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Identification and characterization of novel glomerulus-associated genes and proteins

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To my family

ABSTRACT

The kidney is responsible for sieving the circulating blood to eliminate water-soluble waste products and potentially toxic substances from the body. The filtration step occurs in specialized filtration units called glomeruli. Some renal diseases are related to specific glomerular defects, but it is highly likely that the present knowledge gained from previous studies only represents a small proportion of genes and proteins that have important roles for normal kidney function.

To identify other genes with roles for glomerular filtration function, our group developed GlomBase, which is a glomerular transcript database in which over 300 genes are highly glomerulus specific. Among those genes, several genes with highest glomerular expression were chosen for further analysis, but this thesis is mainly based on studies on three of them, dendrin, adenylate cyclase type I (Adcy1), and Crumbs homolog 2 (Crb2).

Dendrin is a cytosolic protein previously identified only in the brain. However, we localized dendrin in the kidney specifically to the glomerular podocytes. Furthermore, we generated a polyclonal antibody against this novel glomerular protein. We detected that the earliest dendrin expression during glomerular maturation is at the capillary loop stage, and that it is located in the cytoplasmic face of the podocyte slit diaphragm. Unexpectedly, inactivation of the dendrin gene in mouse did not generate any obvious phenotype. Dendrin -/- mice were born at an expected Mendelian ratio and macroscopically all organs appeared normal. By the age of 1.2 years, no signs of renal impairment have been observed in the dendrin-/- mice. Under kidney challenging conditions, dendrin -/- mice show no difference when compared with dendrin +/+ mice. Even though dendrin does not seem to be crucial for the integrity of the glomerular filtration barrier, we do find two proteins that interact with dendrin, and their biological role in podocyte is still under investigation. These results are out scope of this thesis.

Adcy1 is one out of nine members of the adenylate cyclase protein family which catalyze the formation of the secondary messenger cAMP. cAMP is involved in a wide variety of cellular signaling processes, including regulation of actin cytoskeleton assembly through PKA. Adcy1 has previously been thought to be expressed only by certain neuronal cells in the brain, but we localized Adcy1 expression to the glomerular podocytes as well. During glomerulogenesis, the Adcy1 expression was detected first at the stage when maturing podocytes develop foot processes. To study the role of Adcy1 gene in the kidney *in vivo*, we analyzed the kidneys of Adcy1-/- mice (mice generated

by other investigators, that without severe phenotype except mild behavioral abnormalities). We found the glomerulogenesis to proceed normally in Adcy1-/- mice, and in mature mouse, no signs of renal impairment was detected. However, challenging of the kidney with albumin overload caused severe albuminuria in Adcy1-/- mice, whereas wild type mice showed only moderate albumin leakage to the urine. Thus, Adcy1 may in fact be a susceptibility gene for proteinuria.

Crb2 is yet another novel podocyte specific protein we identified. Its Drosophila homologue Crumbs is an essential component for epithelial cells organizing apical-basal polarity and adherent junctions. In the mouse, it is expressed only in brain, kidney and heart. In the kidney, it is specifically located in the glomerular podocyte slit diaphragm. Interestingly, inactivation of this gene led to arrest the embryonic development after E7.75 and embryonic lethality, which demonstrates the importance of this gene during early embryonic development. The *Crb2*-/- embryos show defects in neuroepithelium and epithelial mesenchymal transition (EMT) at the primitive streak. The function of Crb2 protein in the glomerulus will be explored later by my colleagues in studies of conditional knockout mice with podocyte specific inactivation of the *Crb2* gene.

In summary, the discovery and characterization of novel glomerular genes and proteins presented in this thesis has increased our knowledge of glomerular biology as well as on the role of a glomeral gene in early embryogenesis.

LIST OF PUBLICATIONS

I. Takemoto M, He L, Norlin J, Patrakka J, Xiao Z, Petrova T, Bondjers C, Asp J, Wallgard E, Sun Y, Samuelsson T, Mostad P, Lundin S, Miura N, Sado Y, Alitalo K, Quaggin SE, Tryggvason K, Betsholtz C. Large-scale identification of genes implicated in kidney glomerulus development and function. *Embo J*, 25: 1160-74, 2006.

II. Patrakka J*, Xiao Z*, Nukui M, Takemoto M, He L, OddssonA, Perisic L, Kaukinen A, Al-Khalili Szigyarto C, Uhlén M, Jalanko H, Betsholtz C, Tryggvason K. Expression and subcellular distribution of novel glomerulus-associated proteins dendrin, ehd3, sh2d4a, plekhh2 and 2310066E14Rik. *J Am Soc Nephrol*, 18(3): 689-97, 2007. (*equal contribution)

III. Xiao Z, Takemoto M, Chan G, Storm D, Betsholtz C, Tryggvason K, Patrakka J. Glomerular Podocytes Express Type I Adenylate Cyclase – Inactivation Leads to Susceptibility to Proteinuria. *Nephron Exp Nephrol.* In press.

IV. Xiao Z, Patrakka J, Nukui M, Lijun Chi, Dadi Niu, Betsholtz C, Vainio S, Tryggvason K. Crumbs homolog 2 (Crb2) is required for normal epithelial-mesenchymal transition (EMT) of gastrulation process during early embryonic mouse development. *Submitted* 2010

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LIST OF ABBREVIATIONS

Adcy1	adenylate cyclase type 1
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD2AP	CD2 associated protein
CNF	congenital nephritic syndrome of the Finish type
Crb2	Crumbs homologue 2
E	embryonic day
ECM	Extracellular matrix
EM	electron microscopy
FSGS	focal segmental glomerulosclerosis
GAG	glycosaminoglycon
GBM	glomerular basement membrane
GlomBase	golmerular transcript database
GlomChip	glomerular cDNA microarray chip
IHC	immunohistochemistry
Ig	immunoglobulin
ISH	*in situ* hybridization
Kb	kilo base pairs
LMX1B	LIM homeobox transcription factor 1-beta
LPS	lipopolysaccharide
NHERF2	sodium-hydrogen exchange regulatory cofactor 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PKA	protein kinase A
RT-PCR	reverse transcript PCR
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBE	buffer of Tris base, boric acid, EDTA
TPV	talin, paxillin, and vinculin
TRPC6	transient receptor potential channel 6
WT1	Wilms' tumor gene/protein 1
ZO-1	zonula occludens-1

1 INTRODUCTION

1.1 KIDNEY

Kidneys are bean-shaped organs responsible for excretion of urine that contains waste products and potentially toxic substances from the circulating plasma. The urine excretion is performed in the basic structural and functional unit of the kidney called nephron. The nephron comprises two parts: the glomerulus and the tubular system. Normally, vertebrates have two kidneys. Each human kidney contains about one million glomeruli, located in the kidney cortex (Fig. 1 A, B). The glomeruli are filtration units that sieve circulating plasma and produce daily about 180 liters of primary urine, which mainly contains water and small molecules like amino acids, glucose, ions, and small peptides. Subsequently, the primary urine passes through the tubule system that is mostly located in the renal medulla (Fig. 1A), where 99 % of the primary urine is resorbed back into the plasma. In humans, the final daily urine excretion is normally 1-1.5 liters, and does not contain proteins of the size of albumin or larger.

Kidney failure commonly affects human health and leads to death. It may be caused by mutations in the kidney protein genes, or by systemic diseases that cause kidney disorders, such as diabetes, hypertension and systemic lupus erythematosus. Around 75% of the kidney diseases may lead to end stage renal disease, which means permanent loss of kidney function. Kidney failure can generate various types of symptoms elsewhere in like swelling, while the kidney symptoms are usually proteinura or hematuria, which are largely caused by the failure of a specific glomerulus function.

1.2 GLOMERULUS

The glomerulus is a capillary tuft surrounded by the Bowman's capsule (Fig. 1B). The incoming blood enters the capsule through an afferent arteriole and the unfiltered plasma exits through an efferent arteriole. The glomerular capillaries are supported and interconnected by an extracellular mesangial matrix and mesangial cells, and the glomerular tuft is surrounded by the urinary space. The filtration occurs in the capillary wall. Normally, only water and low molecular weight molecules (smaller than albumin) can pass through the filter.

Many diseases affect the kidney function by causing glomerular damage, which exhibits as leakage of large proteins or blood into the urine, and distortion of the glomerular filtration barrier.

1.3 GLOMERULAR FILTRATION BARRIER

The glomerular filtration barrier consists of three layers of the glomerular capillary wall: a fenestrated endothelium, the glomerular basement membrane (GBM), and the slit diaphragms located between the foot processes of the epithelial podocytes (Fig. 1C, D).

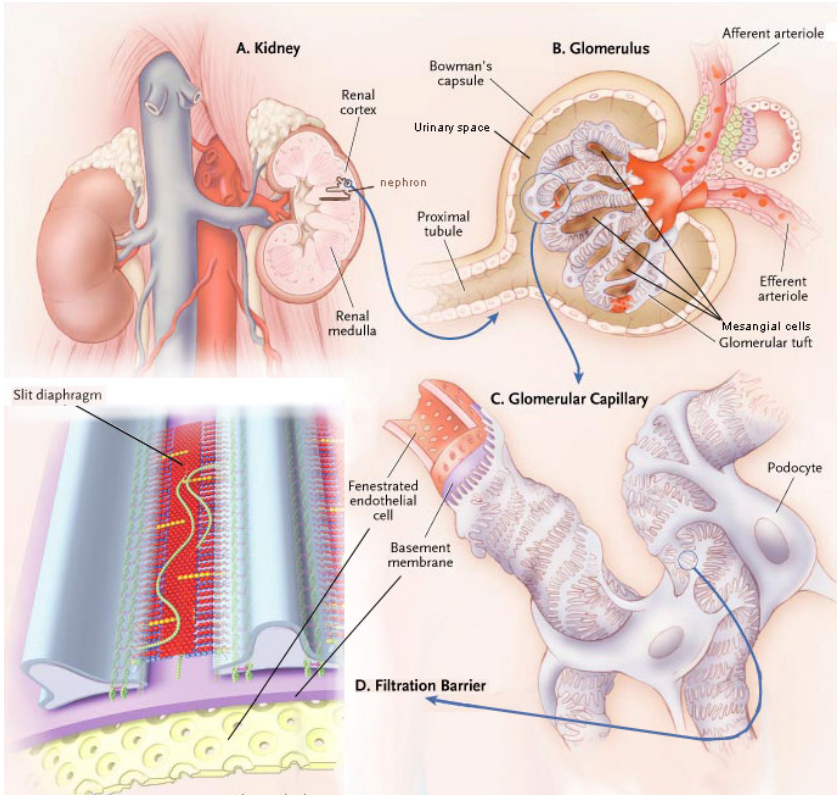

Fig. 1. Glomerular filtration barrier (adapted from Tryggvason and Patrakka, 2006)

The glomerular filtration barrier has for long been believed to be a charge and size-selective filter [1-5]. How individual components of the glomerular filtration barrier contribute to the filtration function has been, and still is, a matter of debate. In the following, the role of components of the glomerular capillary wall in the filtration will be discussed.

1.3.1 Fenestrated endothelium

The innermost layer of the glomerular capillary wall, the fenestrated endothelium has numerous openings of 70-100 nm in diameter. Thus, except for the blood cells, it has been generally considered not to hinder passage of plasma macromolecules into the GBM matrix. On the other hand, the glomerular endothelial cells have been shown to contain negatively charged sialoproteins and proteoglycans on their surface [6], which could potentially contribute to a primary anionic barrier that diminishes amount of macromolecules being passed through. However, there is no direct evidence for such a role of the endothelial glycocalyx. Studies have been presented showing that injection or perfusion of glycosaminoglycon (GAG) -degrading enzymes like hyaluronidase, heparanase, and chondroitinase lead to proteinuria [1, 4, 7]. However, since these enzyme can pass into the GBM, which also contains GAGs, it cannot, as yet, be stated that the negative charges of the endothelial glycocalyx have a more significant role than those in the GBM or on the podocyte for glomerular permselectivity [8].

1.3.2 Glomerular basement membrane (GBM)

The GBM is an amorphous extracellular sheet-like structure of about 300-350 nm in thickness (in humans). It is a fused basal lamina produced by both capillary endothelial cells and epithelial podocyte cells. It is a key structure maintaining the structural integrity of the filtration barrier against the considerable hydrostatic pressure that is generated within the glomerular capillaries [9]. The main components of the GBM are type IV collagen, laminins, proteoglycans (perlecan and agrin), fibronectin, and nidogen/entactin [10-12].

Type IV collagen forms a highly cross-linked three-dimensional network that provides structural support to the GBM. In the fetus, the trimeric type IV collagen molecules contain only α1 and α2 chains (α1: α1: α2), which are replaced by α3, α4 and α5 chains in adults. Mutations in adult type IV collagen cause Alport's syndrome, which is a progressive hereditary kidney disease characterized by hematuria, sensorineural hearing loss, and ocular lesions with structural defects in the GBM [11, 13]. However, the patients has only mild proteinuria, indicating that the type IV collagen network does not really contribute to the size and charge-selectivity of the filtration barrier [14].

All laminins are thought to exist in basement membranes as α:β:γ heterotrimers. In the GBM, laminin forms a network that is interconnected with the type IV collagen

network through interactions with nidogen/entactin [15]. As in the case of type IV collagen, the fetal laminin-511 (α5:β1:γ1) is replaced by adult type laminin-521 (α5:β2:γ1) after birth. Deletion of the laminin β2 gene in mice results in massive proteinuria at birth and death within a month [16]. In 2004, mutations in the human laminin β2 chain were shown cause Pierson's syndrome, an early lethal autonomic recessive form of congenital nephrotic syndrome associated with diffuse mesangial sclerosis and microcoria [14, 17]. Absence of the laminin α5 chain results in breakdown of the glomerular basement membrane (GBM) and failed glomerular vascularization [18]. Podocyte-specific inactivation of the laminin α5 chain gene results in varying degrees of proteinuria and rates of progression to nephrotic syndrome [19]. Together, these data have demonstrated that laminin-521 is an important component of GBM of the filtration barrier.

Proteoglycans, such as perlecan and agrin, have anionic sites on their heparan sulfate and chondroitin sulfate side chains [20-21], and the anionic charges have been thought to contribute to the filtration barrier. However, heparan sulfate -deficient perlecan mice do not have structural defects in the GBM nor do they have proteinuria [22-23]. On the other hand, the mice are prone to proteinuria when challenged with an albumin overload [24]. Mice deficient for agrin in the GBM do not have abnormal glomerular filtration function, even when challenged with albumin overload, although the anionic charge of GBM is severely altered in these mice [22]. Recently, these two mouse lines were crossed, and the offspring were shown not to develop proteinuria [25]. Together, these results strongly suggest that the negative charges of the GBM itself do not play as an important role in the filtration function as previously thought.

1.3.3 Podocyte proteins and involvement in diseases

Podocytes are unique epithelial cells. They are terminally differentiated and highly polarized. They look like octopuses embracing each other with their foot processes, practically wrapping the glomerular capillary (Fig. 1C). The podocyte cell body faces the urinary space, and from there it extends out primary processes, which in turn, divide into secondary foot processes (Fig. 1C). The interlacing foot processes are bridged by the final filtration barrier, the slit diaphragm (Fig. 1D), where proteins form a zipper-like structure with a constant width of around 40 nm. This filter contains lateral pores that are smaller or of the size of albumin [14, 26-27]. A schematic drawing of podocyte foot process assembling and their components is showed below (Fig. 2).

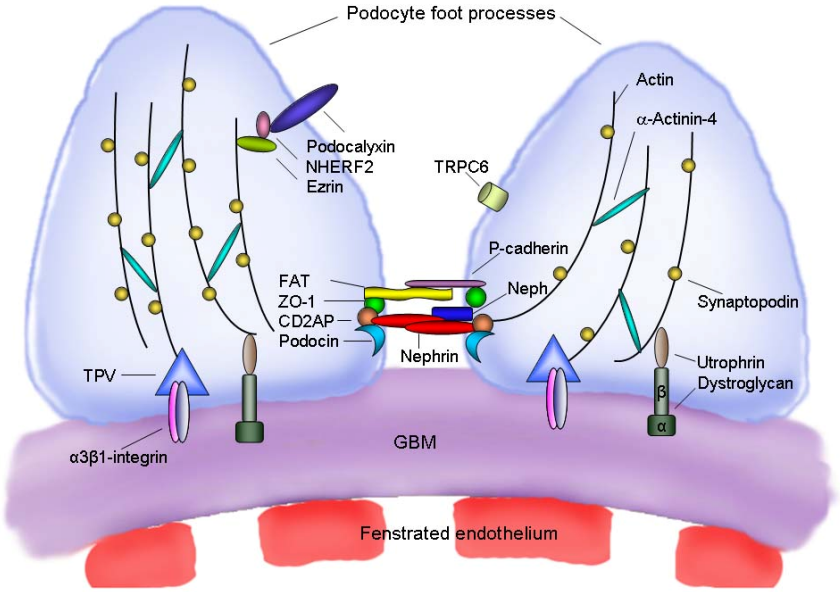

Fig. 2. Schematic drawing of two podocyte foot processes located on the outside of the GBM. Key foot process proteins contributing to the filtration barrier and their interrelationship are illustrated.

1.3.3.1 Transcription factors

LMX1B

Mutations in LMX1B cause Nail-patella syndrome, which is a nephrotic syndrome characterized by thickening and splitting of the GBM accompanied with skeletal and nail dysplasia [28]. LMX1B mutant mice show similar phenotype to patients of Nail-patella syndrome [29]. In the kidney, LMX1B is specifically expressed in the podocytes, and regulate the expression of many genes critical for podocyte differentiation and function, including podocin, CD2AP, and type IV collagen [30-32].

WT1

WT1 is a transcription factor encoded by the Wilms' tumor suppressor gene WT1. It has an important function in kidney organogenesis and differentiation [33]. Dominant mutations of this gene cause Denys-Drash and Frasier's syndromes [34]. Denys-Drash syndrome has renal symptoms characterized by diffuse mesangial sclerosis, usually before the age of one year, and patients frequently develop Wilms' tumor [35-36].

Frasier syndrome displays glomerular symptoms consisting of childhood proteinuria and nephrotic syndrome, characterized by unspecific focal and segmental glomerular sclerosis, progressing to end-stage renal failure in adolescence or early adulthood [37]. In podocytes, WT1 has many targets, *e.g.* nephrin and podocalyxin [38].

1.3.3.2 Foot process cytoskeleton

The unique structure of podocyte foot processes is maintained by a cytoskeleton network of actin filaments. Many actin-associated proteins bind and modify the function of the cytoskeleton in podocyte foot process. Furthermore, the actin cytoskeleton is connected to different plasma membrane domains of podocytes through various linker proteins. α-actinin-4 and synaptopodin are the two most well known cytoskeletal components of podocyte foot processes act the dynamic regulation of foot process structure.

α-Actinin-4

α-actinin-4, encoded by *ACTN4*, is a cytoplasmatic actin bundling protein. It regulates the actin cytoskeleton and cellular motility [39]. It is highly expressed in podocytes, where it crosslinks with F-actin filaments in the foot processes. Mutations in *ACTN4* cause human focal segmental glomerulosclerosis (FSGS), characterized by childhood onset of mild proteinuria and slow progression to FSGS and end-stage renal disease [40]. α-actinin-4 deficient mice develop a recessive phenotype characterized by proteinuria, glomerulosclerosis, and retraction of podocyte foot processes [41].

Synaptopodin

Synaptopodin is an actin binding protein highly expressed in podocytes [42]. Synaptopodin-deficient mice have normal glomerular filtration function, but they develop heavier proteinuria and delayed recovery compared with wild type mice when the podocytes are challenged with lipopolysaccharide (LPS). These mice also show impaired recovery from protamine sulfate-induced podocyte foot process effacement [43]. Synaptopodin is a regulator of Rho GTPases in podocytes and it induces stress fibers by blocking the proteasomal degradation of RhoA [44]. A recent study showed that dephosphorylation of synaptopodin by calcineurin (protein phosphatase) abrogates its interaction with 14-3-3, and promotes synaptopodin degradation; phosphorylation of synaptopodin by PKA or CaMKII (protein kinases) promotes protein 14-3-3 binding,

which protects synaptopodin degradation, thereby contributing to the intact glomerular filtration barrier [45].

1.3.3.3 Negatively charged surface molecules

Podocalyxin

All there layers of the glomerular filter are negatively charged. In podocytes, the charge is mainly provided by podocalyxin. It is a transmembrane protein located at the apical and lateral surface of podocyte foot processes above the slit diaphragm [46-47], and it is one of the major podocyte sialoproteins of the glycocalyx. By repelling adjacent foot process away from each other, podocalyxin helps to maintain the filtration slits open [48], and possibly prevent an adherence of podocytes to the Bowman's capsule. It associates with the podocyte actin cytoskeleton through interaction with ezrin (an actin-binding protein) and NHERF2 [49-51]. The importance of podocalyxin is highlighted by the fact that podocalyxin knockout mice develop anuric renal failure and die within 24 hours of birth. These mice do not develop secondary foot processes, and the slit diaphragms are replaced by impermeable tight junctions [52].

1.3.3.4 Podocyte-GBM adhesion proteins

For normal glomerular filtration function, it is important to keep an intact filtration barrier where the podocyte foot processes are anchored to the GBM. This is facilitated by interactions between podocyte basal surface receptors and their ligands in the GBM. Through this interaction, they provide physical adhesion and also mediate various intracellular signals associated with cytoskeletal proteins and intracellular kinases.

α3β1-integrin

Integrins are heterodimeric receptors composed of different α and β chains that bind various extracellular matrix components. α3β1-integrin is the most abundant integrin in podocytes. It binds to several components of the GBM, including type IV collagen, laminin, fibronection, and entactin/nidogen [53-55]. Blockage of the integrin β1-binding domain leads to foot processes fusion, proteinuria and detachment of podocyte from the GBM [54, 56]. α3-integrin knock out mice die within 24 hours after birth with lung and kidney defects, with disorganized glomeruli and absence of foot processes [57]. Integrins are linked with the actin cytoskeleton through talin, paxillin, and vinculin (TPV) [58]. It is also bridged with a slit diaphragm signaling through integrin-

linked kinase (ILK). Podocyte specific ablation of ILK cause aberrant distribution of nephrin, foot process effacement, heavy albuminuria and animal death after 10 weeks [59].

Dystroglycan

Dystroglycan (DG) is another receptor on the basal surface of podoctyes that binds GBM ligands like laminin, agrin, and perlecan. It is connected to the podocyte cytoskeleton through utrophin [60-61]. The role of DGs in podocytes is unknown.

1.3.3.5 Slit diaphragm

The slit diaphragm consists of many proteins that are connected with cytoskeleton and also involved in signaling pathway. The importance of the slit diaphragm is highlighted by the fact that mutations in the genes for many of its component result in slit diaphragm disassembly and proteinuria. Well known slit diaphragm proteins include nephrin, podocin, CD2AP, Neph, FAT and ZO-1.

Nephrin

In the early 1970s, the hypothesis of the slit diaphragm being the size-selective molecular sieve was raised (Karnovsky and Ainsworth, 1972; Rodewald and Karnovsky, 1974) However, the molecular composition of the slit diaphragm was obscure until 1998, when studies on congenital nephrotic syndrome of the Finnish type (CNF) revealed mutations in a novel gene *NPHS1* that encodes nephrin, a specific component of the slit diaphragm [62-63]. Nephrin is a transmembrane protein of the Ig-superfamily. Its extracellular domains from adjacent foot processes are believed to interact in the center of the slit to form the zipper-like backbone of the slit diaphragm [64]. Patients with nephrin mutations usually lack the podocyte slit diaphragm and exhibit massive proteinuira already at birth. Inactivation of *Nphs1* in mice causes a similar phenotype and neonatal death [65]. Thus, nephrin is critical for the maintenance of the normal slit diaphragm structure. Nephrin is not only a structureal component of the silt diaphragm, but it is also involved in signaling pathways. Tyrosines in the intracellular part of nephrin can be phosphorylated by Fyn (a member of Src family kinases), and the initiated signaling seems to promote antiapoptosis [66], binding to Nck and oligomerization of actin filaments. This is supported by the report that Fyn deficient mice develop proteinuria and podocyte effacement [67]. Recent findings also showed the phosphorylated nephrin link to the Nck adaptor protein, and thereby

regulate the actin cytoskeleton formation. Selective deletion of Nck from podocytes in mice causes defects in the formation of foot processes and congenital nephrotic syndrome [68-71].

Podocin

Podocin is an integral membrane protein of the stomatin protein family. Mutations in the podocin gene, *NPSH2*, cause corticosteroid-resistant nephrotic syndrome characterized by early childhood onset of proteinuria and rapid progression to end-stage renal disease [72-73]. Podocin-deficient mice have fused podocyte foot processes and lack slit diaphragms [74]. These mice develop massive proteinuria antenatally and die a few days after birth. Podocin is required for proper targeting of nephrin into the slit diaphragm, and this may have a pathogenic role in the development of *NPHS2* kidney disease. Podocin has been shown to interact with Neph1, nephrin, and CD2AP [75]. Thus, podocin is critical for the glomerular filtration function.

CD2AP

CD2AP is an intracellular protein first found as an adaptor protein in T-cells [76]. However, CD2AP knockout mice die at 6-7 weeks of age due to a nephrotic syndrome-like disease with podocyte abnormality [77]. CD2AP heterozygous mice develop glomerular changes at 9 months of age and increased susceptibility to glomerular injury by nephrotoxic antibodies or immune complexes (Kim et al., 2003). CD2AP mutations have been found in a few patients with FSGS [78]. CD2AP interacts with nephrin and has been shown to serve as a linker between nephrin and the podocyte cytoskeleton [79-82]. Furthermore, CD2AP seems to be associated with endocytosis to clean up the plasma proteins [78], and also involved in signaling of TGF-beta-induced apoptosis in podocytes [83].

Neph1, Neph2, Neph3

Neph1, Neph2, and Neph3 are transmembrane proteins belonging to the Neph protein family. They are located in the slit diaphragm and interact with nephrin through their extracellular domains [84-87]. Neph1 and Neph2 can also form homodimers, which however do not interact with each other [88]. Mice lacking Neph1 show severe proteinuria and die within 8 weeks of birth [89] similarly to the nephrin knockout mice. All Neph proteins interact with Podocin and the tight junction protein ZO-1 [90-91]. They are also involved in the podocyte slit diaphragm signaling [91-93].

FAT

FAT proteins are very large transmembrane proteins belonging to the cadherin family. In mammals, there exist at least three FAT proteins, FAT1, FAT2, and FAT3. In glomeruli, FAT1 and FAT2 are located in the slit diaphragm [94-95]. Mice lacking FAT1 show glomerular, forebrain and eye defects, and they die within 48 hours probably due to loss of the slit diaphragms [96]. FAT1 regulate actin dynamics and controls cell-cell interactions through its binding to Ena/VASP proteins [97-98], which are regulators of the actin cytoskeleton and cell migration [99]. The function of FAT2 is not clear yet, as FAT2 deficient mice show normal longevity and are fertile [95].

ZO-1

ZO-1 is a member of the membrane-associated guanylate kinase (MAGUK) protein family. It is widely expressed and located at the cytoplasmatic face of intercellular junctions. In typical polarized epithelial cells, ZO-1 is located in the tight junctions that define the apical and basal lateral membrance. In the podocyte, ZO-1 is located at the cytoplasmic face of the slit diaphragms [100]. As mentioned above, ZO-1 binds to Neph family proteins [90]. It has also been reported that Nephrin forms a multiprotein complex with cadherins, p120 catenin, and three scaffolding proteins, including ZO-1, and possibly in that way it connects the slit diaphragm to the actin cytoskeleton and signaling networks [101]. However, the role of ZO-1 in podocytes is still unknown.

P-cadherin

P-cadherin is a transmembrane protein that has been reported to be located in the slit diaphragm and colocalized with ZO-1 [102]. P-cadherin knockout mice do not develop renal deficiency [103]. Thus, this protein is not considered to be important for glomerular filtration.

VE-Cadherin

VE-cadherin (also known as cadherin-5) belongs to the cadherin protein family. It is predominantly expressed in endothelial and plays an important role controlling vascular organization. Inactivation of VE-cadherin gene causes embryonic lethality due to the defects on vascular organization and remodeling [104]. However, a recent study showed that VE-cadherin is also expressed in the podocyte. It is identified as a slit

diaphragm-associated molecule, and effectively links the coexpression and coregulation of nephrin and ZO-1 [105].

TRPC6

TRPC6 is a member of the transient receptor potential (TRP) superfamily of non-selective cation channels. In the glomerulus, it is expressed by capillary endothelial cells, mesangial cells, and podocytes. Mutations in its gene have been shown to cause FSGS [106-107]. TRPC6 is involved in signaling pathways and appears to be a receptor-operated channel leading to calcium influx, and can be activated by G protein-coupled receptors [108] and directly by diacylglycerol [109]. In the podocyte, it has been speculated to be a component and downstream target of the nephrin signaling platform involved in monitoring the integrity of the slit diaphragm [110].

In addition to these podocyte components, there are numerous other highly specific podocyte proteins the role of which in the filtration barrier is not known. In general, the actual glomerular filtration process is not properly understood. A popular view is that the GBM functions as a coarse first filter, and that the podocyte slit diaphragm is the second ultrafilter that hinders the passage of proteins larger than albumin [14, 111]. There also exists another hypothesis stating that the GBM is a gel with size-selective properties determined by permeation and diffusion, but not filtration. According to that hypothesis, the slit diaphragm is essential for normal structure and to retain water passage but not other molecules [112], which the author considers to explain why there is no clogging during filtration.

Regardless of the debate, the importance of the podocyte cell for the filtration barrier is unassailable. The genes and proteins mentioned above represent only some of the podocyte components that have been extensively studied or which have been shown to be important for filtration function and directly related with deferent renal diseases. It is very likely that many novel as yet undiscovered podocyte components have important roles for the normal filtration function. Thus, it is of great interest and importance to explore the unknown side the kidney glomerulus, especially the podocyte.

As described above, the glomerulaus is injured in many systemic renal diseases. The glomerulus is a unique “micro-organ” dedicated to renal ultrafiltration, therefore toxic or otherwise damaging extrarenal substances can cause glomerular insults. The

involvement of those accounts for a large proportion of kidney diseases, but that aspect is outside the scope of this thesis project.

2 AIMS OF THE STUDY

The overall goals of this project are to apply a large-scale approach to characterize the transcriptome of mouse glomeruli in order to identify novel gene products of major importance for glomerular function and pathology. By finding and characterizing these novel proteins, this study is likely to increase our knowledge on glomerulus biology, and will provide new understanding of the pathogenesis of proteinuria.

The specific aims of this project are to:

1. Identify transcripts and proteins that have previously not been associated with glomeruli or renal disease
2. Characterize the expression pattern of novel glomerular transcripts by using northern and *in situ* hybridization methods.
3. Express novel glomerulus-associated proteins of interest in order to produce antisera for characterization of the protein expression using immunohistochemical methods.
4. Determine the biological role of the proteins by studying the phenotypes of knockout mice.

3 METHODS

3.1 REVERSE TRANSCRIPTION (RT) AND RT–PCR

For generating glomerular and "rest of kidney" cDNA, mouse glomeruli and kidney tissue devoid of glomeruli (rest of kidney) were isolated as previously described [113]. Total RNA was then isolated by RNeasy mini Kit (QIAGEN) and 1 µg was reverse transcribed by Superscript III Reverse Transcriptase (Invitrogen). The generated cDNA was diluted, 10-fold with Tricine-EDTA buffer for storage.

For studies on tissue distribution, multiple mouse tissue cDNAs were used as template (Mouse MTC™ Panel I; Clontech Laboratories, Palo Alto, CA), The PCR reaction was done by HotStarTaq DNA polymerase (QIAGEN) with gene specific primers described in original publication. The PCR program was: 1 cycle of 95 °C/15 min; 30 cycles of 95°C/1 min, 55 °C/1 min, 72 °C/1 min; 1 cycle of 72 °C/10 min. The PCR products were analyzed in 1% TBE agarose gel and photographed.

For confirming silence of *Crb2* expression in *Crb2* knock out mice, total RNA was extracted from littermate control and null mutant embryos at E8, followed by RT from 1 µg total RNA. Then, PCR reaction was done by using 1 µl of the RT product as template and 3'-UTR primer pair was used (see manuscript/publication) to detect gene expression.

3.2 NORTHERN BLOT

To study tissue distribution, a specific cDNA probe was amplified, labeled with ^{32}P-dCTP with the Prime-It® RmT Random Primer Labeling Kit (Stratagene, la jolla, CA), and hybridized with Mouse MTN® Blot (Clontech Laboratories, Palo Alto, CA). The Blots were exposed to a PhosphorImager SF screen (Molecular Dynamics) and analyzed with ImageQuant software (Molecular Dynamics).

3.3 SOUTHERN BLOT

To genotype Adcy1 mice, extracted genomic DNA was digested with Bgl II and separated in 0.8 % TBE or TAE agarose gel and transferred onto nylon membrane. The cDNA probe was excised from the plasmid (provided by collaboration group) with Bgl II and Acc65 I (Fermentas) and gel purified. The labeling and hybridizing followed the same procedure as for Northern Blots.

3.4 *IN SITU* HYBRIDIZATION

To localize gene expression in the kidney, *in situ* hybridization experiments were carried out on mouse kidney cryosections. Gene specific probes were transcribed by T7 or SP6 polymerases and labeled with ^{35}S. The procedure has been described previously [114].

3.5 WHOLE MOUNT IN SITU HYBRIDIZATION EXPERIMENT

To study *Crb2* embryonic expression and analyze *Crb2* null embryo with different marker genes, the embryos were fixed in 4 % PFA prepared in PBS and experiment were performed following protocols simplified from standards procedures [115]. 3'-UTR Probe (see publication IV) was used for study *Crb2* expression. Probes for marker genes were provided by other groups (see publication IV). Single-stranded RNA probes were labeled with digoxigenin-UTP according to the manufacturer's instructions (Roche).

3.6 PRODUCTION OF POLYCLONAL ANTIBODY

Mouse dendrin residues 1-330 were cloned into a pET-28a(+) expression vector separately, and the recombinant proteins were purified (see publication II) and d NZW rabbits were immunized for generating polyclonal antibody.

3.7 IMMUNOHISTOCHEMISTRY

For kidney analyses, samples were collected and snap-frozen in OCT. For studies on embryos, samples were either snap-frozen in OCT, or fixed in 4% paraformaldehyde (PFA) in PBS, followed by infiltration of 10 % sucrose in PBS, and embedded in OCT. Cryosections (8-10 µm) were postfixed with cold acetone (–20 °C) followed by blocking in 5 % normal goat serum. The primary antibodies were incubated overnight at 4 °C or 1-h at RT, followed by a one hour incubation with the secondary antibody. For double-labeling experiments, the incubations were performed sequentially. Some sections were double/triple stained with DAPI with 1:2000 diluted in PBS.

3.8 IMMUNOELECTRON MICROSCOPY

For immunoelectron microscopy, the samples from mouse renal cortexes were fixed in 3.5 % PFA plus 0.02 % glutaraldehyde prepared in PBS. After fixation, the samples were embedded in 10 % gelatin, infiltrated with 2.3 M sucrose in PBS, and frozen in liquid nitrogen. Immunolabeling experiments were done as described previously [116].

3.9 MOUSE STRAINS

Dendrin and *Crb2* mutant mice were generated by replacing their entire coding exons 1-2 and 1-13, respectively, with eGFP lox-Ub1- EM7- Neo- lox cassette (Regeneron Pharmaceuticals, Inc). In each strain, heterozygous mice were mated to obtain wild-type, heterozygous, and null mutant mice. Both dendrin and *Crb2* mouse strains were backcrossed with C57bl/6 for more than nine generations. All mouse work was approved by the regional ethical committee.

3.10 GENOTYPING

Adcy1 genotyping was done by Southern blot (see above). *Crb2* genotyping was done by PCR using genomic DNA extracted from toe samples or embryonic yolk sac samples. The PCR reaction was done by HotStarTaq DNA polymerase (primer pairs sequences see publication II and IV).

3.11 WESTERN BLOTTING

Proteins were extracted from normal mouse glomeruli and "rest of kidney" [113]. The Western blotting was done following standard procedures. Anti-β-actin antibody was used as a loading control.

3.12 MOUSE CHALLENGE MODELS

3.12.1 Protein overload

Bovine serum albumin (BSA) was used as overload protein, by daily injecting of 400 µl (0.5 g/ml) intraperitoneally for four consecutive days. Urine samples were collected before each injection and every 24 hours after the last injection. Measurement of proteinuria was done by both ELISA and SDS-PAGE gel (Invitrogen).

3.12.2 LPS- induced Proteinuria

LPS (0.5 mg/ml) was injected once intraperitoneally (20-26 µl/g mouse body weight), and urine was collected at the time of LPS injection, also 12, 24, 36, 48, 60, and 72 hours after the injection. Urine samples were analyzed by SDS-PAGE gel and PAGE-Blue stain (Fermentas).

4 RESULTS AND DISCUSSION

The results presented in this thesis are based on work carried out in collaboration with Christer Betsholtz's group. The initial collaboration led to the development of GlomBase that initially contained over 6,000 glomerular genes identified by microarray analysis. These genes were normalized against Rest of the kidney (non-glomerular kidney tissue) and Brain capillary fragments, which grouped out a category containing 143 glomerular-upregulated genes [117]. Many of these genes were taken for further analysis, including dendrin, adenylate cyclase type 1 (Adcy1), and crumbs homolog 2 (Crb2). Their positions (according to expression activity) in the category and ratios after normalizations are shown below [117], together with podocyte markers podocin and nephrin for comparison.

Table 1. Microarry analysis showed that the expression of dendrin, adcy1, and Crb2 are upregulated in the glomerulur versus "rest of the kidney", and the normalization by comparing their glomerular expression versus brain capillary transcripts, which represents most of the endothelial transcripts, showed that these genes are more likely to be podocyte specific. Nphs1 (encode nephrin) and Nphs2 (encode podocin) are known important pococyte components and their top position in the list suggests that other top genes may also be important for the kidney filtration function.

Position in the list	Gene annotation	Ratio $\log_2$(Glo/R-of-Kid)	Ratio $\log_2$(Glo/Brain-Capi)
2	Nphs2 (Podocin)	3.4	5.08
4	Nphs1 (Nephrin)	2.97	4.2
13	Dendrin	1.92	4.12
47	Adcy1	1.53	2.45
103	Crb2	1.76	1.28

4.1 DENDRIN

4.1.1 Identification of dendrin as a novel glomerular component

Dendrin is a cytosolic protein that does not contain any know functional domain, and previously it has only been identified in the brain [118-119]. However, our microarray data showed that it is strongly upregulated in kidney glomeruli [117]. The tissue distribution was confirmed by RT-PCT and Northern blot experiments, that dendrin is

expressed in brain and kidney. The transcript size is approximately 3.5 kb. Further, by *in situ* hybridization of newborn mouse kidney samples, we found dendrin specifically located in glomerular podocytes. By generating a dendrin polyclonal antibody, we could confirm by immunofluorescence that dendrin is podocyte specific, and based on immuno-EM located in the cytoplasmic face of the slit diaphragm. Western blot result shown that mouse dendrin is an 81 kD protein only expressed in the glomeruli. Thus, dendrin was demonstrated to be a novel glomerular protein.

4.1.2 Dendrin knock out mice are viable, fertile, and do not develop notable kidney defects (data not shown)

The mouse dendrin gene was knocked out to further study its function in glomeruli. The entire coding region of two exons was replaced with eGFP lox-Ub1- EM7- Neo-lox cassette (Regeneron Pharmaceuticals, Inc). The elimination of dendrin in knockout mice was confirmed by immunohistochemistry (IHC). However, the dendrin knockout mice were born at expected Mendelian frequencies and macroscopically all organs appeared to be normal. Urine samples of littermate wild type and knockout mice were collected until age one year and three months, but no proteinuria was detected, or any morphological impairment in the kidney by light microscopy. Nephrin and synaptopodin are expressed normally in the knockout mice. Challenging experiments with LPS, BSA overload, and anti-GBM models did not reveal significant difference between littermate wild type and knockout mice.

Dendrin mice were crossed with nephrin +/- mice and the dendrin-/-nephrin+/- mice were normal as compared to dendrin+/+nephrin+/-. Dendrin mice were also crossed with Fat2 mice, and the mice were followed until age of one year and 2 months. Results showed that dendrin-/-fat2-/- did not show notable difference with dendrin-/-fat2 +/+ mice.

Recently, it is reported that dendrin contains two nuclear localization signals (NLS1 and NLS2) [120]. Another publication showed that the NLS1 is necessary and sufficient for the nuclear import of dendrin in podocytes and the relocation promotes podocyte apoptosis [121]. However, the mechanism behind it and the detailed biological role of dendrin still remain a mystery. However, the above finding, “dendrin relocation promotes apoptosis”, or “nuclear dendrin is harmful”, at least is consitent with our observation that under stress models (LPS, protein overload), deletion of dendrin does not lead to a more severe phenotype than wild type mice, because the

knockout podocyte does not have dendrin to promote the apoptosis, or further damage to the filtration barrier.

Since dendrin has such a restricted expression pattern (only in the brain and kidney), and a specific slit diaphragm location in the kidney, we still speculate that dendrin has some unknown functions for the filtration barrier. But it is not possible to predict the compensating proteins to dendrin, because dendrin does not have any known domains and homologous counterparts. We, therefore, did microarray experiment to examine changes in gene expression profiles. The result did not bring up any well known podocyte genes, but revealed a 1.5-fold upregulated gene Gadd45a (growth arrest and DNA damage - 45a) in dendrin knockout glomeruli. This gene product has also been shown to interact with dendrin using the Yeast 2 hybrid system, and their direct interaction has been proved by co-immunoprecipitation experiments using transfected HEK293 cells (unpublished observations). Gadd45a encodes a protein involved in cell cycle regulation [122-124], DNA repair and genomic stability [125-126]. It is usually induced under stress condition, and arrests cell cycle at G2/M checkpoint and promote apoptosis of damaged cells. Its function has been most studied in from the point of view of controlling proliferating cancer cells, while it is considered to be protective in normal cells [127]. Thus, the upregulation of Gadd45a in dendrin knockout glomeruli can be interpreted as a cell stress responce induced by dendrin deletion. However, the upregulated Gadd45a did not seem to promote podocyte apoptosis since the dendrin knockout kidney could function as normally as wild type ones. Thus, the biological roles of dendrin and its interacting proteins remain unclear and need further studies.

Moreover, the Yeast 2 hybrid experiment using dendrin as bait also fished out another dendrin interacting protein, WTIP (Wilm's tumor interacting protein). It has also been shown to directly interact with dendrin by co-immunoprecipitation experiments using transfected HEK293 cells. Their biological role in the glomerulus is under investigation and these results are outside of the scope of this thesis.

4.2 ADENYLATE CYCLASE TYPE 1 (ADCY1)

4.2.1 Identification of Adcy1 as a novel glomerular component

Adcy1 is one out of nine members of the adenylate cyclase protein family, which catalyze the formation of the secondary messenger cAMP. Previously, Adcy1 has been

reported to be neurospecific with expression solely in the brain, in an area important for long-term memory formation [128-129]. However, our microarray experiments identified Adcy1 as a highly glomerulus specific transcript in the kidney. By RT-PCR, we could validate that, except for the brain, Adcy1 is also expressed in the kidney, and Northern blot hybridization also showed its expression to be in the brain, kidney, and also heart. The transcription size is approximately 11.5 kb. Since Adcy1 has nine homologous family members, it is not possible to detect Adcy1 at the protein level due to a lack of isoform specific antibodies. However, the glomerular specificity was confirmed by an *in situ* hybridization experiment, where Adcy1 was mainly expressed in the podocytes, starting from the capillary stage of glomerular development. Thus, we identified Adcy1 as a novel podocyte protein.

4.2.2 Inactivation of Adcy1 results in susceptibility to proteinuria

Earlier analysis of Adcy1-/- mice revealed only a behavior phenotype in the form of memory defects [128, 130]. To investigate the role of Adcy1 in glomerular podocytes, we analyzed the potential kidney phenotype of Adcy1-/- mice. By light and electron microscopy, the kidney morphology of newborn, 8-week-old, and 1-year-old Adcy1-/- mice appeared normal without any podocyte abnormality. The expression of the podocyte proteins nephrin, podocin, synaptopodin, podocalyxin, wt1, and dendrin was unchanged in Adcy1-/- mice, as judged by intensity of immunofluorescence staining. Urine samples were collected from 10-week and 1-year old Adcy1-/- mice, and no significant proteinuria was detected in Adcy1 knockouts using albumin-ELISA or SDS-PAGE analysis. This indicated that the kidney filtration function in Adcy1-/- mice remained intact under normal circumstances.

The intact glomerular function could be due to redundancy and compensation by other Adcy family members expressed in the glomerular podocyte, and RT-PCR from glomerular cDNA did reveal expression of the other Adcy isoforms, except for Adcy2 and Adcy8. These Adcys are more widely expressed in the body and are not upregulated in the kidney glomeruli according to our microarray data. Moreover, their expression level did not obviously change in Adcy1-/- glomeruli. But the existence of these Adcy proteins, at normal expression level, might be sufficient to compensate for the absence of Adcy1 in the Adcy1-/- mice, and thus to preserve normal kidney function.

To further study if the apparently normal kidney function of Adcy1-/- mice is as sturdy as in wild type mice, we did challenge experiments to Adcy1-/- mice and

littermate controls. By challenging the kidney with LPS, no obvious difference was observed between Adcy1-/- and wild type mice. But when challenging the kidney with the BSA overload method, Adcy1-/- mice developed massive proteinuria within 24 hours which lasted in a severe form for two days after the last injection. In contrast, wild type mice only developed slight proteinuira and then returned to baseline much quicker. This indicates that under pathological conditions, Adcy1 -/- mice are more prone to develop proteinuria, and that the Adcy1 gene may in fact be a susceptibility gene for proteinuria. Proteinuria is an important hallmark for podocyte injury. Many congenital or acquired renal diseases develop proteinuria to different extent, and usually there is more proteinura the more severe the podocyte injury is. Thus, the highly podocyte upregulated Adcy1 may have a function of protecting from podocyte injury, which in the absence of Adcy1 leads to frailer kidney function.

Adcy1 is responsible for catalyzing cAMP that is used for intracellular signal transduction, largely through the activation of protein kinases A (PKA). PKA is involved in a variety of cellular functions, including intermediary metabolism, ion channel conductivity, and transcription. Growing literature also suggests that PKA plays a central role in cytoskeletal regulation and cell migration [131]. Podocyte injury usually alters the dynamics of the actin cytoskeleton, resulting in foot process effacement and proteinuria. Hence, the heavier proteinuria of Adcy1 -/- mice under challenging condition is possibly due to a lower amount of cAMP catalyzed in a stress situation. Less PKA is activated in the podocytes, which affects the normal cytoskeleton dynamics and leads to more severe podocyte injury. However, this hypothetical mechanism was not proved in our studies.

4.3 CRUMBS HOMOLOG 2 (CRB2)

4.3.1 Identification of Crb2 as a novel glomerular component

Crumbs is essential for organizing epithelial cells apical-basal polarity and adherent junctions in Drosophila [132-134]. In mammals, there exist three Crumbs homologues: Crb1, Crb2, and Crb3. Results of microarray experiments (see above) showed that Crb2 is the only crumbs homologue that is upregulated in glomerulur. RT-PCR and Northern blot analyses showed that in mouse tissue Crb2 is mainly expressed in brain and kidney. The transcript is approximately 7.5 kb. Furthermore, *in situ* hybridization experiments and immunofluorescence staining using polyclonal antibody against extracellular Crb2 protein showed that it is located in podocyte foot process where it

colocalizes with synaptopodin. By immuno-EM, Crb2 was specifically located in the podocyte slit diaphragm region. Thus, Crb2 was identified as a novel podocyte component. Recently, its zebrafish homologue *crb2b* was knocked down, and the *crb2b* morphants showed podocyte foot process disorganization and loss of slit diaphragms [135].

4.3.2 Inactivation of *Crb2* results in early embryonic lethality

To further study Crb2 function in the kidney glomerulur, the entire coding region of 13 exons was replaced with an eGFP lox-Ub1- EM7- Neo- lox cassette (Regeneron Pharmaceuticals, Inc). Heterozygous mice were intercrossed to obtain null mutant mice, but no -/- offspring was detected after embryonic day (E) 14.5, indicating homozygous embryos die earlier. Genotyping of embryos from E7 to E12.5 showed that the embryos developed at Mendelian frequencies, which means that mutant mortality does not occur prior to these stages. However, the *Crb2* -/- embryos already developed severe defects during this period. The loss of *Crb2* transcripts in mutants was confirmed by RT-PCR. The heterozygous offspring were viable and fertile and did not reveal any differences from wild type mice.

4.3.3 *Crb2* -/- mice exhibit holistic defects during early embryonic development

The malformation was clearly evidenced at E7.75. The *Crb2*-/- head fold and primitive streak were retarded, the notochord was discontinuous, and the somites were also abnormal. From E8 and onwards, *Crb2*-/- embryos exhibited holistic defects in head, heart, somites, and gut formation. The null embryo was posteriorly truncated, and failed in early organogenesis. The extra embryonic tissue development was also abnormal, namely amnion, allontois and yolk sac. The *Crb2*-/- embryo could grow ectopically until E10.5, and were completely resorbed by E12.5.

4.3.4 Crb2 is necessary for normal gastrulation process

In mammals, gastrulation occurs after implantation, followed by organogenesis. The gastrulation starts at the primitive streak, which morphologically is a thickening of the embryonic ectoderm located in the posterior margin of the embryo. The epiblast at the primitive streak undergoes epithelial-mesenchymal transition (EMT) to geneate mesoderm cells that migrate laterally and arterially in between the ectoderm and

endoderm. These layers later undergo organogenesis and develop into certain bodily systems.

The *Crb2*-/- embryo could develop the primitive streak, suggesting ability to establish the primary axis of the embryo. The *Crb2* -/- primitive streak could generate a thin layer of mesoderm until the late streak stage (E7.5). However, the following gastrulation was significantly retarded. The EMT process is disrupted without Crb2 function, as the epithelium caretaker E-cadherin expression persisted to the new transited mesoderm cells, and the degraded basement membrane between the abnormal ectoderm and mesoderm indicated a wider transiting window of the mutant primitive streak. Moreover, the transited mesoderm cells attack in the posterior embryo and express less fibronectin, a typical extracellular matrix protein that controls cell migration. These findings indicate that Crb2 has an important function in EMT at the primitive streak during mid-gastrulation of embryonic development.

4.3.5 Crb2 is crucial for maintaining normal neuroepithelium morphology

Crb2-/- neuroepithelium is poorly developed and the neural tube closure did not occur. By E8.25, compared to controls, most of the columnar neuroepithelium cells visualized by Pan-cadherin were disordered in the *Crb2*-/- ectoderm showing a pseudostratifed structure. The basement membrane marker perlecan expression in the null embryo showed a discontinuous pattern beneath the neuroepithelium, showing parts of the basement membrane broke down, but not in the controls. By E9.5, the Pan-cadherin labeled Crb2-/- neuroepithelium partially disassembled that they lost their sheet like structure and mixed with mesodermal cells. The disassembled neuroepithelium does not show increased signal by the Tunel assay, indicating that this phenotype is not due to apoptosis in the end stage embryonic development of *Crb2*-/- embryos.

To summarize, Crb2 was in this study identified as a novel kidney glomerular protein. Inactivation of the *Crb2* gene in mice resulted in early embryonic lethality, indicating its important role during mid-gastrulation process. The function of Crb2 in the glomerulus will be explored by my colleagues in studies of conditional knockout mice with podocyte specific inactivation of the Crb2 gene.

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