Sertoli cells of mice [10]. Furthermore, podocyte-specific expression of CD2AP protein rescued CD2AP KO mice from embryonic lethality, which uncovered a hidden phenotype characterized by infertility and testicular abnormalities [117]. Recently, the importance of CD2AP in Sertoli-germ cell adhesion to facilitate germ cell migration has been shown [186]. A transcription factor for nephrin, WT1, is expressed in mature Sertoli cells [187] and mutations in *WT1* gene cause infertility and genital abnormalities in both mouse and man [188-191].

Pubertal development and testicular function have also been reported for male CNF patients [65]. Two patients showed low levels of inhibin B, which suggests impaired Sertoli cell function. One patient had an increased follicle-stimulating hormone (FSH) and a decreased inhibin B level with a small right testicle. The other patient had a decreased level of inhibin B and markedly increased luteinizing hormone (LH) and FSH levels [65]. Inhibin B is a glycoprotein produced predominantly by Sertoli cells and regulates pituitary FSH release by a negative feedback loop [192]. The regulation of inhibin B is complex with changes in the pattern of secretion occurring during development, and many factors such as FSH, testosterone, Sertoli cell proliferation and germ cell complement are likely to contribute to its overall production. Systemic inhibin B concentrations seem to reflect the spermatogenic status with high levels of inhibin

B observed in normal, fertile individuals and lower levels of inhibin B in individuals with severe damage to the testis as a result of germ cell depletion [192].

Cell-cell interactions between Sertoli and germ cells in the testis are crucial for mechanical adhesion and tissue morphogenesis. Importantly, these interactions play a major role in spermatogenesis that involves a series of complex biochemical, molecular and cellular events [193]. However, structural and functional aspects with regard to cell-cell interactions between Sertoli cells and germ cells are still poorly understood.

4.2. CNS

Podocytes share some similarities with neuronal cells. Both cells are highly polarized, have a common cytoskeletal organization and a common machinery for process formation [194, 195]. They also share several proteins that have restricted expression, such as nephrin [5, 59], densin [78], Nck1/2 [159] and many others [196-203].

During development of the CNS, neurons and glia cells are produced from common progenitor cells, radial glia cells, in the ventricular zone (VZ) of the neural tube [204, 205]. In mouse brain development, nephrin expression has for the first time been observed in radial glial cells in the VZ of the fourth ventricle and along the entire spinal cord at embryonic day nine [59]. Filtrin expression has been localized in the same CNS region as nephrin during the mouse brain development at embryonic day 12.5 [206]. Moreover, WT1 is expressed in the roof of the fourth ventricle of the brain during mouse development whereas in the newborn mouse expression is restricted to the kidney glomeruli [207]. Recently, Neph1 and Neph2 have been shown to be expressed in both developing and mature mouse brain [203]. Both these proteins showed a similar expression pattern during neuronal development starting at embryonic days 12 and 11, respectively. Expression of Neph-proteins was strongest in areas of high migratory activity as noticed in nephrin and filtrin expression earlier. These observations suggest that migrating early postmitotic neural precursors in the VZ, might form a neuroepithelial cell-like network with bipolar morphology, and communicate with their neighbouring cells through adherens junctions similar to podocytes [206].

In the newborn mouse brain, nephrin expression was found in the cerebellum, glomeruli of the main olfactory bulb, the hippocampal dentate gyrus and radial glia cells. This suggest a role for nephrin in granular cell and in Purkinje cell migration [5]. However histological analysis did not reveal any apparent morphological changes in the cerebellum of newborn nephrin KO mice [5]. In the adult mouse brain, Neph1 and Neph2 were predominantly seen in the olfactory nerve layer and the glomerular layer of the olfactory bulb, in the hippocampus, and in Purkinje cells of the cerebellum [203]. At the ultrastructural level, Neph1 and Neph2 were detectable within the dendritic shafts of pyramidal neurons. To a lesser extent, there was also synaptic localization of Neph1 within the stratum pyramidale of the hippocampal CA1 and CA3 region on both pre- and postsynaptic sites. There it co-localized with the synaptic scaffolder calmodulin-associated serin/threonin kinase, and both Neph1 and Neph2 interact with its PDZ domain via their cytoplasmic tail [203].

Nephrin and Neph1 KO mice die during their first day of life, which hampers studies on the potential effects of protein loss on brain development and function. Despite the apparent normal development of CNF patients after kidney transplantation [208], the role of nephrin in these patients is still unclear. However, muscular dystonia, mild ataxia and athetosis in CNF patients have been reported [66], but these symptoms may also be a complication of the congenital nephrotic syndrome and massive protein loss itself. Nephrin absence in the brain might be compensated by other cell adhesion molecules of the immunoglobulin superfamily like Neph1, Neph2 and filtrin, which are also expressed in the CNS [203, 206, 209-211]. The function of nephrin antisense transcript reported in mouse brain remains to be clarified in human brain [75].

4.3. Pancreas

Nephrin is expressed in the pancreas, particularly in the insulin producing β-cells of the islets of Langerhans [5, 11]. Recently many other nephrin associated proteins have been identified in the endocrine part of pancreas, like densin [80], filtrin [73, 80, 212], FAT, alpha-actinin-4 [213] and ZO-1 [214, 215]. Also podocin and CD2AP have been found in the pancreas, but these are more likely to be expressed in the exocrine part [213]. Interestingly, the podocin protein detected

by immunoblotting was smaller than the fragment found in the kidney [213]. This might indicate unspecific binding of the antibody, an unknown tissue-specific splicing variant or different post-translational modification of the podocin protein in the pancreas.

The precise role of nephrin and its associated protein complex in the pancreas still remains unsolved. There are no reports of CNF patients, who developed diabetes before or after kidney transplantation. In addition, in glucose tolerance tests, CNF patients after kidney transplantation did not show any difference in serum insulin or glucose levels compared to controls [65]. However, it is speculated that lifelong treatment with immuno-suppressants for preventing rejection of the transplanted kidney protects these patients against the development of type I diabetes.

5. Conventional KO mouse models in podocyte research

Experimental rat models have been widely used in glomerular injury research. The advantages of rat models are that they have been carefully characterized during the past decades, can be performed in adult animals and allow collection of time-dependent tissue samples after disease induction. Most of these models are based on chemical or antibody induced glomerular failure. The disadvantages of these models are that their effect is systemic, their action site and mechanism are relatively poorly known, they have side effects in other tissues, their therapeutic window is narrow, and in different genetic strains the dose is difficult to estimate.

After complete mapping of the genetic code and improvements in techniques for using ES cells, the number of different genetically modified mouse models has increased expotentially. The mouse is an excellent model to study human biology and disease, because of the similarities between genes and cellular functions in mammalian species. Conventional gene deletion in ES cells can be done by using homologous recombination or gene-trapping techniques as was previously described in the generation of nephrin deficient mouse-lines [5, 6]. The gene-trapping approach is based on random mutagenesis caused by random integration of a strong splice acceptor vector into the genome by using

endogenous splicing sites [77]. In homologous recombination a designed genetic vector is introduced into the specific genetic locus of mouse ES cells to cause a defined mutation [216]. Conventional gene KO mouse models of selected podocyte specific genes, their kidney phenotype and overall survival time are summarized in Table 2.

Table 2. *Conventional KO mouse-lines of selected podocyte-specific genes.*

Gene/ protein	Gene locus	Protein location	KO Phenotype	Kidney Phenotype	Glomeruli Phenotype	Effacement	Existing SDs	Proteinuria started	Life span	Ref.
Nphs1/ Nephrin	19q13.1	SD	Smaller	Enlarged	Fibrotic, hypercellular	Yes	No	From birth	24h	[5, 6]
Neph1/ Neph1	1q21-25	SD	Smaller	Normal	Dilated juxtamedullary glomeruli	Yes	No	From birth	3-8 weeks	[92]
Nphs2/ Podocin	1q25-31	SD	Smaller	Normal in size, hemorrhages	Diffuse mesangial sclerosis	Partial	No	From birth	Few days	[104]
CD2AP/ CD2AP	6p12	Near SD	Smaller after 3w	Normal	Increase size and cellularity	Partial	Yes	2 w	6-7 weeks	[108]
ACTN4/ α-act-4	19q13	Podocyte	Smaller after 6w	Smaller	Focal and segmental glomeruloscl.	Partial	Yes	Mild	> 10 weeks	[134]
TRPC6/ TRPC6	11q21-22	Podocyte	Elevated blood pressure	Not reported	Not reported	Not reported	Not reported	Not reported	Normal	[141]
Fyn/ Fyn	6q21	SD	Normal	Normal	Mesangial expansion and hypercell.	Partial	Mostly	No proteinuria	> 60 weeks	[144]
Nck1/ Nck1	3q21	Podocyte	Normal	Normal	Normal	No	Yes	No proteinuria	Normal	[159]
Nck2/ Nck2	2q12	Podocyte	Normal	Normal	Normal	No	Yes	No proteinuria	Normal	[159]

6. Novel conditional genetic mouse models in targeted podocyte research

Research into the role of the impaired glomerular filtration barrier and its specific components in mature kidney has been partly hampered by a lack of viable animal models because of early postinatal lethality (Table 2). To overcome this problem, new strategies are needed where transgenic gene expression is tightly regulated in a tissue and time specific manner during embryogenesis or in adult mice.

6.1. Podocyte specific gene expression and promoters

Expression of a gene of interest in a cell-specific manner is carried out by microinjection of a transgene expression cassette into the pronucleus of a developing zygote or oocyte resulting in random integration of the transgene into

the mouse genome [216, 217]. Transgene expression vectors contain two major components: the promoter driving transgene expression under spatial control and the transcription unit including the Kozak consensus sequence, coding exons and a termination signal (poly-A-tail) [216, 218, 219].

Table 3. *Kidney-specific promoters.*

Promoter	Renal expression	Extra-renal expression	Reference
Nephrin	Podocytes	Brain	[221]
Podocin	Podocytes	None	[222]
Kidney androgen promoter 2	Proximal tubules	Brain	[223]
γ-Glutamyl transpeptidase	Cortical tubules	None	[224]
Na/glucose cotransporter	Proximal tubules	None	[225]
Aquaporin-2	Collecting ducts	Testis, Vas deferens	[226]
Hox-B7	Collecting ducts, ureteric bud, Wolffian duct, ureter	Spinal cord, dorsal root ganglia	[227]
Ksp-cadherin	Renal tubules, collecting ducts, ureteric bud, Wolffian duct, mesonephros	Müllerian duct	[228, 229]
Tamm-Horsfall protein	Henle's loop	Testis, brain	[230]
Renin	Juxtaglomerular cells, afferent artelioles	Adrenal gland, testis, sympathetic ganglia	[231, 232]

Several kidney-specific promoters have now been characterized and are summarized in Table 3. By using these promoters, it is possible to determine whether cell-specific modification of candidate genes expression protects or leads to kidney disease. Interestingly, many of these kidney specific promoters drive expression also in the testis and CNS. Despite the growing list of kidney-specific promoters, only two promoters have been characterized that direct expression in the glomerulus and in the podocytes. A 1.25 kb fragment of the human nephrin promoter and 8.3 kb, 5.4 kb, 4.125 kb, and 1.25 kb fragments of the murine nephrin promoter direct expression in the podocytes and the CNS and pancreas

[67, 116, 220, 221]. A 2.5 kb fragment of the promoter from a second gene, podocin, directs expression only in podocytes without any noticeable influence on extra-renal tissues [222].

6.2. Site-specific recombinases in the podocytes

In addition to over-expression studies, the same promoters can be used to delete or down-regulate a gene in the selected cells by using site-specific recombinase systems. Two different recombinase systems have been used successfully *in vivo*; the Cre-loxP from the bacteriophage P1 [233] and the FLP-FRT from the budding yeast Saccharomyces cerevisiae [234, 235] (Figure 5).

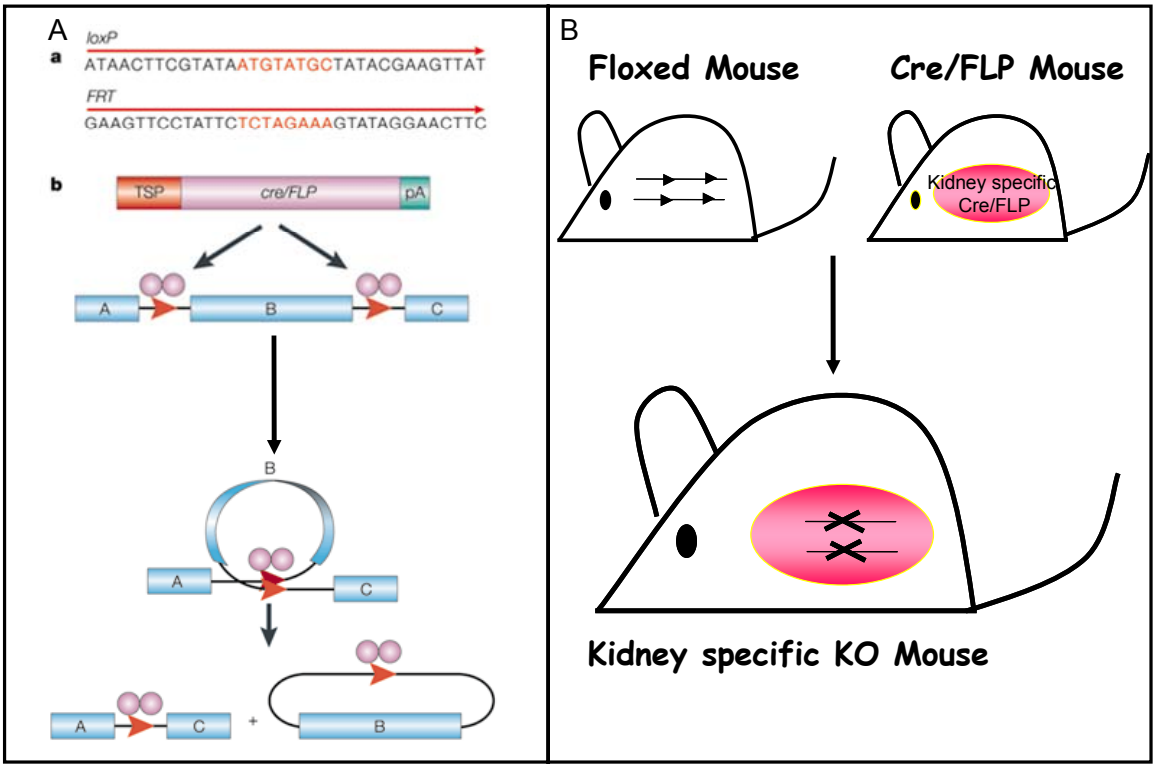

Figure 5. *Two different site-specific recombinase systems have been used successfully in vivo; the Cre-loxP from the bacteriophage P1 and the FLP-FRT from the budding yeast Saccharomyces cerevisiae.* ***A****) Both recombinases Cre and FLP recognize a 34-bp consensus sequence known as loxP and FRT, respectively, and induce recombination between two of these sites.* ***B****) Cross-breeding between a mouse-line containing a loxP/FRT flanked gene and a mouse-line expressing Cre/FLP recombinases under a tissue-specific promoter, results in littermates with selected genes deleted in a spatial manner.*

The Cre-loxP system has been most widely used in the generation of targeted gene deletion in the mouse. Cre recombinase is a 38 kDa protein that

recognizes a 34 bp DNA target called locus of X-over of P1 (loxP) and was discovered in the bacteriophage P1 [233]. When two loxP sites are located on the same DNA molecule, Cre causes inversion or excision of the intervening DNA segment depending on their respective orientation. Two transgenic mouse-lines are needed to perform tissue specific KO. The first mouse-line expresses Cre recombinase under the control of a tissue specific promoter. The second carries loxP sites around the gene of interest (floxed gene). Cross-breeding these two lines removes the selected gene in a tissue specific manner [236]. The Cre-loxP system can also be used to activate the over-expression of genes by excision of a "floxed STOP codon" placed between a highly active promoter and the gene of interest [237, 238].

An alternative approach for the Cre-loxP system is the FLP-FRT system. FLP recombinase recognizes a 34 bp consensus sequence known as FLP recombination target (FRT) and induces recombination between two of these sites [234, 235]. The activities of Cre and FLP have different temperature sensitivities *in vitro*. Cre was shown to be active over a wider range of temperatures than FLP, with a maximal performance at 42°C and therefore offered a theoretical advantage for use *in vivo* [239]. To improve the activity of FLP, an FLP mutant was developed by introducing four amino acid changes (Flpe) [240]. FLPe has activity in ES cell cultures equivalent to that of Cre and thus should be just as useful for *in vivo* experiments. Combining both FLP and Cre systems may allow an investigator to target cell lineages that would otherwise be inaccessible.

In the glomerular research field, only the Cre-loxP system has been used for targeted gene deletion in mice. Several different podocyte specific Cre mouse-lines have been characterized. One is under the control of the 4.125 kb fragment of murine nephrin [220] and another one under the control of the 2.5 kb fragment of human podocin [241]. Recently expression of the nestin gene in podocytes was reported [242]. Nestin is also expressed in CNS, pancreas and heart [242-246]. Transgenic mice expressing Cre recombinase under the control of the rat nestin promoter are commercially available from Jackson Laboratories [244].

However, so far only a few conditional models using these Cre-lines in podocytes have been published. A nephrin-Cre mouse-line has been used to

study the role of vascular endothelial growth factor A deficiency in renal disease [247-249]. A podocin-Cre mouse-line has been used to study the role of intergrin linked kinase deletion [250, 251] and HIV-1 Nef over-expression in podocytes [252].

6.3. Inducible systems

In order to study the role of specific genes in the adult mouse or in various state of disease, a temporal control of gene expression in addition to tissue specificity is needed. To achieve this goal tetracycline-sensitive systems have been successfully employed in the kidney. The binary tetracycline-controlled transcriptional activation system is a powerful tool in achieving temporal control of transgene expression in mammals [253]. For this purpose, two different tetracycline-controlled activators are widely used in transgenic mice (Figure 6A).

The original transcriptional activator (tTA) activates transcription of the target gene in the absence of tetracycline after binding to the tetracycine operator sequence (Tet-O) located in the 5' region of the target gene (Tet-off system) [254]. The reverse tetracycline-controlled transcriptional activator (rtTA) binds to Tet-O and activates transcription of the target gene in the presence of tetracycline (Tet-on system) [253]. A major problem of Tet-on system is leakiness in uninduced animals [255, 256], but improved rtTA versions have been developed for avoiding this leakage. These constructs require far lower tetracycline concentration for the induction together with minimal background expression of Cre recombinase in the absence of tetracycline [257]. Furthermore the tetracycline-controlled transcriptional silencer (tTS) is a transcriptional repressor specially designed for use with the Tet-on system and prevents unregulated gene expression, when tetracycline is absent [256, 258, 259].

Mice carrying Tet-off or Tet-on transcriptional regulation can be bred with a mouse-line that contains Tet-O activator together with the cytolomegalovirus (CMV) minimal promoter driving Cre recombinase and a mouse-line with floxed genes of interest to achieve temporal and spatial gene deletion. Addition of tetracycline or its derivative doxycycline to the drinking water initiates the expression of Cre recombinase and deletes the targeted gene selectively in the preferred tissue. Although widely used *in vivo*, a disadvantage of the tetracycline

system is that to delete the gene of interest in a temporal manner the generation of triple-transgenic offspring is required (Figure 6B).

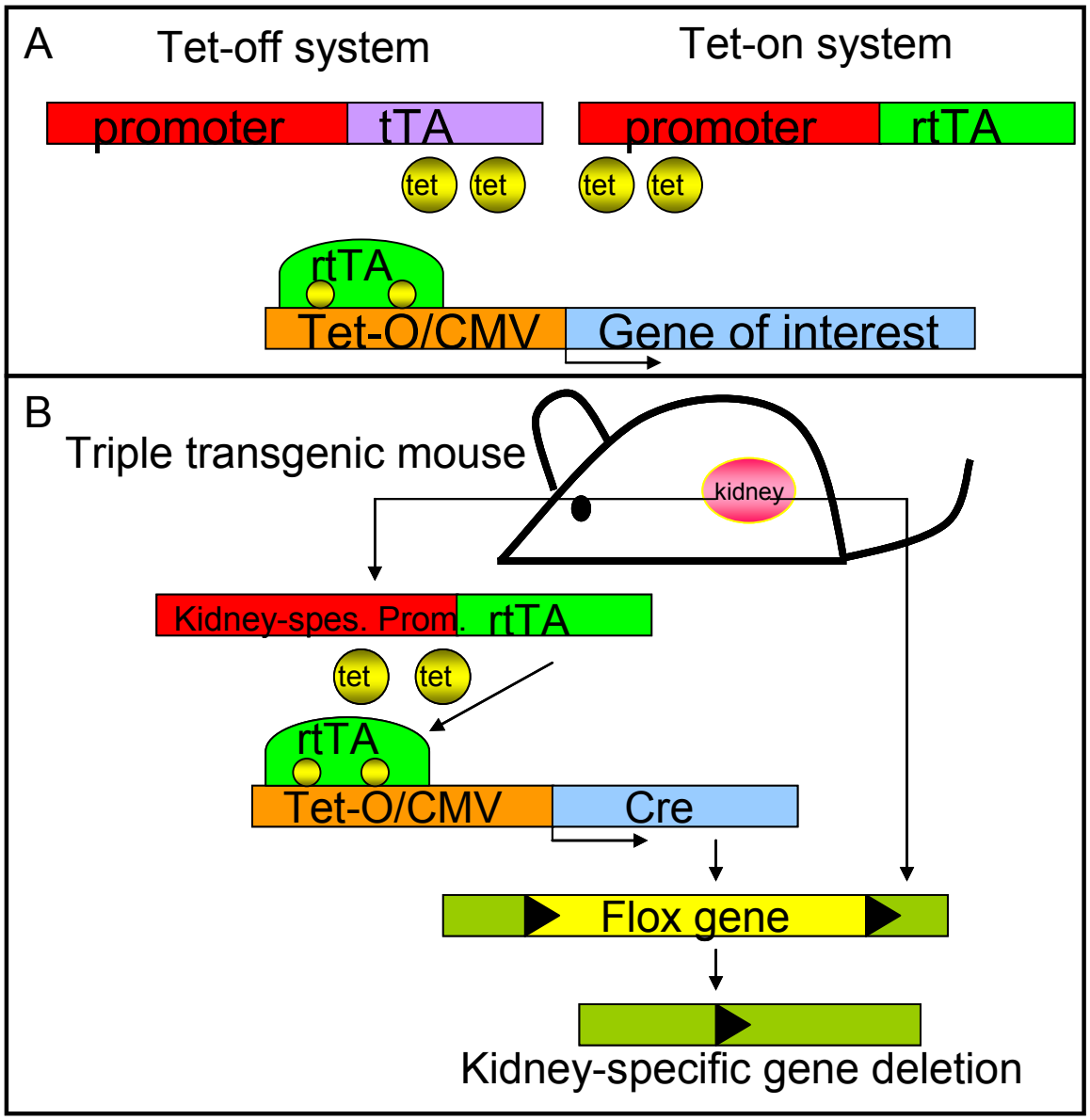

Figure 6. *Two different time-specific tetracycline inducible systems have been used successfully in vivo.* ***A****) The original transcriptional activator (tTA) activates transcription of the target gene in the absence of tetracycline after binding to the Tet operator sequence (Tet-O), located in the 5' region of the target gene (Tet-off system). The reverse tetracycline-controlled transcriptional activator (rtTA) binds to Tet-O and activates transcription of the target gene in the presence of tetracycline (Tet-on system).* ***B****) To knock-out the gene of interest in a temporal manner the generation of triple-transgenic offspring is required. The first mouse-line contains the tissue-specific promoter to drive expression of the rtTA cassette. These mice can be bred with a mouse-line that contains Tet-O activator together with the CMV minimal promoter driving Cre recombinase and a mouse-line with the floxed genes of interest. This generates offspring from which the selected gene can be deleted by tetracycline administration in a spatial and temporal manner.*

"All-in-one" strategies have been developed to avoid the generation of triple-transgenic offspring. In this strategy, two expression units of the Tet-off system [260] or the Tet-on system [261, 262] on a single DNA fragment can be integrated in one cloning step. However, whether this "all-in-one" system is functional in transgenic mice remains to be fully elucidated. Another technique that overcomes this problem is the development of ligand-regulated forms of Cre recombinase by fusing a mutant estrogen receptor (ER) ligand-binding domain to the COOH terminal of enzymes. These fusion proteins are induced by the application of a synthetic estrogen antagonist 4-OH tamoxifen but are insensitive to endogenous ß-estradiol. Three different mutant estrogen receptors have been reported: mouse ER^{TM} [263], human ER^{T} [264] and human ER^{T2} [265]. $CreER^{T2}$ has been reported to be 10 times more sensitive than $CreER^{T}$ [266].

Inducible gene deletion has been used in mouse for many targets including the liver [267, 268], heart [269, 270], kidney collecting ducts cells [271] and prostaglandin receptors [272]. In kidney glomeruli research, several inducible mouse-lines have been characterized. The first tetracycline inducible transgenic-line consisted of the podocin promoter to drive expression of the rtTA cassette in podocytes. The limitation of this mouse-line, is that the generation of triple-transgenic offspring is required [273]. Previously, a tamoxifen inducible mouse-line has been generated that expresses Cre recombinase in podocytes but also in a variety of other tissues including striated/smooth muscles, some pancreatic cells, and cultured primary fibroblasts derived from the ear [274]. This mouse-line was used successfully to over-express the Pax2 transcription factor in adult mice and to reveal its role in the development of glomerular diseases in mature podocytes [178]. The $CreER^{T2}$ system has also been used in renal epithelial cells under the control of the Ksp-cadherin promoter [275]. However, there is still a great need to generate simpler and faster systems to delete selected genes only in the podocytes in a temporally controlled manner without extensive breeding steps and gene deletion in other tissues.

6.4. RNA Interference

Although mouse mutants lacking a specific gene are often useful for analyzing gene function, the traditional procedure used to generate mutant mice by

homologous recombination in ES cells is costly and time-consuming. RNAi technique provides an alternative approach by inducing sequence-specific mRNA degradation [276]. RNAi is a highly conserved mechanism throughout evolution and can be found from plants to humans. RNAi is the process of sequence-specific, post-transcriptional gene silencing initiated by double-stranded (dsRNA) that is homologous in sequence to the silenced gene. The presence of dsRNA in the cytoplasm activates Dicer enzymes that cleave the RNA into short pieces [276]. The mediators of sequence specific mRNA degradation are 21 and 22-nucleotide small interfering RNAs (siRNAs) [277]. These siRNAs are incorporated into a multiprotein RNA-inducing silencing complex (RISC). The antisense strand guides RISC to its homologous target mRNA resulting in cleavage.

Chemically synthetized siRNA has been used succesfully for mRNA degradation in mammalian cultured cells. However, despite its downregulation efficiency, the effect is only transient and can not be used effectively for gene silencing *in vivo* [277, 278]. To overcome these problems, techniques have been developed using DNA-vectors that can integrate into the host genome and express substrates in a stable manner, which can be further converted into siRNAs *in vivo* [279-281]. The RNA Pol III H1- and U6-RNA gene promoters were shown to efficiently produce RNA from a DNA template in cultured cells [280]. Meanwhile, it has been shown that Dicer can process short (shRNA) or long hairpin dsRNA structures resulting in the generation of appropriate siRNAs [281]. Produced siRNA can then effectively silence target mRNA expression making it possible to downregulate genes by using only one vector that produce the hairpin structure [279-281]. Some of these constructs can silence gene expression in a stable manner in transgenic mice [281].

However, the Pol III promoter is active in all tissues and the use of the RNA Pol II promoter is essential for developing a method to efficiently generate partly tissue-specific knockdown mice. One of the difficulties using a long dsRNA transcribed from the Pol II promoter is the interferon response [282]. In mammals, the introduction of long dsRNAs into the cells leads to an inhibitory response mediated through release and synthesis *de novo* of interferon similar to a viral infection. This leads to a block in translation and sequence-nonspecific mRNA degradation [278]. Pol II transcripts are transferred to the cytosol immediately after

transcription, where they induce interferon synthesis [282]. To prevent the interferon response, the pDECAP (DEletion of CAP structure and poly(A)) vector which express a long ds-RNA regulated by the Pol II promoter has been used [281]. This efficiently blocks the export of long dsRNA to the cytosol [281]. The pDECAP transcript lacks both the 5´-cap structure and the 3´-polyA tail which prevents export of dsRNA to the cytosol and non-specific mRNA degraration caused by interferon response [281]. Because the ribonuclease(s) such as Dicer which cleave long ds-RNA are localized in both the nucleus and the cytoplasm [283], the long dsRNA expressed by this vector is expected to be processed into siRNA already in the nucleus. siRNA then moves to the cytosol and induce the degradation of target mRNA [273]. In the future, spatial and temporal control of dsRNA expression in the kidney should be possible by using a combination of inducible Cre mouse-lines, permitting rapid and high throughput analysis of gene function in a time and cost effective manner.

Aims of this thesis

The generation and analysis of various genetically modified mouse models were used to assess the biological function of nephrin. Specifically the aims of this thesis were:

i) To generate novel podocyte-specific constructs that provides an inducible tool for *in vitro* and *in vivo* gene targeting.

ii) To generate a podocyte-specific inducible Cre recombinase mouse-line that allows conditional and targeted gene deletion in adult mouse kidneys.

iii) To study biological roles of nephrin in podocytes and in extra-renal tissues using nephrin KO mice rescued by podocyte-specific trasgenic rat nephrin expression.

iv) To study the role of SD components in the testis.

v) To generate a construct, with a long hairpin structure of dsRNA that allows stable densin and filtrin gene silencing *in vitro and in vivo*.

Materials and methods

1. Human and mouse testis and brain samples (III)

Frozen human testis samples were obtained from two adult patients who underwent prostate cancer surgery at the University of Helsinki (Department of Obstetrics and Gynecology). A frozen sample of normal brain tissue adjacent to a tumor in the temporal lobe was taken from a young adult during tumor surgery at the University of Helsinki (Department of Neurosurgery). The use of these human samples was approved by the local ethics committee and informed consent was obtained from the patients.

Mouse testis samples were obtained from adult (20 weeks old, total body weight approximately 25 g) male C57BL/6 mice. After cervical dislocation, mouse testes were immediately dissected and snap frozen in liquid nitrogen and stored at -80°C until use. Brain tissues were dissected to serve as positive controls for densin expression. All animal experiments had the approval of the local committee for laboratory animal welfare of the University of Helsinki.

2. Constructs and cloning

2.1. Doxycycline inducible Cre recombinase construct (I)

The p2.5PodocinpnlacF plasmid containing 2.5 kb of the genomic sequence of the human podocin promoter located 5' to the translation initiation codon was kindly provided by Dr. Lawrence Holzman [222, 241]. The “Core” construct was provided by W.H. Lee [262]. An EcoRI-HindIII fragment from the original rtTA of the core construct was replaced with the optimized version, rtTA-M2 [257]. However, several attempts to clone promoters into the designated cloning site failed due to the instability of the construct. Therefore the two functional EcoRI-ScaI fragments from the Core construct were reconstituted in a pBR322 based low copy backbone after modification of the polylinker, generating NotI restriction sites instead of ScaI on both sides of the transgene. Finally unique SalI and SmiI sites

were introduced at the position of the EcoRI site. The resulting construct was stable and designated as RRC-M2.

The unique XbaI site in p2.5PodocinpnlacF was replaced by a SalI using a linker (sense: 5'-ctagcagatctaagcagtcgaca-3' and antisense: 5'-ctagtgtcgactgcttagatctg -3'). The resulting SalI-NcoI fragment of the podocin promoter was cloned into the SalI-SmiI digested RRC-M2 construct as a SalI-blunt fragment. The NcoI end was blunted using T4 polymerase (New England Biolabs). The identity of the clones was verified by restriction mapping and sequencing. The final construct was designated JRC-CRE (Figure 7).

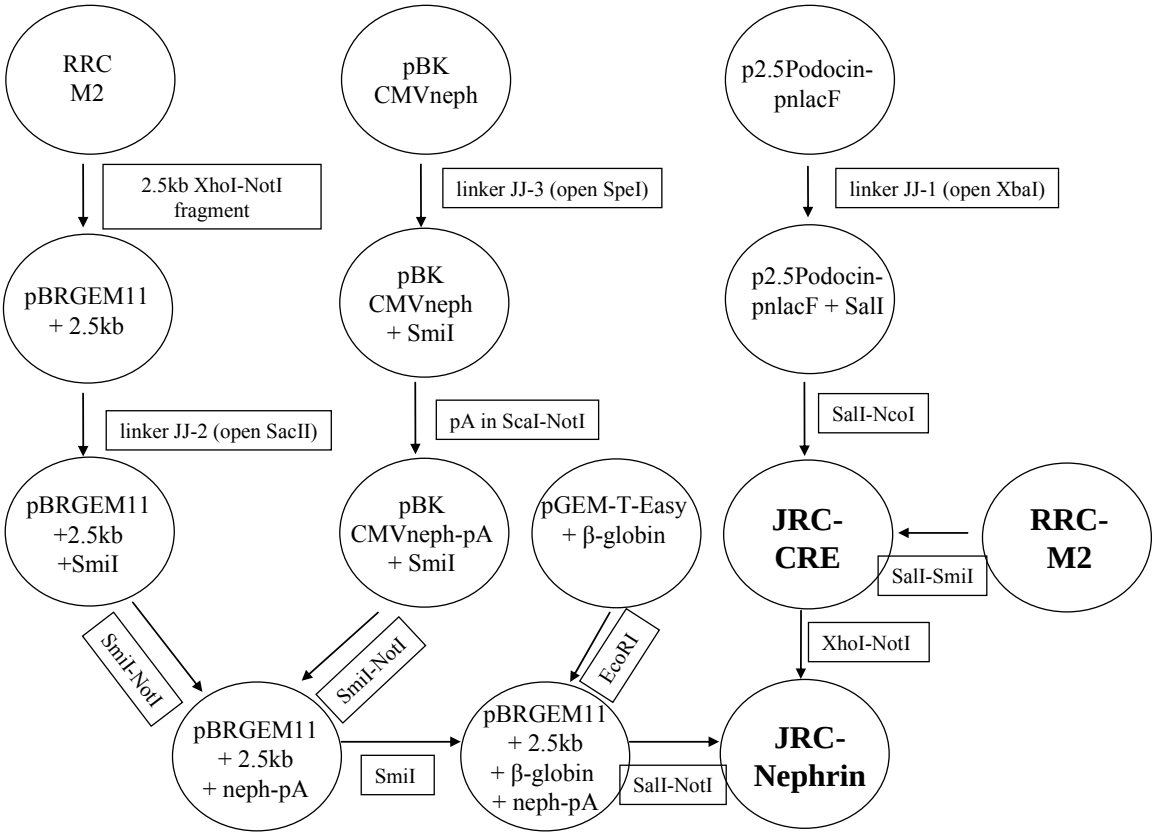

Figure 7. *Different steps for cloning JRC-CRE and JRC-Nephrin constructs.*

2.2. Doxycycline inducible rat nephrin construct (II)

The original RRC-M2 plasmid was digested by XhoI-NotI digestion and this 2.5 kb fragment was subcloned to the pBRGEM11 plasmid. A linker (sense: 5'-ctagcagatctaagcagtcgaca-3' and antisense: 5'-ctagtgtcgactgcttagatctg -3') containing a SmiI site was cloned into the pBRGEM11 plasmid opened by SacII

digestion. In parallel, a linker (sense: 5'-ctagcagatctaagcagtcgaca-3' and antisense: 5'-ctagtgtcgactgcttagatctg -3') containing an additional SmiI restriction site was cloned into the SpeI site of the pBKCMVneph plasmid, containing 3.7 kb of the sequence of rat nephrin complementary DNA (cDNA). The polyA tail (pA) was cloned to the 3' end of the rat nephrin cDNA by ScaI-NotI digestions. Subsequently the construct was cut with SmiI-NotI and the resulting 4.3 kb fragment containing both rat nephrin cDNA and pA was cloned into the SmiI-NotI sites of the RRC-M2 vector. From the pGEM-T-Easy (Promega) vector containing 572 bp of the sequence of the β-globin intron amplified by polymerase chain reaction (PCR) was released by EcoRI digestion. The cut fragment was blunted using T4 polymerase. The resulting blunted fragment of the β-globin intron was cloned into the SmiI site of the RRC-M2 vector containing rat nephrin cDNA and pA.

Finally, the SalI-NotI fragment containing TetO-CMV-B-globin-rat nephrin-pA sequence was cloned into the XhoI-NotI sites of the JRC-CRE construct containing the podocin promoter. The final construct was designated JRC-Nephrin (Figure 7).

2.3. Long hairpin RNAi construct (Unpublished data)

For RNAi contructs cloning, a recently published and academically free pDECAP vector donated by Dr. Ishii was used [281]. The DNA from three different fragments of the densin gene and two different fragments of the filtrin gene were cloned to p-DECAP vector followed by inverted repeats; 3´-sense 500 bp 5´-spacer 12 bp - 5´-antisense 500 bp-3´.

The constructs were transformed to SURE® 2 supercompetent cells (Stratagene). These cells have been engineered to allow the cloning of certain DNA segments, which are difficult to clone in conventional *E. coli* strains. The SURE strain lacks components of the pathways that catalyze the rearrangement and deletion of non-standard secondary and tertiary structures, including cruciform (caused by inverted repeats) and Z-DNA, which occur frequently in eukaryotic DNA and impede the cloning of eukaryotic DNA in conventional strains.

3. In vitro assays and micro-injection (I, II, Unpublished data)

For *in vitro* testing of the constructs, the human embryonic kidney cell-line A293 (American Type Culture Collection) was cultured in RPMI-1640 medium (Gibco Biocult) containing 10% fetal calf serum, penicillin (100 U/ml; NordCell) and streptomycin (100 µg/ml; NordCell). The cells were grown in 24-well plates to 70% confluency. Transfections were performed by Fugene 6 reagent™ (Roche Molecular Biochemicals) according to the manufacturer's protocol.

The JRC-CRE or JRC-Nephrin constructs were co-transfected with the pCAG20-1 vector containing a β-actin promoter to drive tTA2, as no podocin promoter activity was detected in A293 cells. Transfected cells were cultured for 24 hours in the presence or absence of 2.0 µg/ml doxycycline hyclate (Sigma-Aldrich). The cells transgenic mRNA and protein expression were analyzed using assays that are later described in this section.

Tested constructs were purified by digestion of the plasmid and subsequent gel electrophoresis followed by DNA extraction using the QIAEX II Gel Extraction Kit (Qiagen). The purified construct DNA was introduced into the pronuclei of fertilized oocytes from FVB/N mice by microinjection using standard procedures. The resulting founders were identified by PCR on tail genomic DNA (50 to 100 ng), using different primer sets and 35 cycles at 95°C (30 s), 54°C (30 s), and 72°C (30 s), and a final extension at 72°C for 5 min. The reaction was performed with HotStar Taq DNase polymerase (Qiagen) in a total volume of 20 µl, including 400 nM of both primers. Samples were analyzed by standard agarose gel electrophoresis (1.5% gel).

The RNAi constructs (250 ng of DNA), reporter vector (psiCHECK-1; Promega; 250 ng of DNA) and control plasmid (pGLBasic/SV40P; Promega; 100 ng of DNA) were co-transfected to A293 cells using Fugene reagent™. The RNAi Renilla luciferase reporter vector psiCHECK-1 was modified by placing a mouse densin or filtrin full-length cDNA to the front of the luciferase gene. The silencing efficacies of the used RNAi constructs to Renilla luciferase activity were measured and normalized by using the co-transfected plasmid pGLBasic/SV40P as a control with Firefly luciferase. After transfection (24-48 hrs), the cells were assayed for luciferase activity with Dual-Luciferase Reporter Assay System (Promega) and

measured with a luminometer (Thermo Scientific). The results are presented as relative light units (RLU).

4. Cross-breedings

4.1. JRC-CRE x Z/AP reporter-line (I)

To analyze transgenic Cre recombinase localization and activity, JRC-CRE transgenic founders were bred with lacZ/human placental alkaline phoshatase (Z/AP) reporter mice [284]. The Z/AP reporter mouse-line carries a ß-geo cassette flanked by two loxP sites and an alkaline phosphatase gene [284]. Alkaline phosphatase (AP) cannot be expressed in this transgene unless the ß-geo cassette is excised. Thus, these mice express AP after Cre-mediated recombination. Double transgenic mice were identified by PCR on tail genomic DNA. Offspring carrying both transgenes (JRC-Cre x Z/AP) were administrated 0.2 mg/ml doxycycline in their drinking water for 14 consecutive days.

4.2. Transgenic JRC-Nephrin x Nephrin KO mice (II)

Mice from selected JRC-Nephrin founder-lines were cross-bred with nephrin deficient heterozygous mice [6]. The F1 offspring were intercrossed to generate F2 offspring that lack a functional endogenous nephrin gene while containing the inducible podocyte-specific rat nephrin transgene. Transgenic mice were identified by PCR on tail genomic DNA as described earlier [6]. The classification for the different genotypes was as follows: 1) JRC-Nephrin x wild-type control (Nephrin/WT/WT), 2) JRC-Nephrin x heterozygous KO (Nephrin/WT/KO) and 3) JRC-Nephrin x homozygous KO (Nephrin/KO/KO) mice. Doxycycline was administered to the F1 females via drinking water (2.0 mg/ml in 5% sucrose) from conception on and during pregnancy. After birth, doxycycline administration was continued via drinking water (2.0 mg/ml in 5% sucrose) for one to six weeks.

5. Phenotypic and histological characterization

5.1. Determination of urinary protein level (II)

Spot urine samples were collected from at least five mice of each genotype. The urinary protein levels were semiquantified with the Uristix (Bayer) protein assay as indicated on the Uristix test strip package.

5.2. Determination of blood glucose level (II)

Blood samples from mice were collected from at least three individual mice of each genotype. Glucose levels were quantified with the Accutrend Sensor (Roche Diagnostic Corporation) system by using Accu-Chek test-strips (Roche) according the manufacturer's protocol.

5.3. Primary behavioral screening (I and II)

Ten-weeks-old JRC-CRE and five-weeks-old JRC-Nephrin x KO male mice from different genotypes was used for behavioral characterization. The employed battery of behavioral observational tests was a modification of the Irwin procedure [285], during which a total of 40 separate measurements were performed for each animal (for a full method description, See Shirpa Protocol):

http://www.mgu.har.mrc.ac.uk/facilities/mutagenesis/mutabase .

5.4. Histological characterization (II)

The dissected tissues from one- and six-weeks-old JRC-Nephrin x KO mice of each genotype were fixed in 4% formaldehyde and embedded in paraffin. Two to three micrometer thick sections were cut, deparaffinized and stained with hematoxylin-eosin using standard procedures. The slides were observed with an Olympus Provis microscope.

For electron microscopic examination, kidney cortex samples from one and six-weeks-old JRC-Nephrin x KO mice were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for two hours. The samples were postfixed in 1% osmium tetroxide (OsO_4) in the same buffer for one hour and stained *en-bloc* in 1% uranyl acetate in 10% ethanol for one hour, after which they were dehydrated in ethanol and embedded in LX112. Semithin sections were

stained with toluidine blue. Thin sections were stained with uranyl acetate and lead citrate and examined with a Jeol JEM 1200EX electron microscope.

6. mRNA expression (I, II, III)

mRNA expression was measured using the reverse transcription (RT)-PCR method. After cervical dislocation, mouse tissues were dissected immediately, snap frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted from homogenized lysate of frozen samples using the RNeasy Mini Kit (Qiagen) according to the manufacture's instructions. cDNA was prepared from 1 µg RNA with the M-MLV reverse transcriptase (Promega) using random primers (Roche) (RT+). To confirm the RNA origin of the PCR signals each sample was also analysed without the reverse transcription reaction (RT-). Tissue cDNA was amplified by PCR using the primers presented in Table 4. The PCR reaction was carried out as described above with 35-40 cycles.

Table 4. *Oligonucleotide primers used in RT-PCR:s.*

Copied cDNA	Forward Primer 5' -3'	Reverse Primer 5' -3'	Size of Product	Used in study:
Cre recombinase	gaccaggttcgttcactca	tagcgccgtaaatcaat	420 bp	I
Nephrin	cctggagctaccctgcata	ggacttggtaaggcagcaaa	317 bp	I
Rat Nephrin	caggtacagcctggaaggagatc	tcctctgatccctcattcacat	335 bp	II
Mouse Nephrin	aggtacagcctggaaggagaca	tcctctgatccctcattcacgc	335 bp	II
Podocin	ccagcttcgatacttgcaca	ctttgcccattcgcctataa	210 bp	II
Synaptopodin	gctgctggagcactgggc	ttggagagcctggctttg	298 bp	II
CD2AP	gttgggactgtttccctcaa	tttctttggctgtgcaactg	172 bp	II
FAT1	gtgacggacgttgaggaaat	actgctgttctgtggtgtcg	182 bp	II
Fyn	cgaactacaacttccac	ctggagccacgtaattgctg	279 bp	II
Nck1	aaggacaccttaggtattgg	agagaacctacatgatcacc	311 bp	II
Nck2	gaacctcaaggacacactag	cgtaggctcaggaagctggg	342 bp	II
Beta-actin	ttccttcttgggtatggaat	ggccaggatggagccaccga	250 bp	II
Densin	atgctttccctgacaactgg	gtgtgtctgtgggtggactg	208 bp	III
Beta-actin	aaccgcgagaagatgacccagatcatgttt	agcagccgtggccatctccttgctcgaagtc	351 bp	III

7. Protein expression

7.1. Immunoblotting (I, II, III)

Samples of frozen tissues were lysed on ice with a glassware homogenizer in RIPA lysis buffer (150 mmol l^{-1} NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], 50 mmol l^{-1} Trizma base [pH 8], 0.02% NaN3) with a protease inhibitor mixture (Complete Mini, EDTA-free, Roche Diagnostics), centrifuged (5 min at 14000g), and the protein concentration of the supernatant was measured with the BCA Protein Assay Kit (Pierce). Samples were boiled for 5 min at 100°C in reducing 1 x LSB-buffer (4 x LSB: 125 mmol l^{-1} Tris-HCl [pH 6.8] 20% glycerol, 0.04% SDS, 10% 2-mercaptoethanol, 0.001% bromophenol blue). The proteins were resolved by SDS-polyacrylamide gel electrophoresis on 10% gels and were transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 3% bovine serum albumin (BSA), 0.05% Tween-20 in PBS at 4°C overnight or with Odyssey reagent (LI-COR) 1h at RT. The membranes were incubated with the same antibodies as used in the immunofluorescence staining at 4°C overnight. The membranes were then incubated either with affinity-purified peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories; 1:50000) or with goat anti-mouse or anti-rabbit secondary antibodies conjugated to AlexaFluor 680 (Molecular Probes; 1:10000) or IRdye 800 (Rockland Immunochemicals; 1:10000). The Supersignal West Pico Chemiluminescent Substrate (Pierce) or the Odyssey infrared imaging system (LI-COR) was used to detect the bound antibodies.

7.2. Immunofluorescence staining (I,II,III)

Immunofluorescence staining for selected antibodies was performed on frozen mouse sections (6 μm). The sections were air-dried, fixed with acetone (–20°C) for 2-10 min, washed with PBS and blocked with CAS Block solution (Zymed Laboratories Inc.) for 10 min. The sections were incubated at 4°C overnight with the primary antibody in ChemMate Antibody Diluent (DakoCytomation). After washing, the sections were incubated with affinity-purified TRITC-conjugated goat anti-rabbit IgG (1:200; Jackson Laboratories) and/or affinity-purified FITC-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories; 1:200) in

PBS for 30 minutes, mounted with Vectashield Mounting Medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The slides were observed with an Olympus Provis microscope equipped with filters to detect fluorescence.

7.3. Alkaline phosphatase substrate staining (I)

For testing the presence of AP, the JRC-CRE x Z/AP and WT control mice either received (n=3) or did not receive doxycycline (n=3). A list of the tissues collected is provided in the original publication I (Table 2).

Prior to staining slides were re-fixed in cold PBS containing 0.2% glutaraldehyde for 10 min. After fixation the slides were washed in PBS for 5 min and endogenous AP was inactivated by incubating the slides in PBS at 75°C for 30 min. The slides were rinsed with PBS, washed in AP buffer (100 mM Tris–HCl, [pH 9.5], 100 mM NaCl, 10 mM MgCl2) for 5 min, followed by incubation with BM purple (Roche) for 1 hr at 4°C to detect AP activity. Gurr Aquamount (BDH) was used for mounting. The slides were observed with an Olympus Provis microscope.

8. Statistical analyses (I, II, III)

The results are presented as means ± standard deviation. Statistical analyses were carried out using SPSS for Windows 12.01 (SPSS Inc.). Analyses were performed using a one-way analysis of variance. When statistically significant differences ($P < 0.05$) were found, post-hoc comparisons were carried out using the least significant difference test (LSD).

Results

1. A novel inducible mouse-line for podocyte-specific gene deletion (I)

In order to generate a mouse-line with doxycycline inducible Cre recombinase expression only in podocytes, a Tet-on construct with podocin promoter called JRC-CRE was cloned as detailed in materials and methods (Orig. I, Fig. 1). Prior to pronuclear injection, the inducible construct was tested *in vitro* in A293 cell culture. Cre recombinase expression was localized within the nuclei and was tightly regulated by doxycycline administration. The construct was injected into FVB/N mouse oocytes and 16 out of 122 offspring were found to carry the transgene. To achieve an inducible mouse-line in which transgene is transferred to F1 littermates, these founders were back-crossed with C57Bl/6 WT mice. All founders were fertile and transmitted the transgene to their F1 offspring. The phenotype of F1 littermates was normal and remained normal after a two-week administration period of 0.2 mg/ml doxycycline compared to WT controls.

1.1. Doxycycline dependent expression of Cre recombinase in the adult mouse

To prove time- and tissue-specific Cre recombinase mRNA expression in the adult mouse kidneys, F1 offsprings at the age of eight to ten weeks were treated with doxycycline, and mRNA expressions were analyzed by RT-PCR. These results showed that expression of Cre recombinase mRNA was observed only in the kidney cortex and only after doxycycline treatment (Orig. I, Fig. 2).

To determine the specific localization of Cre recombinase protein expression in the kidney cortex, renal cryosections from F1 littermates were examined by Cre antibody immunofluorescence staining before or after doxycycline treatment. These studies indicated that expression of Cre recombinase was prominent in the periphery of the glomeruli, which is consistent with the localization of the podocytes. Cre recombinase protein expression was absent in the WT and in the JRC-CRE controls not treated with doxycycline (Orig.

I, Fig. 3). Cre recombinase protein expression was not observed in the other tissues analyzed including heart, liver and pancreas (Orig. I, Fig. 4).

1.2. Functionality of inducible Cre recombinase in podocytes

To test Cre recombinase capability to catalyze recombination between two loxP sites, cross-breeding with a Z/AP reporter mouse-line was performed. Cre recombinase expression excises the ß-geo cassette from reporter mouse-line genomics and induces AP expression. This was tested in a set of tissues from all founder-lines by using AP substrate staining. Three founder-lines out of sixteen showed Cre recombinase expression in podocytes without doxycycline administration. Two founder-lines out of sixteen expressed Cre recombinase in heart tissue after two weeks of doxycycline administration. For further studies one founder-line was selected because it had a high Cre recombinase expression level only in podocytes, and inaddition it lacked leakiness without doxycycline administration.

1.3. Localization of Cre recombinase expression in podocytes

To determine the localization of human placental AP protein expression as a marker for Cre activity, the kidney tissues of reporter mice were examined by immunofluorescence microscopy with antibodies against AP, synaptopodin and WT1. Double stainings showed that expression of AP co-localized with synaptopodin protein, but not with WT1 podocyte-specific nuclear staining. A total of one hundred glomeruli from several sections were counted. All glomeruli positive for synaptopodin also showed high expression for AP in doxycycline treated double transgenic mice. AP protein expression was not observed in double transgenic mice without doxycycline or in WT controls (Orig. I, Fig. 5). Nevertheless it might be that all podocytes do not express Cre recombinase upon induction, but the present data suggest that the percentage is very substantial.

2. A novel inducible mouse-line for podocyte-specific nephrin over-expression (II)

To evaluate the role of transgenic over-expression in adult mouse kidneys, a podocyte-specific inducible rat nephrin mouse-line was generated. The construct

expressing doxycycline inducible rat nephrin (Orig. II, Fig. 1) under the control of podocin promoter was cloned and tested in an A293 cell-line, which showed no endogenous nephrin expression. The introduced rat nephrin expression was tightly regulated by doxycycline administration and was localized preferentially in the cell membrane and in the developing cell protrusions (Figure 8). Similar findings have also been reported with the rat nephrin transgene and HEK293T cells. On the basis of those results, it was suggested that morphological changes including cell protrusions and spikes, are mediated by nephrin tyrosine phosphorylation and by Nck interaction [148] .

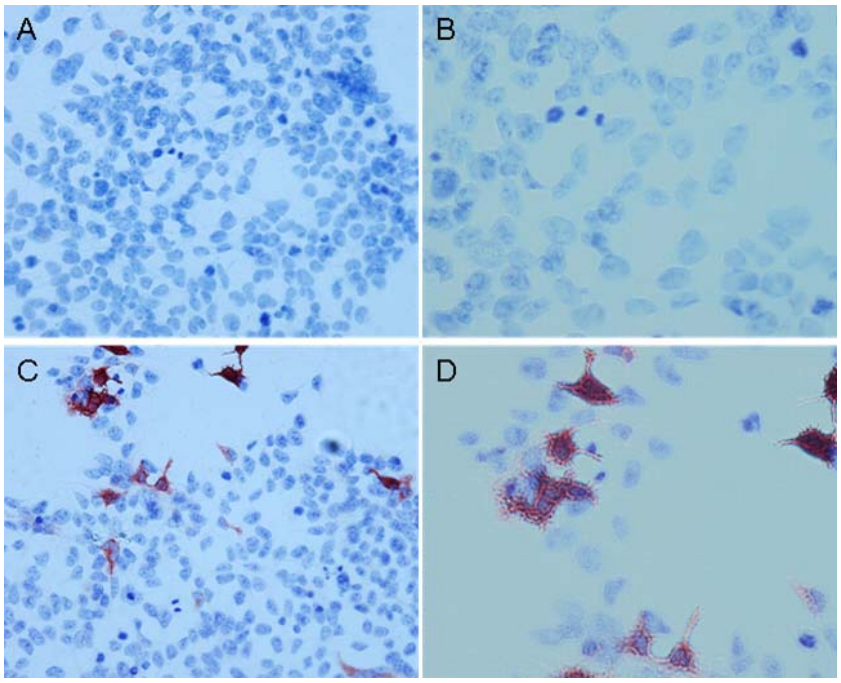

Figure 8. *Inducible rat nephrin expression in A293 cells. A293 cells were co-transfected with the JRC-Nephrin construct and pCAG20-1 construct containing the β-actin promoter driving the tTA2 expression. Doxycycline (2 µg/ml) was added to the wells 24 h before fixation of the cells to shut-down nephrin expression.* ***A, B****) Doxycycline 2 µg/ml treated co-transfected A293 cells do not show any nephrin staining.* ***C, D****) Nephrin expression in co-transfected A293 cells without doxycycline treatment. Magnification: x100 (A and C) and x200 (B and D).*

After *in vitro* testing the construct was injected into FVB/N mouse oocytes, which resulted in 10 out of 90 pups carrying the transgene as detected by PCR. In order to achieve a mouse-line with doxycycline inducible rat nephrin expression only in podocytes, these founders were cross-bred with C57Bl and ICR WT mice.

Eight out of 10 founders transmitted the transgene to their offspring (F1). All founders and their F1 offspring were fertile and their tissue phenotypes were normal. After a two-week administration period of 0.2 mg/ml doxycycline, the phenotype of these F1 offspring remained healthy with normal body weight as noticed in WT controls.

2.1. Doxycycline regulated expression of rat nephrin in podocytes

Nephrin mRNA expression was studied in the kidney cortex of F1 adults carrying the transgene at the age of 10 to 12 weeks by RT-PCR. Expression of rat nephrin mRNA was observed in F1 offspring from five out of seven founder-lines. The expression was firmly regulated by the administration of doxycycline and observed only in the kidney cortex (Orig. II, Fig. 1). Transgenic rat nephrin expression was not found in other extra-renal tissues obtained from F1 littermates, which included testis, cerebellum, cortex, pancreas, heart, liver and ovaries. Doxycycline induced expression of the rat nephrin showed similar expression pattern as endogenous nephrin and was localized only in the podocytes. The expression of transgenic rat nephrin did neither alter the expression of the other podocyte-specific genes or their protein localization.

3. Inducible nephrin transgene expression in podocytes rescues nephrin deficient mice from perinatal death (II and unpublished data)

Conventional nephrin KO in mice leads to early lethal phenotype [6]. In order to investigate whether inducible transgene expression of rat nephrin only in podocytes can rescue nephrin KO mice from perinatal lethality, a podocyte-specific rat nephrin mouse-line was crossed with nephrin KO mouse-line. Cross-breedings in the presence of doxycycline resulted in an F2 generation of 72 pups of which 14 (10 males and four females) carried the rat nephrin transgene and were homozygous for the KO allele lacking endogenous nephrin expression (Nephrin/KO/KO) as verified by PCR. Three offspring were sacrificed at the age of one week. Two female pups died at the age of seven to 14 days. Three males died at the age of 38 and 40 days and showed severe proteinuria (> 3.0 g/l) with

histological changes in the kidney morphology that included glomerular changes and tubular dilatations with proteinaceous material. Five males and one female survived until the age of six weeks and were sacrificed for further analysis.

3.1. Impaired locomotor activity in Nephrin/KO/KO mice

Deficiency of endogenous nephrin was associated with significant impairments in locomotor activity in comparison with other genotypes as summarized in Table 5. Behavioral evaluation in the viewing jar showed decreased spontaneous activity in Nephrin/KO/KO mice compared to other genotypes ($P < 0.05$). In an open arena, elevated pelvic and tail dragging during locomotion was observed in all Nephrin/KO/KO mice ($P < 0.001$). Nephrin/KO/KO mice had also reduced locomotor activity in the novel environment ($P < 0.05$). No statistically significant behavioural differences between Nephrin/WT/WT and Nephrin/WT/KO genotypes were found.

Table 5. *Differences between JRC-Nephrin x Nephrin KO mice in the primary behavioral screening.*

Mean ± S.D. or number of mice exhibiting of the observed behavior			
	Nephrin/WT/WT	Nephrin/WT/KO	Nephrin/KO/KO
	n=3	n=3	n=5
Spontaneous Activity	2.33 ± 0.57	2.67 ± 0.58	1.20 ± 0.45 *
Locomotor Activity	24,67 ± 6.43	23.67 ± 7.57	11.80 ± 5.45 *
Elevated Pelvic	0	0	5***
Dragging Tail	0	0	5***

*Statistically significant differences after LSD test: * $P < 0.05$; *** $P < 0.001$.*

3.2. Podocyte specific rescue of nephrin results in severe tissue phenotypes

All Nephrin/KO/KO mice that survived for six weeks were notably sick, smaller in size and had significantly lower body weights (Figure 9 A and B). They all suffered from severe proteinuria at the age of three weeks (> 3.0 g/l). Blood glucose levels were decreased dramatically in Nephrin/KO/KO littermates ($P < 0.001$) and were also decreased in Nephrin/WT/KO ($P < 0.05$) compared to Nephrin/WT/WT controls (Table 6). The tissue/body weight ratios of kidneys, brain, heart and

testicles were increased in Nephrin/KO/KO littermates compared to other genotypes, which may be due to the smaller body weight (Table 6).

Table 6. *Phenotypic parameters measured from six-weeks-old JRC-Nephrin x Nephrin KO mice***.**

					Average Tissue Weight Compared to Body Weight (%)				
Geno-type	**n**	**Body Weight (g)**	**Protein in Urine (g/l)**	**Glucose in Blood (mmol/l)**	**Kidney**	**Brain**	**Heart**	**Testis**	**Spleen**
Nephrin/ WT/WT	3	25.4 ± 5.3	0.3-1.0	13.2 ± 1.6	2.24 ± 0.14	1.97 ± 0.11	0.66 ± 0.13	0.79 ± 0.12	0.50 ± 0.12
Nephrin/ WT/KO	3	25.7 ± 4.8	0.3-1.0	10.3± 1.2*	2.15 ± 0.07	1.86 ± 0.21	0.67 ± 0.03	0.81 ± 0.11	0.63 ± 0.07
Nephrin/ KO/KO	3	15.2 ± 6.4***	≥ 3	5.0 ± 1.7***	2.56 ± 0.11*	4.02 ± 0.30***	1.01 ± 0.23*	1.31 ± 0.34*	0.46 ± 0.30

*Statistical significant differences after LSD test compared Nephrin/WT/WT group: * P < 0.05; *** P < 0.001.*

Glomerular morphology of the Nephrin/WT/WT mice was normal at both one and six weeks of age as analyzed by light microscope after hematoxyllin-eosin staining. The kidneys of one-week-old Nephrin/KO/KO mice didn't show any major morphological differences compared to Nephrin/WT/WT littermates. However, the kidneys of six-weeks-old Nephrin/KO/KO mice showed striking morphological differences compared to age matched Nephrin/WT/WT littermates. The abnormalities in the kidney include a remarkable increase in glomerular extracellular matrix and sclerosis, abundance of partly collapsed glomeruli together with cystic lesions filled with proteinaceous material and tubular dilatations (Orig. II, Fig. 2).

In electron microscopy analysis, varying levels of podocyte damage was seen in both the one-week-old and six-weeks-old Nephrin/KO/KO mice (Orig. II, Fig. 3). This is in contrast to the Nephrin/WT/WT littermate controls that showed normal podocyte structure. Podocyte abnormalities seen in the Nephrin/KO/KO mice in both ages, usually consisted of a distinct loss or fusion of foot processes. The six-weeks-old Nephrin/KO/KO mice glomeruli also showed

glomerulosclerosis, mesangial expansion and glomerular basement membrane thickening. SDs were still partly existing in Nephrin/KO/KO mice in both analyzed ages despite the foot process effacement, although these were completely missing in conventional nephrin KO newborns [6].

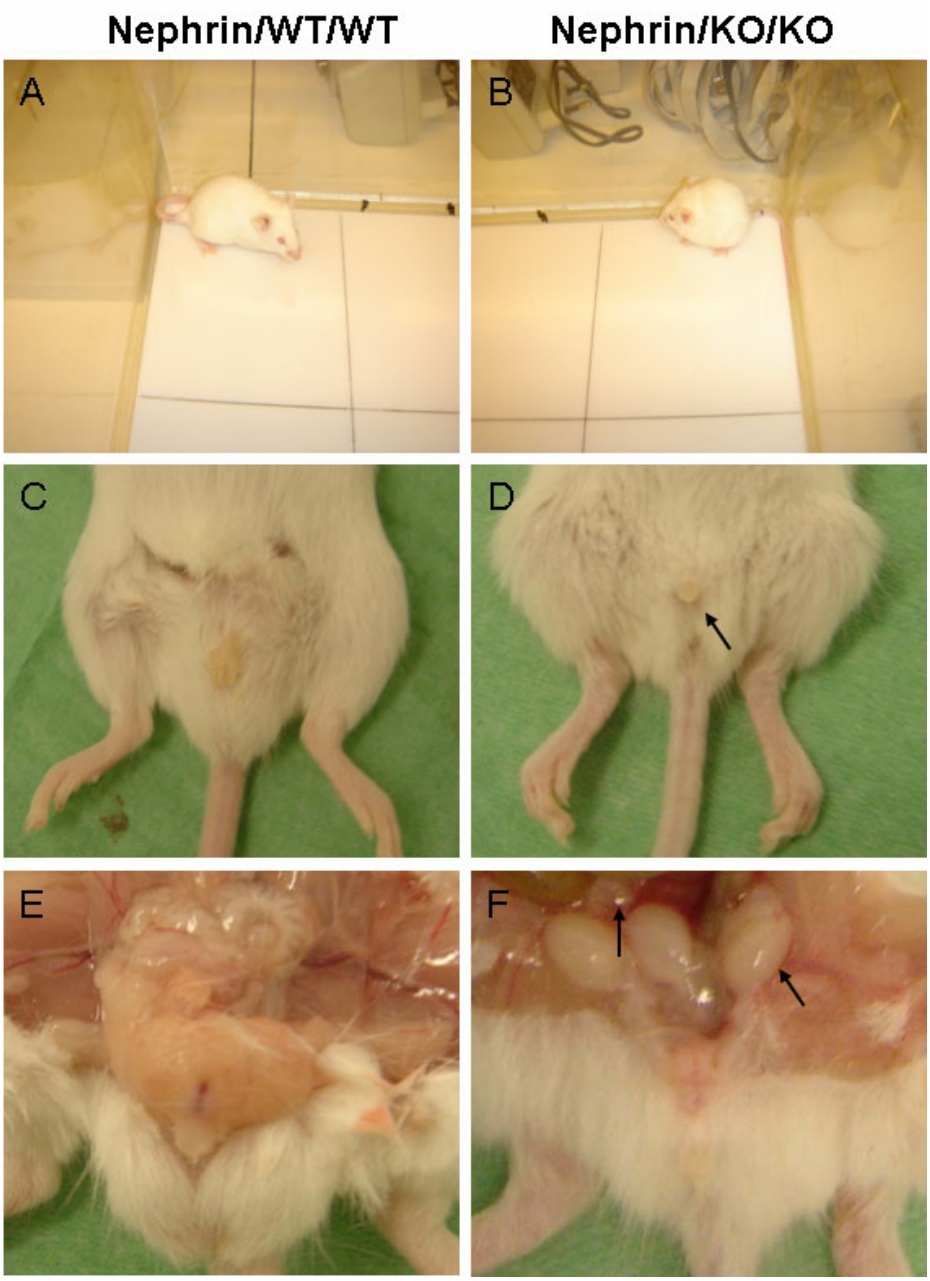

Figure 9. *Endogenous nephrin deficiency causes genital track malformations in Nephrin/KO/KO mouse: Left panel: Nephrin/WT/WT mouse; Right panel: Nephrin/KO/KO mouse. Panels **A** and **B** show the differences in body size; Panels **C** and **D** show the smaller size of the male genitals of Nephrin/KO/KO males and undeveloped scrotum; Panels **E** and **F** show the abnormalities in the male reproductive tract development in Nephrin KO/KO mice. Testicles of these mice were not descended and the formation of seminal vesicle and prostate were impaired.*

All Nephrin/KO/KO males were infertile and their genitals showed anatomical and developmental malformations. Notably, their penis size was decreased, testicles had not descended and seminal vesicle formation was severely impaired (Figure 9 C to F). Histological changes were analyzed from sites of previously reported nephrin expression. The qualitative changes found included, a decreased number of Leydig cells and spermatids in the testis, absence of the lumen of the seminiferous tubules in the testis, a loss of elongated shape the Purkinje cells in the cerebellum and an increase in cell number and density in the islets of Langerhans in the pancreas (Figure 10). No histological differences between Nephrin/WT/WT and Nephrin/WT/KO mice in observed tissues were found. This indicates that one functional nephrin allele is enough to produce a relatively normal phenotype.

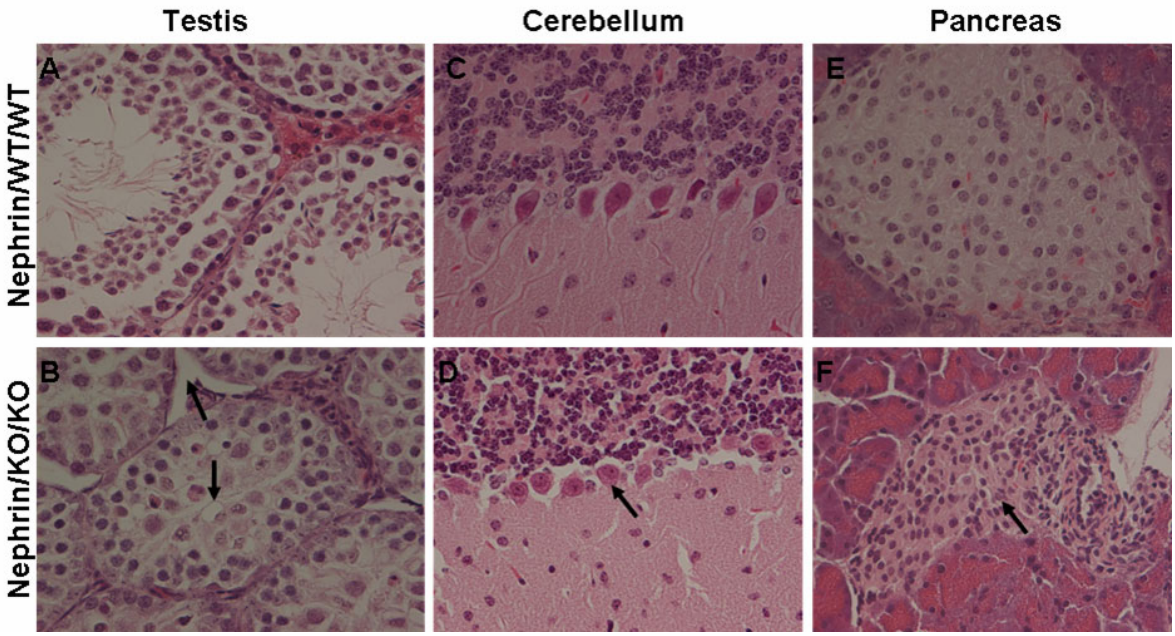

Figure 10. *Histology in JRC-Nephrin x KO mouse tissues. Left panel: Nephrin/WT/WT mouse; Right panel: Nephrin/KO/KO mouse. Histological changes found in sites of previously reported nephrin expression included:* ***A, B****) A decreased number of Leydig cells and spermatids in the testis and absence of the lumen of the semiferous tubules.* ***C, D****) A loss of elongated shape of the Purkinje cells in the cerebellum.* ***E, F****) An increase in cell number and density in the islets of Langerhans. No difference between Nephrin/WT/WT and Nephrin/WT/KO genotypes was found. Magnification: x200.*

3.3. The nephrin transgene is highly expressed at mRNA and protein level in Nephrin/KO/KO mice

To ensure that progressing ESRD in Nephrin/KO/KO mice is not due to partial transgenic rat nephrin expression the following studies were performed. The expression of the transgenic rat nephrin mRNA in the kidneys was similar in all genotypes at the age of one and six weeks as determined by RT-PCR. The expression of endogenous mouse nephrin mRNA was totally absent in Nephrin/KO/KO littermate kidneys as expected, while in all other genotypes it was indistinguishable (Orig. II, Fig. 4A).

Immunofluorescence microscopy showed that nephrin was expressed similarly in all glomeruli of all genotypes at the age of one week (Orig. II, Fig. 4C). Also nephrin protein localization and expression levels remained normal in six-weeks-old Nephrin/KO/KO mice compared to other genotypes (Orig. II, Fig. 4D). This indicates sufficient complementation of the transgenic nephrin protein. Surprisingly immunoblotting results show an increased level of the phosphorylated nephrin form (~180 kDa) in six-weeks-old Nephrin/KO/KO mice compared to other genotypes (Orig. II, Fig. 4B). On SDS-PAGE rat nephrin runs as a doublet, presumable because of differential glycosylations, and phosphorylated nephrin consistently corresponded to the upper band of size ~180 kDa [148].

3.4. Podocyte specific rescue of nephrin alters mRNA and protein expression profiles in the kidney

In order to investigate whether the morphological abnormalities found in the kidney, were related to changes in other podocyte-specific mRNA and protein expression patterns, other analyses were performed. Many nephrin-associated proteins mRNA and protein expression levels were analyzed including podocin, synaptopodin, CD2AP, FAT1, Fyn, Nck1 and Nck2. Both subtypes of Nck protein were selected for analysis, because of their unknown distribution and role in podocytes.

Results by RT-PCR showed that expression of podocin and Nck2 mRNA levels were decreased in six-weeks-old Nephrin/KO/KO mice compared to other

genotypes and one-week-old offsprings. Semi-quantification showed a decrease in podocin (52 ± 5%) and in Nck2 (43 ± 4%) compared to Nephrin/WT/WT controls (Orig. II, Fig. 5). Other significant alterations in mRNA expression levels of the measured genes were not found.

Selected proteins expression and localization was analyzed in the kidney cortex using double stainings with synaptopodin. Immunofluorescence stainings with podocin, Fyn, Nck1 and Nck2 antibodies in one-week-old mice didn't reveal any appreciated differences in protein expression and localization between the genotypes. However, in six-weeks-old Nephrin/KO/KO mice several abnormalities in the protein localization were found. Double stainings with synaptopodin showed marked decrease in podocin protein in Nephrin/KO/KO mice podocytes compared to Nephrin/WT/WT controls. In addition, there was also a decreased in podocin mRNA expression level as described earlier. Fyn kinase expression and localization was also changed in Nephrin/KO/KO mice. Compared to Nephrin/WT/WT mice Fyn protein levels were increased specifically near to the podocyte foot processes and SD area. These changes may be related to the previously noticed increase in nephrin phosphorylation in six-weeks-old Nephrin/KO/KO mice (Orig. II, Fig. 6).

Also in the Nck family protein subtype characterization differences between genotypes were found (Orig. II, Fig. 7). Nck1 was found to be expressed in tubules, but some staining was found in the glomeruli as well. However Nck1 did not co-localize with synaptopodin and was noticed more in the mesangial parts of the glomerulus. No apparent differences between the genotypes were found. Instead of Nck1 expression, Nck2 subtype staining was detected to co-localize with synaptopodin indicating its expression specifically in podocytes. However some tubular expression was also noticed, but it was much less in comparison to the Nck1 staining. Interestingly, Nck2 expression in podocytes was totally absent in Nephrin/KO/KO mice. Taken together, these findings may indicate that Nephrin/KO/KO mice develop a kidney disease before adulthood. This may result from the alteration of expression in the proteins that are responsible for nephrin tyrosine phosphorylation and intracellular signaling.

4. Expression of SD proteins in extra-renal tissues (III)

4.1. Expression of densin

In the original publication III, densin expression in the testis and brain were evaluated based on previous reports on other podocyte specific molecules. RT-PCR with densin-specific primers showed mRNA expression in human and mouse testicular tissues (Orig. III, Fig. 1). In human brain cortex and in mouse brain cortex and cerebellum, densin mRNA expression was detected, this is in agreement with previous observations of rat brain tissues [78] and confirms inter-species close conservation in the brain. These results were verified by sequencing the amplified PCR products.

Positive staining for densin protein was also detected in lysates of human and mouse testis by immunoblotting (Orig. III, Fig. 2). The detected size in the testis (210 kDa) of densin was similar to that detected in human kidney glomerular lysates [79] and in the mouse brain (180 kDa) similar to the originally detected in the rat forebrain [78].

4.2. Localization of densin and other SD components in the testis

Expression of densin in mouse testis was clearly localized in the cell membranes of stained cells (Orig. III, Fig. 3). The localization of densin-positive cells close to the basement membrane of the seminiferous tubules suggested them to be Sertoli cells. This was confirmed by the distinct Sertoli cell-type nucleus appearance of the stained cells as observed with DAPI staining. Identification of Sertoli cells with antibody to WT1 [191] in serial testicular sections confirmed that the cells stained for densin are indeed Sertoli cells.

Immunofluorescense staining for β-catenin and cadherins showed that these molecules are predominantly localized in the cell membranes of Sertoli cells in the mouse testis (Orig. III, Fig. 4). This is consistent with earlier reports on the expression of β-catenin and the members of the cadherin family, such as N-cadherin, in the adherens junctions between Sertoli cells and germ cells [286]. Some similarity between the staining patterns of β-catenin and cadherins and

densin was detected, but compared to stainings for β-catenin and cadherins, densin expression was more tightly restricted to Sertoli cells.

Double staining experiments using a monoclonal β-catenin antibody evaluated potential overlapping antigen distribution of densin and β-catenin. While β-catenin was detected in the cell membranes of a number of testicular cell-types, double staining with β-catenin and densin showed partial co-localization in cell membranes of a particular cell population, most likely Sertoli cells. The specific staining pattern suggests an expression of densin in the cell-cell junctions between Sertoli cells and germ cells.

In contrast to the expression patterns of densin, β-catenin and cadherin, the expression of ZO-1 was found to be localized in the basal compartment of the seminiferous epithelium at the site of the blood-testis barrier. This finding is in agreement with earlier detailed studies on ZO-1 expression in rat and mouse testis [180]. The staining pattern of densin in comparison with ZO-1 does not exclude the possibility of densin being expressed in the blood-testis barrier, although densin does not appear to be restricted to the blood-testis barrier.

5. Long hairpin RNAi construct results in unspecific gene silencing in vitro (Unpublished data)

The purpose of this part of the study was to generate RNAi constructs with a long hairpin structure of dsRNA that allows specific, long-term densin and filtrin gene silencing *in vitro and in vivo*. A pDECAP vector was used to clone in the DNA from three different fragments of the densin gene and two different fragments of the filtrin gene. To test the downregulation efficacy of the RNAi construct, the endogenous levels of densin and filtrin mRNA and protein were analyzed in mouse insulinoma cells (MIN6) and in mouse kidney cortex collecting duct cells (M-1). However the expression levels of both gene's mRNA and protein were below the detection limit as measured by RT-PCR and immunoblotting.

To measure the silencing in an artificial manner, a modified version of a previously reported luciferase-based assay was used [281]. The luciferase-based assay was selected because of it's high sensitivity and the fact that it had been

used in the original pDECAP article [281]. The mouse densin or filtrin full-length cDNA was cloned into the front of the Renilla luciferase reporter gene of the psiCHECK-1 vector. The modified psiCHECK-1 vectors were then co-transfected to A293 cells with the RNAi constructs and the control plasmid pGLBasic/SV40P with the Firefly luciferase. In the nucleus of the A293 cells, the RNAi constructs will be cleaved to siRNA molecules that are targeted against filtrin or densin cloned psiCHECK-1 vector. This will silence Renilla luciferase activity that is detected with a luminometer.

Table 7: *Down-regulation efficiency of RNAi constructs to filtrin and densin mRNA levels measured with a luminometer assay.*

RNAi/ psiCHECK ratio *Incubation time*	1:1 24 h	1:1 46 h	1:2,5 46 h	1:5 46 h
psiCHECK / Filtrin				
pDECAP	1	1	1	1
pDECAP / FILT I	0,61	0,6	0,69	0,91
pDECAP / FILT II	0,62	0,76	-	-
pDECAP / DENS I	0,66	0,49	-	-
pDECAP / DENS II	0,61	1,13	0,96	1,08
pDECAP / DENS III	0,70	0,88	-	-

psiCHECK / Densin				
pDECAP	1	-	1	1
pDECAP / FILT I	0,71	-	0,65	0,78
pDECAP / FILT II	0,76	-	-	-
pDECAP / DENS I	0,72	-	-	-
pDECAP / DENS II	0,60	-	1,34	1,49
pDECAP / DENS III	0,94	-	-	-

Table 7 summarizes the fold changes in RLU by two filtrin constructs and three different densin constructs compared to an empty pDECAP control. Different transfection ratios of the RNAi construct and the psiCHECK-1 vector at different incubation times were tested. The most reliable downregulation efficiency was obtained with a 1:1 RNAi/psiCHECK ratio and a 24 h incubation time. Figure 11 shows the effect of the RNAi constructs to down-regulate the psiCHECK reporter vector activity with a mouse filtrin (A) or densin (B) cDNA. These results indicate that expression of long filtrin and densin ds-RNA using the pDECAP

vector degrades significantly at about ~30-40% of the mRNA expression of both genes. However no speficity for filtrin or densin was observed (Figure 11).

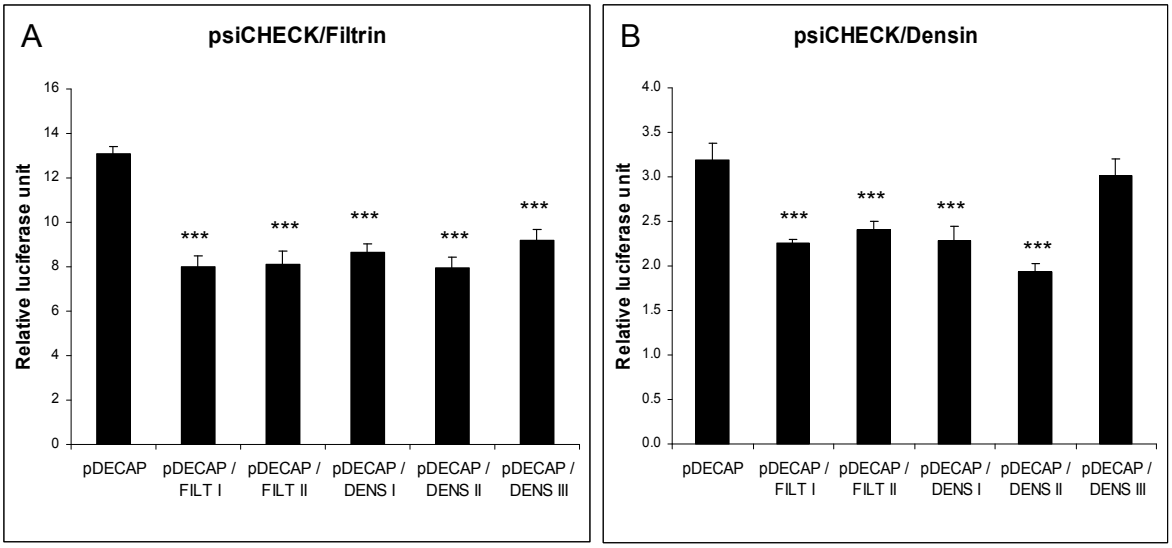

Figure 11. *The effect of RNAi constructs to down-regulate either the psiCHECK reporter vector with a mouse **A**) filtrin or **B**) densin cDNA. The RNAi contructs was co-transfected with psiCHECK reporter plasmids into A293 cells. After 24 hours, the cells were harvested and the luciferase activities measured. The activities produced by the studied constructs were normalized against the co-transfected control plasmid (pGLBasic/SV40P) activities. The RLU values represent means ± SD of three individual samples. *** The difference to the control with empty expression vector (pDECAP) is statistically significant P < 0.001.*

Discussion

The aim of this thesis was to provide and test alternative approaches to circumvent general problems in conventional gene targeting. Mouse models allowing gene manipulation in a specific tissue and cell-type of the adult mouse are expected to provide more reliable functional test platforms for diagnostic and therapeutics in human diseases compared to the classical ubiquitous gene KO mouse-lines. In this thesis, several novel mouse-lines were generated by using recent technological advances in the field. All mouse-lines that were developed and their proposed future use are summarized in Table 8.

Table 8: *Summary mouse-lines generated in this thesis project.*

Approach	Mouse-line	Purpose	Remarks and Future Perspectives
Transgenic	JRC-CRE	Inducible podocyte-specific gene deletion.	Cross-breedings with selected floxed-lines.
Cross-Breeding	JRC-CRE x Z/AP reporter-line	To test Cre recombinase functionality.	Showed podocyte-specific, doxycycline dependent Cre activity.
Transgenic	JRC-Nephrin	Inducible podocyte-specific rat nephrin over-expression.	1) Cross-breedings with nephrin KO mice. 2) Disease-induction studies.
Cross-Breeding	JRC-Nephrin x Nephrin KO	To study if inducible transgene expression of rat nephrin only in podocytes can rescue nephrin KO mice from perinatal lethality.	Rescued nephrin KO mice, but caused severe tissue phenotypes need to be further characterized.
RNAi	-	To down-regulate filtrin and densin expression *in vitro* and *in vivo.*	Unspecific down-regulation *in vitro*. No *in vivo* models.

1. General problems in traditional gene targeting

Even though conventional targeting has provided wide insight into the biological functions of many genes, several disadvantages still exist. Conventional KO mouse models targeted to podocyte key-molecules lacked time- and tissue-specific regulation and often cause early postnatal death (See Table 2, Page 48). Early disturbance of gene function in ES cells often results in embryonic or perinatal lethality. Due to perinatal lethality, conventional KO mouse models of genes expressed in the kidney often fail to reveal a detailed biological function of these genes in adulthood. Many genes are also expressed in multiple cell types and organs, and the resulting KO phenotypes can be complex and difficult to study. In addition, early embryonic lethality caused by gene deletion in a specific organ can fail to reveal a detailed biological function of the gene within in other organs. Therefore, an ideal tool to manipulate gene function would permit tight control of expression in a spatial and temporal manner, which is a challenge in genetic engineering today.

Despite some promising results that show regulation of gene deletion in a spatial manner using the Cre-loxP system, there are still several aspects that need to be considered. Firstly, the degree of Cre-mediated excision during embryonic development is critical as incomplete excision might lead to unexpected results. Secondly, the exact timing of Cre-mediated excision during embryonic development of the tissue or cell of interest is also important and if a gene is expressed prior to the time of excision, a less severe phenotype may develop. Thirdly, the tissue-specific promoter driving Cre recombinase expression may restore cell-specific transcription factors itself, and cause the phenotype. Fourthly, Cre expression might be toxic to the mammalian cells [287, 288]. Toxicity might be caused by Cre-mediated recombination between endogenous sequences that might be targets for Cre [289]. Finally, even with spatial expression, embryonic gene deletion by the Cre/lox system often causes a lethal phenotype. To avoid this, the Cre/lox system requires not only spatial expression but also temporal control of gene expression.

2. Novel inducible podocyte-specific mouse-lines (I and II)

2.1. Novel construct for inducible, cell-specific and single-step gene targeting

The conventional KO or over-expressing mouse models often cause an early perinatal death or complicated phenotype, because of the resulting embryonic expression of the transgene. In kidney research, there is an increased need for cost-effective gene constructs that allows time- and tissue-specific regulation of transgene expression in adult mice. A novel approach, based on a single cell-type specific doxycycline inducible construct for *in vitro* studies, and for the generation of a transgenic mouse-lines is described in the original publications I and II.

Several advantages are gained when using a single inducible transgene vector that confers site-specific expression after doxycycline induction. First, the cell-type specificity can be changed by a single cloning step replacement of one cell-specific promoter for another. Second, any gene of interest can be over-expressed in an inducible and cell-type specific manner by replacing the Cre recombinase gene, with the cDNA of the selected gene. Third, the single construct gives superior speed and economy *in vitro* and *in vivo*, since no multiple rounds of cell transfection or oocyte injections and no further breeding steps are needed. Fourth, the transgene expression can be turned on or off as desired, giving invaluable targeting, e.g. to a particular stage of embryonic development or in adult life thus overcoming the limitations of embryonic lethality or redundancy. Fifth, oral doxycycline administration is easier to perform in mice compared to the intra-peritoneal administration of tamoxifen and it generates less side effects. Sixth, doxycycline penetrates the placenta and expression can be triggered during embryonic development if necessary.

Some disadvantages of doxycycline inducible strategies have also been reported. A major problem of the Tet-on system is the possible leakiness in un-induced animals or in other tissues. In the recent improved rtTA versions, such as the one used in this study (M2), a far lower tetracycline concentration for the induction and thus decreased leakiness occurs [257]. Furthermore, it is impossible to estimate the transgene integration-site and the copy-number

resulting from the oocyte injection procedure. Additionally, when the cDNA is used instead of the genomic sequence, normal post-transcriptional modifications such as alternative splicing may not take place due to lack of introns in the sequence.

2.2. Doxycycline inducible podocyte-specific KO strategy

Site specific recombinases (e.g. Cre-loxP system) are excellent tools to establish tissue-specific gene deletions in the mouse [233]. The cell-type specificity depends on the regulation of the tissue-specific promoters that drive the expression of the Cre recombinase gene. A drawback of these models is that Cre recombinase is expressed during embryonic development and the phenotype after gene silencing during embryogenesis, might resemble the phenotype of a conventional mouse-line. Thus, both an inducible and a cell-type specific Cre recombinase regulation will be needed to silence genes expressed in the adult animal.

The original publication I, describes a transgenic mouse-line with tightly regulated inducible expression of Cre recombinase exclusively in the kidney podocytes in the adult mouse. This viable and fertile transgenic mouse-line was generated by injection of oocytes with a single DNA construct. This construct contains a podocin promoter that drives the expression of rtTA and a Cre recombinase gene whose expression is initiated by rtTA in the presence of doxycycline. Sixteen out of 122 offspring carried the transgene following oocyte injection. This is a relatively high integration percent, even though the modified construct used was relatively large (~11 kb). All founders were fertile and transmitted the transgene to their F1 offspring, enabling further studies and breedings. Three founder-lines out of sixteen showed Cre recombinase expression in podocytes also without doxycycline administration. Two founder-lines also showed Cre activity in the heart tissue after two weeks of doxycycline administration. This notable leaking only in these founder-lines may be a consequence of random integration sites in the genome, number of transgene copies integrated to the genome or a “partial” leakiness of the construct in heart tissue. For further studies, one founder-line was selected on the basis of its high Cre recombinase expression level restricted to podocytes only after doxycycline treatment.

The successful generation of the novel doxycycline inducible Cre recombinase mouse-line will be extremely useful for conditional deletion of essential podocyte proteins in the adult mice. This is a unique model for the further identification of specific gene functions in physiological and pathological conditions *in vivo*. Furthermore, targeted gene deletion in adult mice provides an excellent tool for pharmacologic testing platforms.

2.3. Doxycycline inducible podocyte-specific over-expression strategy

Similar to conventional silencing models, over-expression of specific genes during embryogenesis might lead to a complicated phenotype, because of failings in the exact timing or placing of transgenic expression. The above mentioned single, binary Tet-on construct can also be used in over-expressing particular podocyte-specific proteins in the adult mouse kidney. In the original publication II, a transgenic mouse-line with a podocyte specific, doxycycline inducible expression of trangenic rat nephrin was generated.

Following injection of the construct into oocytes 10 out of 90 pups were found to carry the transgene, which is a relative high integration percentage. Eight out of ten founders transmitted the transgene to their offspring. All founders and their F1 offspring were fertile and their phenotypes were normal. Also the rat nephrin expression was firmly regulated by the administration of doxycycline and observed only in the podocytes. Interestingly, nephrin over-expression in the podocytes did not alter the expression or localization of the other studied proteins.

These results indicate that the inducible construct used is suitable for targeted gene over-expression in the adult mouse and in the various tissues by changing tissue-specific promoters. This novel doxycycline inducible rat nephrin mouse-line will be used to establish, whether nephrin over-expression can protect against proteinuria in ligand induced podocyte injury models, including puromycin or adriamycin aminonucleocide treatments.

2.4. Doxycycline inducible podocyte-specific rescue strategy

In the original publication II, the JRC-Nephrin mouse-line was crossed with a nephrin deficient mouse-line [6] to investigate, whether podocyte-specific

transgene expression of rat nephrin can rescue nephrin KO mice from perinatal lethality.

Cross-breeding of F1 offspring, which carried transgenic rat nephrin expression with heterozygote nephrin KO allele, in the presence of doxycycline resulted in the expected number of 14 out of 72 (19%) F2 littermates. Six of these pups (five males and one female), which lacked endogenous nephrin but carried and expressed the transgenic rat nephrin survived at least six weeks after birth. This result shows that nephrin over-expression merely in the podocytes can rescue nephrin deficient mice from perinatal death. In addition, this study suggests that the lethal phenotype due to nephrin deficiency is mainly caused by loss of kidney filtration function.

All rescued male mice were smaller, infertile and showed impaired locomotor activity and genital malformations (See Figure 9, Page 73). Histological analysis revealed distinct abnormalities in the kidney, testis, cerebellum and pancreas that are reported to express nephrin in WT mice (See Figure 10, Page 74). This mouse model can be used to study nephrin associated complexes in the podocytes after transgenic expression of nephrin originated from other species. Most importantly, this mouse-line allows precise characterization of the extra-renal tissues after embryonic nephrin loss.

3. New insights into the role of nephrin in the podocytes by using inducible mouse-line (II)

All rescued Nephrin/KO/KO mice were smaller in body weight and showed severe proteinuria at the age of three weeks. Interestingly, females seemed to be more sensitive for early death caused by kidney dysfunction and only one survived until six weeks of age. The kidneys from six-weeks-old mice were analyzed by light microscopy and showed distinct abnormalities including an increase in extracellular matrix, focal sclerosis, partly collapsed glomeruli with cystic lesions filled with proteinaceous material and dilatations in the tubular profiles. These kidney abnormalities were not observed in one-week-old mice and in other genotypes, which indicates that morphological changes occurred due to endogenous nephrin loss. However, electron microscopy revealed malformations

in the podocyte fine structures in Nephrin/KO/KO mice even at both studied ages. These mice had distinct foot process effacement but still existing morphologically normal SDs. Interestingly, SDs were not found in nephrin deficient newborn pups [6]. Although Nephrin/KO/KO mice still had existing SDs, and foot process effacement was only partial. The obtained phenotype of these mice resembles the findings from podocyte-specific Nck 2 KO in Nck1 null mice [9].

There are several possible explanations why the podocyte specific expression of rat nephrin was not able to restore kidney function completely. Perhaps, insufficient rat nephrin expression timing or localization at the protein level occurred. At present it is difficult to experimentally determine this, as critical cell level protein expression differences are beyond the ability of current methods. However, our results showed that transgenic rat nephrin expression level and localization appeared similar to endogenous nephrin expression. Nephrin antibody staining was also present in all glomeruli in one- and six-weeks-old Nephrin/KO/KO mice compared to other genotypes, which suggests sufficient complementation in protein expression level.

Differences between the mouse and rat nephrin gene and protein sequence remains a possible reason to explain partial complementation. Rat nephrin cDNA was chosen as a transgene because it shares homology with mouse nephrin, but has minute differences in the coding sequence, enabling distinction from mouse endogenous nephrin by RT-PCR *in vivo*. However, rat nephrin in comparison to mouse nephrin has different tyrosine-based motifs (YDEV) in the cytoplasmic tail. This had not been published at the time rat nephrin was selected as the transgene. These conserved regions have recently been shown to be important for the binding of Nck protein SH2 domain when phosphorylated with Src-family kinases, e.g. Fyn [8, 9]. Interestingly, one of these two tyrosine motifs that are important in Nck binding in human (1176-YDEV) and mouse (1191-YDEV), is not conserved in rat (1187-HDEV) [8, 9, 59].

There is an increasing amount of literature of nephrin phosphorylation and its role in the maintenance and repair of podocyte foot processes and SD (reviewed in Tryggvason et al. 2006). These functions are summarized in Figure 12. With these findings in mind further evaluations of the significance of different

podocyte-specific proteins, which participate in nephrin phosphorylation and intracellular signaling in rescued mice, were performed. Indeed, changes in podocin, Fyn and Nck2 protein expression and localization in Nephrin/KO/KO mice at six weeks of age were found, but not observed in one-week-old Nephrin/KO/KO pups. These findings indicated altered intracellular signaling in the podocytes after birth. Futhermore, mRNA expression of podocin and Nck2 was attenuated in six-weeks-old Nephrin/KO/KO mice. These results showed that Nck2 protein in the mouse is more prominent in podocytes compared to Nck1 localization. Interestingly, Nck2 expression in podocytes was totally absent only in six-weeks-old Nephrin/KO/KO mice, while expression and localization of these proteins were still normal in six-weeks-old littermate controls and one-week-old Nephrin/KO/KO mice kidneys.

These findings indicate that the altered tyrosine residue site in the rat nephrin sequence may not be capable of binding to the mouse Nck2 protein and thus, mediate its regulatory function to the actin cytoskeleton. Changes in Fyn protein expression and localization in six-weeks-old Nephrin/KO/KO mice also occurred. This altered Fyn expression may be related to the increase in nephrin phosphorylation and may occur in order to compensate for the lack of Nck2 binding. Nephrin-Nck interaction after Fyn phosphorylation has been shown to be important in podocyte foot process development and repair after injury [9, 149]. These changes in the expression pattern of key signaling molecules in Nephrin/KO/KO mice are associated with the onset and continuous increase of proteinuria after three and up to six weeks of age. Interestingly, these findings also resembles the phenotype in mice with podocyte-specific Nck deficiency [9].

In conclusion the extracellular domain of rat nephrin protein appears to be efficient in forming the functional SD structure in the rescued nephrin deficient mice, as shown by electron microscopy. Furthermore, these results indicate that nephrin may have a bigenic role in the podocytes, both in the formation of functional filtration slit during development and also in maintaining foot process integrity during adulthood, via Nck2 signaling. However, this remains to be elucidated in greater detail.

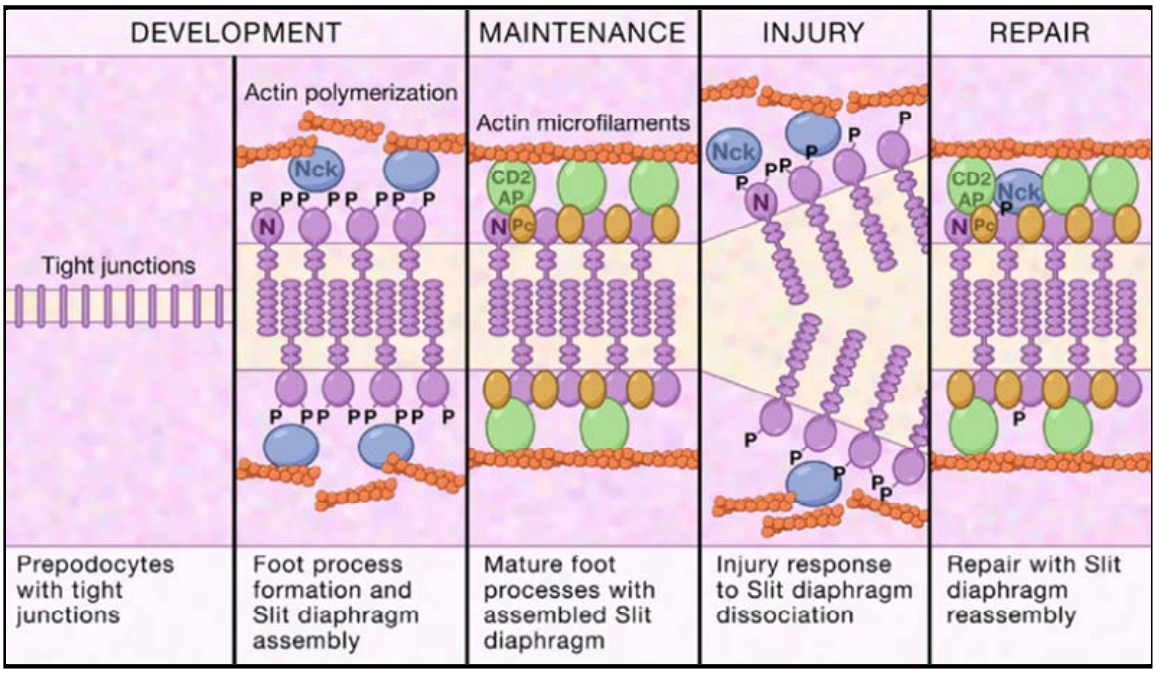

Figure 12. *Nephrin phosphorylation and its role in the maintenance and repair of podocyte foot processes and SD. Prior to formation of the foot processes and the slit diaphragm, prepodocytes covering the glomerular capillaries are connected by tight junctions. During formation of the foot processes, nephrin (N) becomes phosphorylated (P), possibly upon encountering its extracellular ligand, which results in recruitment of Nck adaptor proteins and induction of actin polymerization. Once the slit diaphragm is assembled, the nephrin molecules are dephosphorylated and nephrin is connected to actin through CD2AP, podocin (Pc), and possibly some other proteins. Following injury of the slit diaphragm leading to foot-process effacement and proteinuria, nephrin molecules become clustered, which induces their phosphorylation, Nck association, and actin polymerization. Finally, upon injury repair, the nephrin molecules are dephosphorylated and the slit diaphragm-actin filament complex is restored to the normal mature foot processes. This figure is reprinted with permission from reference 146.*

4. SD components role in extra-renal tissues (II and III)

4.1. Testis

4.1.1. Nephrin deficiency causes abnormalities in testis development

In the original publication II, all rescued nephrin deficient male mice were infertile and compared to WT littermates their genitals showed gross anatomical malformations; penises were smaller, testicles had not descended, seminal vesicle formation was severely impaired, a decreased number of Leydig cells and

a few morphologically normal spermatids in the testis were observed (See Figures 9 and 10, Pages 73 and 74). Remarkably, these findings show strong similarities to the data of the *in vivo* complementation experiments with another protein crucial for podocyte function, CD2AP [117]. Podocyte-specific expression of CD2AP cDNA, allowed rescuing of CD2AP KO mice from post-natal lethality by restoring renal function. This also uncovered a hidden phenotype characterized by infertility and testicular abnormalities [117].

Interestingly structural changes were observed in six-weeks-old Nephrin/KO/KO mouse testicles in structures, which have been shown to originate from the mesonephros during the genitoureteric development. These changes include incorrect structure of seminiferous cords [168, 169] and absence of Leydig cells [172]. During development, extensive cell migration between the mesonephros and the developing testis occurs [167]. Furthermore, the expression of nephrin protein and its regulatory protein WT1, have been shown to be detectable for the first time during the mesonephros stage of development [4, 5, 59, 61, 176]. Recently, the role of another Ig-family protein, filtrin has been shown to be involved in the migration of early postmitotic neural precursors during mouse CNS development [206]. Based on the results of this study, nephrin protein may have a role during the organogenesis of testis and mesonephric cell migration. However these speculations will need further verification.

Currently treatment with early nephrectomy and subsequent dialysis followed by renal transplantation is used to treat CNF patients lacking nephrin in their kidneys [44]. This treatment perhaps together with the immune suppressants act to prevent rejection of the transplanted kidney, and this treatment appears to remove all symptoms with no confirmed later manifestations in other organs [45]. Interestingly, two male CNF patients showed decreased serum levels of inhibin B [65] thus suggesting arrested spermatogenesis [290]. The first successfully treated CNF patients are only now entering their reproductive years. Thus, it is still premature to speculate on possible consequences of nephrin deficiency for fertility in humans.

4.1.2. Densin is also expressed in the testis

The original publication III, reports densin as a novel testicular protein in addition to its expression in the kidney and brain [78, 79]. Firstly RT-PCR analysis showed the presence of densin mRNA in human and mouse testis. Secondly, western blotting confirmed the expression of the densin protein (approx. 210 kDa) in the testis, this same size has also been reported in the kidney [79]. Thirdly, immunofluorescence staining revealed that the densin protein is specifically localized to the cell membranes of Sertoli cells. In consideration to the known/anticipated function of densin in the brain and the kidney it is suggested here, that densin may function as an adherens junction protein between the Sertoli cell and the developing germ cells.

Densin expression was specifically confined to the cell membranes of Sertoli cells, with an expression pattern resembling that of cadherins and β-catenin. Members of the catenin and cadherin families are ubiquitously expressed proteins which form structural and signaling complexes in numerous organs. In the testis, there is accumulating knowledge of the important functional role of the catenin/cadherin complexes particularly in the adherens junctions between Sertoli cells and germ cells, and thus play a role in spermatogenesis [193, 286, 291, 292].These findings may indicate a potential structural/functional interaction of densin with the catenin/cadherin complex in Sertoli cells. In the brain, densin associates with N-cadherin via neural specific catenin, δ-catenin/neural plakophilin-related armadillo repeat protein [88]. Furthermore recent data from the kidney, show that there is an interaction between densin and ß-catenin in the podocytes [91]. Therefore, it is hypothesized that densin may also interact with the catenin/cadherin complexes in the adherens junctions of the testis, however this needs further study. The testis as an extracorporeal paired organ may become an important and feasible site to test the functional relevance of the nephrin complex of proteins.

4.1.3. Hypothetical role of densin in the testis

As stated above, the functional role of densin as a part of nephrin complex of proteins in the testis remains to be clarified. In the brain, there is evidence that densin is involved in establishing cell polarity, assembling signaling complexes in

the postsynaptic membranes at the glutamatergic synapses and in establishment of cell-cell contacts [88, 89]. The role of densin also in the kidney remains to be fully characterized, but preliminary results suggest that densin interacts with several components of the podocyte filtration SD either directly or indirectly and thus is associated with the assembly of SD protein complexes. Collectively, the previous knowledge of the role and functions of densin in non-testicular tissues suggests that densin is an adhesion molecule, important in establishing cell-cell contacts and controlling signaling between Sertoli cells and developing germ cells.

The precise generic functions of junction complexes apparently shared in various tissues remains to be clarified. Meanwhile testis may present itself as one of the most easily accessible tissues, to gain information on the structure and the precise functions of these complexes, as has been suggested earlier [293]. Consequently, advances in the understanding of functions in one tissue may offer valuable clues to understanding other tissues, especially those which share structural similarities.

Spermatogenesis in the seminiferous epithelium of the mammalian testis is a dynamic cellular event, in which Sertoli-germ cell actin-based adherens junctions appear to be involved. The restructuring at the Sertoli-germ cell interface, permits germ cells to traverse the epithelium from basal to adluminal compartment [193]. The current knowledge of the regulation of adherens junction dynamics by interactions between adherens junction integral membrane proteins, phosphatases, kinases, adaptors, and the underlying cytoskeleton network has previously been described [193].

Interference of Sertoli-germ cell interactions in the testis, has been shown to affect germ cell movement within the epithelium [193, 291, 294]. Therefore manipulation of Sertoli-germ cell adherens junctions has been identified as a potential target for a male contraceptive pill. This has led to calls to identify and characterize novel proteins at the various junction types in the testis [294]. Interestingly it has recently been shown (with an intratesticular androgen suppression-induced germ cell loss model) that disruption of adherens junctions, can be limited to the Sertoli-germ cell interface without perturbing the blood-testis

barrier [295]. The identification of densin in the present study has increased our knowledge of the molecular composition of the testicular junctions. Identification of the exact junction types that densin is involved with, as well as deciphering the exact assembly of the densin-linked junctions is warranted. Further work on the functional role of densin is also needed to reveal whether densin has a role in spermatogenesis and as a target for male contraceptives.

4.2. CNS

In the original article II, rescued nephrin deficient mice showed a disarray of the Purkinje cells in the cerebellum (See Figure 10, Page 74). The data from the primary behavioral screening of the rescued mice indicate severe motor defects (See Table 5, Page 71). Interestingly neurological symptoms are frequently found in the nephrectomized, transplanted and treated CNF patients [66]. These findings suggest a role for nephrin in regulating locomotor activity in mice, while more detailed characterization at a cellular and molecular level will be needed to substantiate this hypothesis in humans.

4.3. Pancreas

In the original article II, rescued nephrin deficient mice also showed a strong increase in the number of Islets of Langerhans in the pancreas and decreased glucose levels in the blood (See Figure 10 and Table 6, Pages 74 and 72). The blood glucose levels were relatively high in Nephrin/WT/WT (13.2 mmol/l) and Nephrin/WT/KO (10.3 mmol/l) mice compared to normal glucose levels in mice. However, the blood glucose levels measured in one-week-old Nephrin/WT/WT (5.8 mmol/l) and Nephrin/WT/KO (6.3 mmol/l) pups were normal (Data not shown). This finding indicates that elevated blood glucose levels in six-weeks-old controls may be due to the administration of 5% sucrose to the drinking water after birth. These findings may also indicate changes in pancreas function because of pancreatic nephrin loss during embryogenesis, and the resulting changes in glucose homeostasis. However, an increased incidence of diabetes has not been observed in treated CNF patients (C Holmberg, personal communication). It is speculated that life long treatment with immunosuppressants to prevent rejection of the transplanted kidney protects these patients against the

development of type I diabetes. However, these results are still very preliminary and will need further investigation.

5. Long hairpin RNAi construct pDECAP fails to provide a tool for gene downregulation in vivo (Unpublished data)

Although conventional and conditional KO mouse-lines are useful for analyzing gene function, their generation in ES cells is very expensive and time-consuming. A rough estimation of time required spans between 12-24 months and costs about 50000-100000 euros. Chemically synthesized siRNA [277, 278] or plasmid based RNAi [276] provides an alternative approach by inducing sequence-specific mRNA degradation. Both approaches have been used successfully in gene silencing in podocyte cell-cultures [103, 112, 296, 297]. Very recently, nephrin was silenced with synthesized siRNA in WT podocytes [297]. Nephrin down-regulation abrogated the insulin response, and stable nephrin transfection of nephrin-deficient podocytes rescued their insulin response. This work demonstrated a previously unsuspected role for nephrin in vesicular docking and insulin responsiveness of podocytes [297]. In an other recent study, RNAi expression vector (pSilencer 2.1-U6) specifically targeted against podocin mRNA was transfected into the mouse podocyte clone (MPC5), and the molecular interactions among podocin, nephrin, and alpha-actinin-4 were studied [103]. This study showed that the silencing of podocin also decreased nephrin expression, whereas the expression and distribution of alpha-actinin-4 showed no change. These results suggested that podocin may interact directly with nephrin, but not with alpha-actinin [103]. Together these results show that the RNAi method is feasible when cultured cells for example podocytes are used.

Although the RNAi approach has been used successfully in podocyte cell-lines, no results of downregulation in podocytes *in vivo* have been published. The main problem with synthesized siRNA *in vivo,* is that it produces only transient mRNA degradation. The major difficulty in the use of long dsRNA transcribed from the Pol II or Pol III promoter *in vivo* is that this procedure induces the interferon response. In mammals, the dsRNA transcripts longer than 30 nucleotides are immediately transferred from the nucleus to the cytosol, where they induce

interferon synthesis leading to a block in translation and nonspecific mRNA degradation [278]. To prevent the interferon response, we used the pDECAP vector in our approach. The pDECAP transcript lacks both the 5´-cap structure and the 3´-pA tail and the lack of these structures prevents the export of dsRNA to the cytosol and prevents the interferon response [281]. The ribonuclease(s) such as Dicer which cleave long ds-RNA are localized both in the nucleus and the cytoplasm [283], and the long ds-RNA expressed by this vector is thought to be processed into siRNA already within the nucleus. The processed siRNA moves to the cytosol and causes the degradation of the target mRNA only [273].

The results *in vitro* showed that the expression of long filtrin and densin ds-RNA using the pDECAP vector leads to degradation of ~30-40% of the mRNA levels of both genes (See Table 7 and Figure 11, Pages 79 and 80). However, this downregulation was not target specific. This may be due to; 1) a still existing interferon response because of too long fragments of processed siRNA (more than 30 nucleotides); 2) the dsRNA fragments used are too long (500 bp) and results in too many siRNA molecules; 3) a homology between filtrin and densin mRNA sequences.

However, due to the high cost of the generation and maintenance of mouse-lines by the microinjection procedure or lentiviral transfection, the efficiency of these constructs to downregulate filtrin and densin expression *in vivo* was not examined further. Moreover, a report from another academic group stated that, they didn't either achieve selective gene silencing *in vivo* using the pDECAP vector (H Lohi, personal communication). As a conlusion, there is still a need to resolve some of the remaining practical problems in use of the pDECAP and other RNAi systems *in vivo* in the future.

Future perspectives

These novel mouse-lines provide an excellent tool for conditional, kidney glomerular podocyte specific gene deletion and over-expression in adult mice. Rescuing of nephrin deficient mice from perinatal death has enabled us for the first time, to study consequences of nephrin deficiency in extra-renal tissues in adulthood. Further studies to characterize detailed consequences of nephrin deficiency in the extra-renal tissues at a cellular and molecular level have already been started.

In the kidney research field it is an absolute necessity to study the regulation of gene expression in the adult animal. The availability of different floxed lines of distinct gene sets will allow podocyte-specific deletions by cross-breedings with the Cre-line. The floxed nephrin mouse-line has already been generated with Professor Josef Verbeek, Department of Genetics, University of Leiden. These novel mice will shed more light into the role of nephrin deficiency in the adult kidney and in other tissues.

These mouse-lines and the results obtained from them, will lead to a deeper understanding of the disease mechanisms in the kidney glomeruli. New understanding is urgently needed because of the enormously increasing prevalence of chronic kidney diseases, particularly diabetic kidney damage, which invariably starts with podocyte failure. Dysregulation of SD components, especially nephrin, is emerging as an important mechanism leading to loss of the filtration barrier function in diabetic nephropathy.

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