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**CHARACTERIZATION OF NOVEL PODOCYTE
PROTEINS IN THE GLOMERULAR FILTRATION
BARRIER**

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ABSTRACT

The kidney is considered the most important organ of excretion eliminating the body from metabolic waste products. The nephron is the functional unit of the kidney, composed of a cluster of capillaries named glomerulus, and a tubular system. The blood is filtered in the glomerular capillaries across the glomerular filtration barrier. The barrier is an intricate biological structure composed of the inner capillary endothelial cells, the glomerular basement membrane, and the podocytes. The podocyte is phenotypically and functionally a sophisticated cell governed by a highly organized cytoskeleton. Podocytes play a critical role in the initiation and progression of proteinuric kidney diseases. The reorganization of podocyte cytoskeleton causing foot process effacement is a key factor in the development of proteinuria. Better understanding of the molecular composition and the biological interactions in these cells is a necessity for future specific targeted therapies.

This study is based on a previous genome-wide approach aimed to characterize the transcriptome of the renal glomerulus through large-scale sequencing and microarray profiling. In reference to other well characterized podocyte-specific proteins, the rationale was to identify and characterize novel podocyte-specific proteins that show a similar restricted expression profile outside the glomerulus. In the papers, several such proteins are described.

In paper I, *sult1b1* was localized exclusively in the podocyte Golgi apparatus and *ankrd25* was localized to podocyte foot processes as shown by RT-PCR, Western blotting and immunofluorescence stainings.

In Paper II, *pdlim2* was localized to podocyte foot processes as shown by RT-PCR, Western blotting, immunofluorescence and immunoelectron microscopy. In cultured podocytes, *pdlim2* seemed to regulate actin dynamics. In addition, using Co-immunoprecipitation experiments, *pdlim2* was shown to interact with α -actinin-4 and *amotl1*. In semi-quantitative immunoelectron microscopy, there was a reduced expression of *pdlim2* in podocytes of patients with certain proteinuric renal diseases.

In Paper III: *hip1*, *nfasc* and *olfml2a* were localized exclusively in podocyte major processes as shown with RT-PR, Western blotting, immunofluorescence stainings and immunoelectron microscopy. During glomerulogenesis, the proteins were colocalized with a major processes marker vimentin as shown by immunofluorescence double stainings. The expression of *hip1*, *olfml2a* and *pdlim2* was observed in glomerular crescents indicating the presence of podocytes in these lesions.

In PaperIV, *gprc5b* was expressed exclusively by podocytes as shown with RT-PCR, Western blotting, immunofluorescence and immunoelectron microscopy. *Gprc5b* knockdown in zebrafish exhibited classical features associated with loss of glomerular function such as pericardial edema and dilated Bowman's capsule. Thus, *gprc5b* is essential for an intact glomerular filtration barrier.

LIST OF PUBLICATIONS

- I. Xu X, Patrakka J, **Sistani L**, Uhlen M, Jalanko H, Betsholtz C, Tryggvason K: Expression of novel podocyte-associated proteins sult1b1 and ankrd25. *Nephron Exp Nephrol*, 117(2):e39-46, 2011.

- II. **Sistani L**, Dunér F, Udumala S, Hultenby K, Uhlen M, Betsholtz C, Trygvason K, Wernerson A, Patrakka J: Pdlim2 is a novel actin-regulating protein of podocyte foot processes. *Kidney International*, 80(10):1045-54, 2011.

- III. **Sistani L**, Rodriguez P, Hultenby K, Uhlen M, Betsholtz C, Jalanko H, Tryggvason K, Wernerson A, Patrakka J: Huntingtin interacting protein 1, neurofascin and olfactomedin-like 2a are novel components of podocyte major processes and demonstrate the presence of podocytes in glomerular crescents. *Submitted*, 2012.

- IV. Rodriguez P, **Sistani L**, Oddsson A, He B, Hultenby K, Ebrasi L, Betsholtz C, Wernerson A, Tryggvason K, Patrakka J: Gprc5b is required for the integrity of glomerular filtration barrier. *Manuscript*, 2012.

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LIST OF ABBREVIATIONS

| | |
|--------|---|
| ALP | Actinin-associated LIM protein family |
| Amotl1 | Angiomotin-like 1 |
| CD2AP | CD2-associated protein |
| CKD | Chronic kidney disease |
| CNF | Congenital nephrotic syndrome of the Finnish type |
| ESL | Endothelial surface layer |
| ESRD | End-stage renal disease |
| FSGS | Focal segmental glomerulosclerosis |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GBM | Glomerular basement membrane |
| GFP | Green fluorescent protein |
| GPCR | G-protein-coupled receptor |
| hpf | Hours post fertilization |
| HSPG | Heparan sulfate proteoglycan |
| Ig | Immunoglobulin |
| ILK | Integrin-linked kinase |
| L1-CAM | L1-cell adhesion transmembrane |
| Lmx1b | LIM homeobox transcription factor 1-beta |
| MN | Membranous nephropathy |
| MCNS | Minimal change nephrotic syndrome |
| MO | Morpholino |
| mRNA | Messenger ribonucleic acid |
| NERF2 | Na ⁺ /H ⁺ exchanger regulatory factor 2 |
| RT-PCR | Reverse transcript-polymerase chain reaction |
| SD | Slit diaphragm |
| SRNS | Steroid-resistant nephrotic syndrome |
| SULT | Sulfotransferase |
| TRPC6 | Transient receptor potential channel 6 |
| Wt1 | Wilm's tumor 1 |

1 INTRODUCTION

1.1 RENAL ANATOMY AND PHYSIOLOGY

References used for this section are medical physiology books (Bruce M. Koeppen 2008; Gerard J. Tortora 2009; Rodney A. Rhoades 2009).

The kidneys are paired organs lying outside the peritoneal cavity in the posterior part of the upper abdomen - one on each side of the vertebral column. The kidney has a bean-like shape with the medial border indented by a deep fissure called the hilus. This is where the blood vessels, lymphatics, nerves and ureter enter or leave the kidney. Each adult kidney weighs about 0.5 percent of total body weight corresponding to about 150 grams in adults.

The kidney is considered the most important organ of excretion, eliminating the body's metabolic waste products by filtering the blood from substances that are unwanted, or are in excess. The nephron is the functional unit of the kidney where the composition of blood is regulated through: filtration, secretion, reabsorption, and concentration. The kidneys receive about 20% (1.2 L/min) of the cardiac output through the renal arteries, which is an immediate branch of the abdominal aorta. The renal artery enters the kidney through the hilus and branches off to a network of smaller blood vessels that supply all parts of the kidney.

The blood is filtered in the glomerular capillaries, and in 24 hours, about 180 liters of blood is filtered in the kidney. Most of the glomerular filtrate, also called primary urine, is reabsorbed by the tubular system of the nephron and returned to the systemic circulation through the renal vein that exits through the hilus and empties into inferior vena cava. The glomerular filtrate, or primary urine, is concentrated in the tubular parts of the nephron (Figure 1) into final urine, which is then excreted via the ureter into the bladder and expelled through the urethra.

The kidneys selectively reabsorb important substances that are necessary to maintain the normal composition of the blood such as: amino acids, glucose, vitamins, minerals, ions, water, sodium, chloride, bicarbonate, potassium, magnesium, phosphate, and sulfate. By adjusting the blood composition, the kidneys are able to maintain blood volume and pressure, ensure proper balance of salts, electrolytes, acid-base, and pH levels. The kidneys also eliminate metabolic waste products, such as urea, uric acid, creatinine, and ammonia, as well as various toxins and drugs. In addition, the kidneys

serve as an endocrine organ releasing a variety of hormones including renin, erythropoietin, and calcitriol. Renin is involved in the regulation of blood pressure, sodium and potassium balance. Erythropoietin stimulates the production of erythrocytes in the bone marrow. Calcitriol, which is the active form of vitamin D, stimulates the uptake of calcium in the gastrointestinal tract, a necessary mineral for growth and remodelling of the bones.

1.2 THE NEPHRON

References used for this section are medical physiology books (Bruce M. Koepfen 2008; Gerard J. Tortora 2009; Rodney A. Rhoades 2009).

The nephron (Figure 1) is the basic unit of kidney structure and function that regulates the composition and volume of body fluids. Each human kidney is comprised of approximately one million nephrons. Each nephron consists of a renal corpuscle and a tubular system. The renal corpuscle is composed of a cluster of capillaries named the glomerulus (Figure 2), which is encased in a thin bag-like structure called Bowman's capsule. The space inside the capsule and surrounding the glomerulus is called Bowman's space, or urinary space. The Bowman's capsule continues into the proximal convoluted tubule, which is where the tubular system begins. This system is formed of various segments including the descending and ascending loop of Henle, distal convoluted tubule and collecting duct (Figure 1).

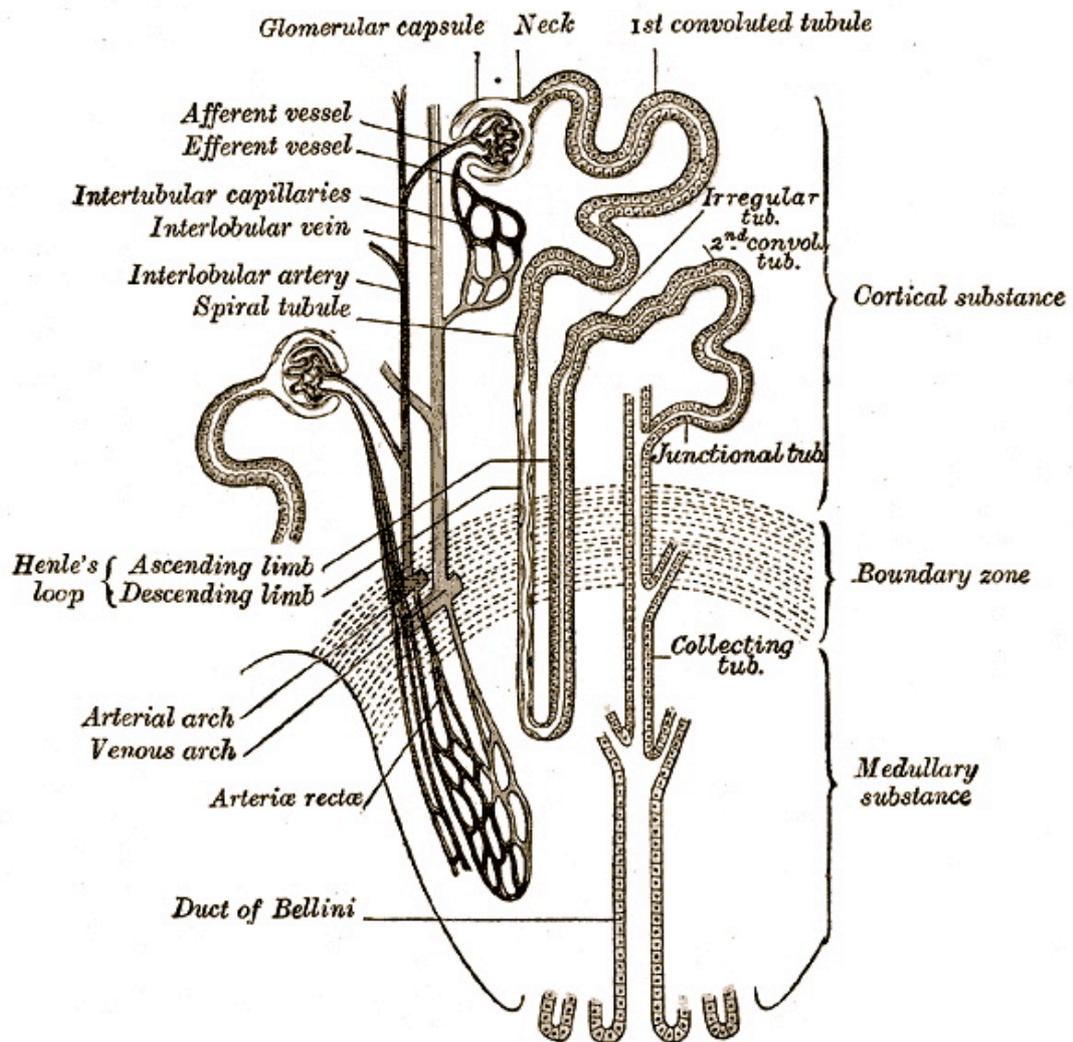


Figure 1 Schematic drawing of the nephron. *Image source: The Bartleby.com edition of Grey's Anatomy of the Human Body from the classic 1918.*

1.2.1 Dynamics of ultrafiltration

Each nephron is supplied with blood through the afferent arteriole, a subbranch of the renal artery, which give rise to the capillary tuft of the glomerulus. The glomerular capillaries then converge into the efferent arteriole where blood can exit the capsule to form a second capillary network that supply the nephron. The driving force for fluid filtration passing through the glomerulus is the Starling forces (the hydrostatic and colloid osmotic pressures). The arterial pressure forces fluid from the porous capillaries of the glomerulus across the filtration barrier into Bowman's space. The composition of the glomerular filtrate is basically the plasma, excluding any significant amount of larger proteins. The plasma-like filtrate diffuses across Bowman's space and travels

through the series of tubular segments of the nephron wherein the ultrafiltrate is effectively processed, and concentrated, through reabsorption and excretion into final urine. About 99% of the glomerular filtrate returns to the systemic circulation through tubular reabsorption and only 1-1.5 liter is excreted as urine.

1.3 THE GLOMERULAR FILTER

The plasma ultrafiltrate sieved through the glomerular capillary bed is almost exclusively free of cellular and macromolecular blood components. The composition of the plasma ultrafiltrate is based on size, charge and molecular configuration (Haraldsson and Sorensson 2004). For example, negatively charged high molecular weight proteins such as albumin are restricted from passing through the filtration membrane.

The glomerular filtration barrier (Figure 3) is a highly complex biological structure, which is essentially composed of three main structures: 1) the glomerular basement membrane in the middle which serves as a foundation and support for; 2) fenestrated endothelial cells at the inside; and 3) the visceral epithelial cells (podocytes), located on the outside of the glomerular capillary facing the urinary space (Tryggvason and Wartiovaara 2001).

The glomerulus contains a third cell type namely, mesangial cells (Figure 2). These cells are specialized vascular smooth muscle cells, some of which have phagocytic properties, and suggested to serve as the “pericytes of the glomerulus” (Schlondorff and Banas 2009). Mesangial cells reside between the capillary loops in the stalk of glomerulus, and provide support to maintain structure and stability to the glomerulus (Schlondorff and Banas 2009).

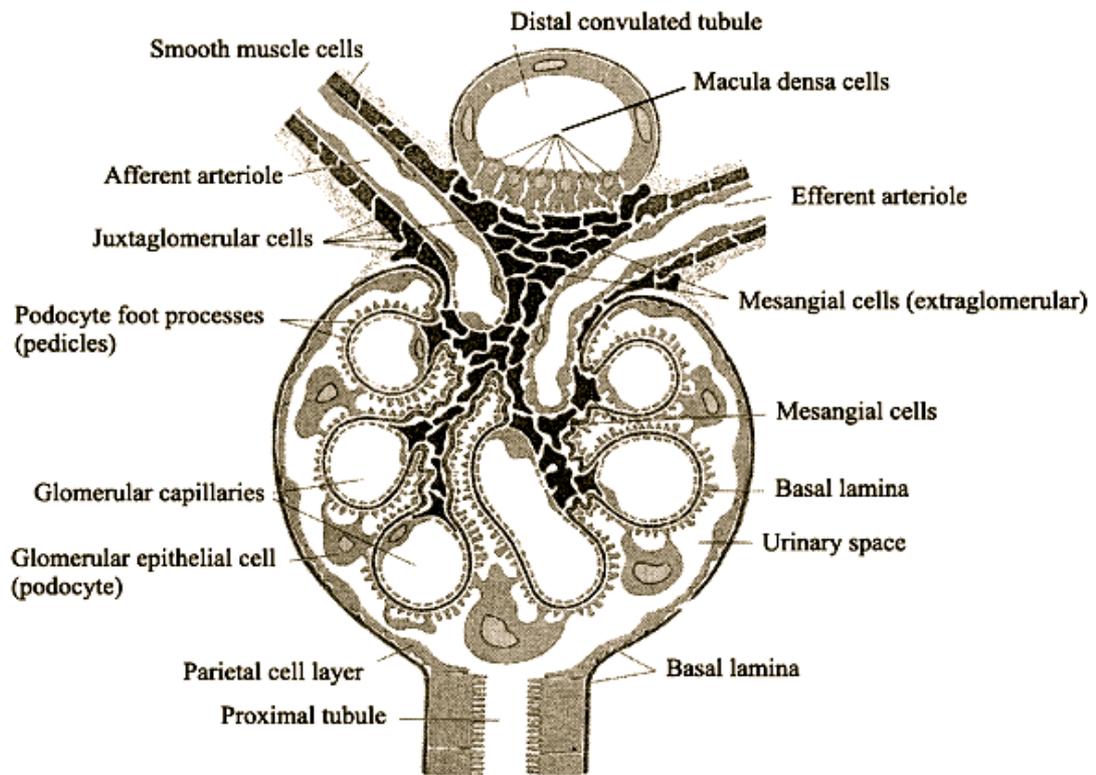


Figure 2 Schematic drawing of the kidney glomerulus. *Image source: modified from Ross and Romell, 1989.*

1.3.1 The glomerular endothelium

The glomerular endothelium forms the innermost layer of the filtration barrier. The endothelium is composed of flat cells containing round or ovoid transcellular holes, 60-70 nm in diameter, named fenestrae (Avasthi and Koshy 1988). The large fenestrae allow for the extensive rate of exchange of water and low molecular weight waste products in the glomerulus (Bulger, Eknoyan et al. 1983; Satchell and Braet 2009). In mature glomeruli, the fenestrae do not seem to contain diaphragms (Ichimura, Stan et al. 2008), and occupy about 20-50% of total capillary surface area (Bulger, Eknoyan et al. 1983).

The endothelial cells at the luminal side are coated with glycocalyx, a negatively charged gelatinous layer about 300 nm thick. The glycocalyx is composed of glycoproteins, proteoglycans, glycosaminoglycans, and trapped plasma proteins, which might possibly contribute to the charge-selective properties of the filtration barrier (Haraldsson, Nystrom et al. 2008; Satchell and Braet 2009). Several studies suggest the vital role of the endothelial cells and surface layer in the glomerular filtration barrier

where loss of anionic sites is associated with albuminuria (Gelberg, Healy et al. 1996; Jeansson and Haraldsson 2003; Bakker, Borghuis et al. 2005; Jeansson and Haraldsson 2006). Similar effects have been reported in *in vitro* studies using monolayers of human glomerular endothelial cells. When disrupting the glycocalyx components, an increased albumin flux is observed (Singh, Satchell et al. 2007; Lennon, Singh et al. 2008) highlighting the important role of endothelial cell layer in charge-selectivity of the glomerular barrier.

1.3.2 The glomerular basement membrane

The glomerular basement membrane (GBM) is a specialized extracellular matrix and a crucial element of the filtration barrier that provides structural support for both podocytes and endothelial cells. During development, endothelial cells and podocytes synthesize separate basement membranes which later fuse to form the unusually thick, 300-350 nm, GBM (Abrahamson 1987; Gubler 2008). As observed in electron microscopy, the mature GBM is subdivided into three layers; starting from base of endothelial cells the layers are termed, lamina rara interna followed by lamina densa in the middle, and lamina rara externa in the outer aspect (Abrahamson 1987). The major components of the GBM include laminin, type IV collagen, heparan sulfate proteoglycans, and nidogen (Gubler 2008).

1.3.2.1 Laminin

Laminins belong to a large family of heterotrimeric glycoproteins present in all basement membranes. They assemble into different ($\alpha\beta\gamma$) isoforms providing a structural function in basement membranes (Miner 2008). Laminins are signaling molecules and play an essential role in organogenesis (Durbeej 2010). During glomerulogenesis, the laminin isoforms found in the GBM are LM-111 ($\alpha:1\beta:1\gamma:1$) and LM-511 ($\alpha:5\beta:1\gamma:1$). As the glomerulus develops the fetal laminins are entirely replaced with the mature isoform, LM-521 ($\alpha:5\beta:2\gamma:1$) (Miner 1998). Laminin networks are connected to type IV collagen networks via nidogen providing structural support to the capillary wall (Timpl 1996). Mutations in laminin have been associated with proteinuria and nephrotic syndrome both in mouse and human. The autosomal

recessive disorder, Pierson syndrome, is caused by mutation in *LAMB2* gene encoding the laminin $\beta 2$ chain. Pierson's syndrome displays diffuse mesangial sclerosis and congenital nephrotic syndrome (Zenker, Aigner et al. 2004). Laminin $\beta 2$ knockout mice show proteinuria, foot process effacement, and die one month of age (Miner, Go et al. 2006). Podocyte-specific mutation in laminin $\alpha 5$ causes proteinuria in mice, which can progress to renal failure (Goldberg, Adair-Kirk et al. 2010). The above reports indicate the important role of laminins in the glomerular filtration barrier function.

1.3.2.2 Type IV collagen

Type IV collagen is a trimer composed of three α -chains arranged in a triple helix molecule. Type IV collagen is exclusively found in the basement membranes wherein the trimers cross-link and assemble into a polymeric network (Yurchenco 2011). The cross-linked molecular structure of collagen IV serves as a scaffold where other molecules such as laminins, perlecan, and proteoglycans can interact (Yurchenco 2011). There are six different genes, *COL4A1*-*COL4A6*, encoding six distinct collagen α -chains ($\alpha 1(IV)$ - $\alpha 6(IV)$) in humans (Khoshnoodi, Pedchenko et al. 2008). During glomerulogenesis the type IV collagen of glomerular basement membrane is formed by the triple helix $\alpha 1.\alpha 1.\alpha 2$. Similar to laminins in the GBM, the embryonic type IV collagen is gradually replaced to the mature type IV collagen $\alpha 3.\alpha 4.\alpha 5$ (Hudson, Tryggvason et al. 2003).

Mutations in *COL4A3*, *COL4A4*, and *COL4A5* genes cause renal disease such as Alport's syndrome, which is clinically characterized by hematuria, mild albuminuria, progressive renal disease and hearing impairment (Barker, Hostikka et al. 1990; Hudson, Tryggvason et al. 2003). Histopathological observations of kidney biopsies in Alport's syndrome patients may first reveal a thinner GBM than normal, however, as the disease progresses the GBM is thickened into multilamellations known as GBM splitting, which is present along with podocyte foot process effacement, glomerular scarring and interstitial fibrosis (Hudson, Tryggvason et al. 2003; Cosgrove, Kalluri et al. 2007).

Type IV collagen $\alpha 3$ -knockout mice display a similar renal phenotype as observed in Alport syndrome including GBM abnormalities and podocytopathy (Cosgrove, Meehan et al. 1996; Miner and Sanes 1996). Given that no significant proteinuria was

observed in type IV collagen deficient patients/animals, type IV collagen does not seem important in the charge- and size-permselectivity of the filtration barrier. Nevertheless, it seemingly has a contributing role in maintaining the structural integrity of the glomerular filtration barrier.

1.3.2.3 Heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPGs) are glycoproteins that consist of a core protein with covalently linked glycosaminoglycan side chains such as heparan sulfate and chondroitin sulfate. HSPGs present in basement membranes include, perlecan, agrin and collagen XVIII (Sarrazin, Lamanna et al. 2011). The glycosaminoglycan side chains are sulfated and negatively charged contributing to the anionic properties of GBM. Perlecan and collagen XVIII are predominantly expressed during glomerulogenesis (Saarela, Rehn et al. 1998; Groffen, Veerkamp et al. 1999), whereas the mature GBM is primarily composed of agrin (Groffen, Ruegg et al. 1998).

Podocyte-specific agrin knockout mice show reduction in GBM anionic sites but do not develop proteinuria (Harvey, Jarad et al. 2007). Similarly, perlecan deficient mice show no proteinuria or renal abnormalities, although they show increase susceptibility to protein-overload proteinuria (Morita, Yoshimura et al. 2005). Collagen XVIII mutant mice show increased mesangial matrix and elevated creatinine levels in the kidney (Utriainen, Sormunen et al. 2004). Moreover, double mutant agrin and perlecan mice develop and maintain a normal filtration barrier (Goldberg, Harvey et al. 2009). Despite the reduction of GBM anionic charge in these mice no structural abnormality of the filtration barrier or proteinuria was detected which indicate that the negative charge of the GBM is not the primary barrier as it was initially suggested.

1.3.2.4 Nidogen/entactin

Nidogens-1 and nidogen-2, also known as entactin, are glycoproteins that are widely expressed in basement membranes and bind to collagen IV, perlecan and laminin (Yurchenco and Patton 2009). Nidogen-1 and -2 are both components of the GBM. Nidogen links laminin and type IV collagen forming a ternary complex, which is the reason nidogen was considered to be required for basement membrane development

(Salmivirta, Talts et al. 2002). However, nidogen-1 and -2 single and double knockout mice develop a normal GBM and only show a mild phenotype in certain basement membranes (Bader, Smyth et al. 2005).

1.3.3 The podocyte

The podocytes constitute the third layer of the glomerular filter and are attached to the GBM on the outer aspect of the glomerular capillary. Podocytes are highly specialized cells with a remarkably sophisticated architecture and a corresponding well-developed cytoskeleton (Pavenstadt, Kriz et al. 2003). Morphologically and functionally the podocyte is subdivided into three major parts: cell body, major processes and foot processes. The podocyte has a large cell body, a prominent nucleus and well-developed organelles. The cell body expands to form large cytoplasmic projections named primary processes (Pavenstadt, Kriz et al. 2003). A single podocyte is able to spread out with its processes over several loops of glomerular capillaries.

The primary processes further divide and form numerous smaller projections known as foot processes. The podocyte foot processes of the neighboring cells interdigitate and cover the loops of capillaries (Pavenstadt, Kriz et al. 2003). The interdigitating foot processes are bridged by specialized cell-cell contacts and form the slit diaphragm (SD). The SD is a dynamic multiprotein structure critical for a sustained filtration barrier function (Tryggvason and Wartiovaara 2005; Tryggvason, Patrakka et al. 2006). Furthermore, the podocyte is supported by a well-organized cytoskeleton. Bundles of microtubules and intermediate filaments support the major processes, whereas the cytoskeleton of foot processes is composed of actin filaments (Drenckhahn and Franke 1988; Pavenstadt, Kriz et al. 2003).

Initially, the GBM was thought to be the principal charge- (Deen, Lazzara et al. 2001) (Tryggvason and Wartiovaara 2005) and size-selective barrier (Caulfield and Farquhar 1974; Kanwar, Liu et al. 1991). However, this view changed with the discovery of the first molecular component of the SD, nephrin. In 1998, Tryggvason and colleagues identified mutations in nephrin in patients suffering from congenital nephrotic syndrome of the Finnish type (CNF) (Kestila, Lenkkeri et al. 1998). With the discovery of nephrin, podocyte biology entered a new era as it gained an increasing

research interest. It is now well recognized that podocytes play a central role in glomerular filtration process (Leeuwis, Nguyen et al. 2010).

Several podocyte proteins have been identified to be essential for the normal glomerular filtration function. These proteins include components of the SD: podocin (Boute, Gribouval et al. 2000), Nephrin (Donoviel, Freed et al. 2001), FAT1 (Inoue, Yaoita et al. 2001), and TRPC6 (Winn, Conlon et al. 2005), linker proteins connecting the SD to the podocyte cytoskeleton including CD2-associated protein (CD2AP) (Shih, Li et al. 1999), and Nck (Jones, Blasutig et al. 2006), as well as actin-associated proteins synaptopodin (Deller, Merten et al. 2000), and α -actinin-4 (Kaplan, Kim et al. 2000).

Podocytes are terminally differentiated epithelial cells with limited ability to regenerate upon injury (Mathieson 2012). Podocytes play a critical role in the ultrafiltration process and are involved in the initiation and progression of proteinuric kidney diseases (Mathieson 2012). The identification of podocyte genes causing glomerular kidney disease has provided valuable insight into understanding the role of podocytes in the glomerular filtration function (Tryggvason, Patrakka et al. 2006).

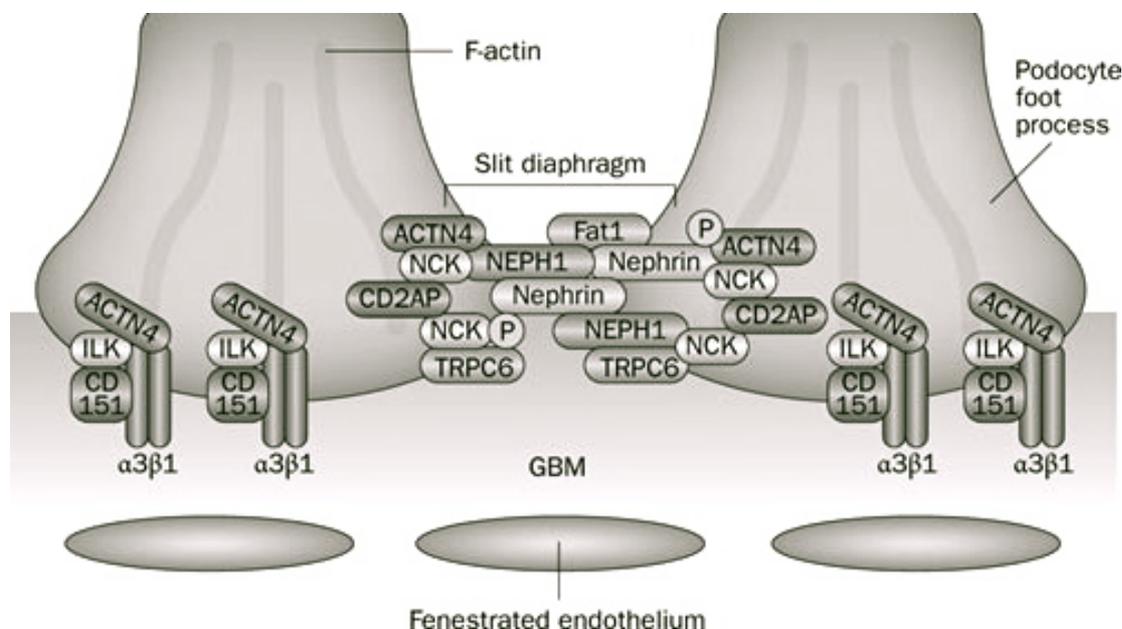


Figure 3 Schematic drawing of the glomerular filtration barrier composed of the fenestrated endothelium, the GBM and a layer of podocyte foot processes. The two neighboring podocyte foot processes are connected via a complex of proteins forming the SD. *Image source: modified from Patrakka and Tryggvason, 2009.*

1.3.3.1 Cell Body: transcription factors

Wt1

Mutations in the gene *Wt1* that encodes the tumor suppressor Wilm's tumor 1 (Wt1) cause Frasier syndrome and Denys-Drash syndrome (Pelletier, Bruening et al. 1991; Klamt, Koziell et al. 1998). Wt1 is a transcription factor essential for normal genitourinary development regulating numerous genes involved in cellular differentiation (Lee and Haber 2001). However, in the adult kidney, Wt1 is present only in podocytes indicating the importance of Wt1 in maintaining podocyte differentiation (Schedl and Hastie 2000). Mutations in Denys-Drash syndrome occur in the zinc finger encoding exons affecting DNA binding affinity; while in Frasier syndrome mutations in intron nine alter the proportion of two alternatively spliced Wt1 transcripts (Barbaux, Niaudet et al. 1997; Kikuchi, Takata et al. 1998; Klamt, Koziell et al. 1998; Lee and Haber 2001).

Patients with Denys-Drash syndrome exhibit early nephrotic syndrome including diffuse mesangial sclerosis with rapid development into renal failure, male pseudohermaphroditism, and Wilm's tumor (Pelletier, Bruening et al. 1991; Niaudet and Gubler 2006). Patients with Frasier syndrome develop gonadoblastoma, nephrotic syndrome caused by focal segmental glomerulosclerosis (FSGS) and progressing renal failure (Gubler, Yang et al. 1999; Koziell and Grundy 1999). Wt1 has been reported to regulate the expression of podocyte proteins such as podocalyxin and nephrin (Palmer, Kotsianti et al. 2001; Guo, Morrison et al. 2004; Wagner, Wagner et al. 2004).

Lmx1b

Mutations in the *Lmx1b* gene that encodes LIM homeobox transcription factor 1 β (Lmx1b) cause the autosomal dominant disorder nail-patella syndrome. The phenotypic characteristics of the disease include: skeletal malformations, nail dysplasia, patellar abnormalities, nephropathy and glaucoma (Dreyer, Zhou et al. 1998; Vollrath, Jaramillo-Babb et al. 1998; Bongers, Huysmans et al. 2005). Renal disease is seen in 40% of nail-patella syndrome patients, and it is an important factor in predicting the prognostic outcome. The pathological changes observed in nephrotic syndrome are a thicker, discontinuous GBM with fibrillar collagen inclusions and foot process effacement (Rasclé, Suleiman et al. 2007).

Lmx1b knockout mice show similar phenotypic characteristics seen in nail-patella syndrome as well as lack of foot processes and SD formation (Chen, Lun et al. 1998; Miner, Morello et al. 2002; Rohr, Prestel et al. 2002). However, Lmx1b heterozygous mice (the genotype in nail-patella patients) do not present a podocyte phenotype (Rohr, Prestel et al. 2002). Several important podocyte genes have been shown to be dysregulated in these mice including podocin, CD2AP and type IV collagen. Although, it is worth mentioning that the dysregulation of these genes have not been reported in nail-patella patients (Suleiman, Heudobler et al. 2007).

1.3.3.2 Podocyte processes

The podocyte primary/major processes are large cytoplasmic projections of the cell body. The major processes, unlike the cell body, almost lack organelles but have an abundant cytoskeleton composed of microtubules, and intermediate filaments such as vimentin (Drenckhahn and Franke 1988; Pavenstadt, Kriz et al. 2003); however, the molecular nature of major processes is still poorly understood. On the other hand, the foot processes are composed of a dense network of actin filaments connecting the neighboring foot processes (Faul, Asanuma et al. 2007).

The plasma membrane of foot processes is subdivided into three compartments including the apical, slit diaphragm and basal surfaces, which are connected via a number of linker proteins and the actin cytoskeleton (Oh, Reiser et al. 2004; Patrakka and Tryggvason 2009). The reorganization of podocyte foot process cytoskeleton in pathological conditions is known as foot process effacement, which is often observed in proteinuric renal diseases (Oh, Reiser et al. 2004). While the molecular composition of podocyte foot processes is well characterized (Patrikka and Tryggvason 2009), a missing focus in the podocyte field is on the role of major processes and its molecular composition in renal diseases. The differences in the morphology of the primary and the foot processes indicate an obvious molecular and functional divergence between these structures.

1.3.3.3 Foot processes: apical surface

Podocalyxin

The apical and lateral surfaces of podocyte foot processes have a negative charge contributing to the overall negative charge of the glomerular filtration barrier. The abundant expression of podocalyxin, a sialylated and sulfated transmembrane protein is responsible for the negative charge present in podocytes (Nielsen and McNagny 2009). During development, podocalyxin is first present only at the apical surface of podocyte foot processes, and as the podocyte differentiates, podocalyxin expression migrates along the lateral surfaces above the slit diaphragm. Podocalyxin has been reported to have antiadhesive properties that maintain the filtration slits open through charge repulsion (Schnabel, Dekan et al. 1989).

Experimental studies suggest the essential role of podocalyxin in the filtration slit structure and function. Although podocalyxin deficient mice have podocytes, they fail to form foot processes and slit diaphragms. These mice also show severe kidney defects, anuric renal failure and neonatal lethality (Doyonnas, Kershaw et al. 2001). Podocalyxin has been shown to associate to actin filaments through interaction with the PDZ-containing protein, Na⁺/H⁺ exchanger regulatory factor 2 (NERF2) (Takeda, McQuistan et al. 2001), and the linker protein ezrin (Orlando, Takeda et al. 2001). The disruption of podocalyxin/NERF2/ezrin complex has been shown in pathological conditions associated with foot processes effacement, suggesting an important role of these proteins in disease (Takeda, McQuistan et al. 2001).

1.3.3.4 Foot processes: slit diaphragm

Podocyte foot processes of neighboring cells interdigitate and connect through specialized cell-cell contacts known as slit diaphragm (SD) (Tryggvason and Wartiovaara 2005). In the 1970s Karnovsky and colleagues first described the SD as a zipper-like structure. It was suggested to contain a central band between two neighboring foot processes that were bridged by even spaced filaments forming a porous size-selective structure (Rodewald and Karnovsky 1974). At present, the SD is considered to be a three dimensional structure composed of a complex of proteins that may resemble a dynamic sieve-like structure with small pores that are 5-15 nm in size, as suggested by Karnovsky and colleagues (Jefferson, Nelson et al. 2011). Besides

contributing to the structure of filtration barrier, the slit diaphragm proteins have an important role in podocyte signaling, and connect to the podocyte cytoskeleton machinery (Pavenstadt, Kriz et al. 2003; Faul, Asanuma et al. 2007).

Nephrin

Mutations in the *NPHS1* gene that encodes nephrin cause congenital nephrotic syndrome of the Finnish type (CNF), highlighting the crucial role of nephrin and the SD in the glomerular filtration barrier function (Tryggvason and Wartiovaara 2001). CNF is an autosomal recessive disorder characterized by absence of SD between podocyte foot processes, and massive proteinuria in *utero* (Lenkkeri, Mannikko et al. 1999). Similarly, the genetic deletion of nephrin in mice causes massive proteinuria, foot process effacement and early death (Putala, Soininen et al. 2001; Rantanen, Palmén et al. 2002).

Nephrin is a 180-kDa transmembrane protein belonging to the immunoglobulin family of cell adhesion molecules. It is thought that nephrin molecules from adjacent foot processes interact in the SD and perhaps in that way form the filter structure proposed by Karnovsky and colleagues (Rodewald and Karnovsky 1974; Khoshnoodi, Sigmundsson et al. 2003). Besides providing structural function, nephrin is thought to participate in signal transduction by phosphorylation of tyrosine residues present in the intracellular domain. Nephrin is also associated with mechanisms regulating the podocyte actin dynamics (Zenker, Machuca et al. 2009). For example, nephrin is tyrosine phosphorylated by Fyn kinase leading to interaction with nck and subsequently stimulating actin polymerization (Verma, Kovari et al. 2006). Experimental studies with Fyn and nck deficient mice were shown to develop a compromised filtration barrier (Yu, Yen et al. 2001; Jones, New et al. 2009).

Podocin

Mutations in the *NPHS2* gene that encodes podocin was identified to be the cause of autosomal recessive steroid-resistant nephrotic syndrome (SRNS) characterized by early onset nephrotic syndrome with resistance to steroid therapy and fast progression to end-stage renal disease (ESRD) (Boute, Gribouval et al. 2000). Podocin is an integral membrane protein exclusively expressed in podocytes. It is a 42-kDa protein that has a hairpin-like transmembrane domain and cytosolic N- and C-terminal domains

(Horinouchi, Nakazato et al. 2003). Podocin is localized in the SD where it has been shown to interact with other SD proteins such as nephrin, CD2AP and Neph1 (Huber, Kottgen et al. 2001; Schwarz, Simons et al. 2001; Sellin, Huber et al. 2003). Podocin is thought to serve as a scaffold protein essential to maintain the structural integrity of the slit diaphragm (Schwarz, Simons et al. 2001). Podocin deficient mice present a severe phenotype including foot process fusion, deficient formation of SD, proteinuria, and perinatal lethality (Roselli, Heidet et al. 2004).

Neph1

Neph1 is a nephrin-like transmembrane protein of the immunoglobulin superfamily and it has been localized in the SD (Donoviel, Freed et al. 2001). Deletion of *neph1* in mice has been reported to cause proteinuria, foot process effacement, and early postnatal death suggesting an important role in maintaining the structure of the filtration barrier (Donoviel, Freed et al. 2001). Neph1 has been reported to interact with both nephrin and podocin in the SD (Barletta, Kovari et al. 2003; Sellin, Huber et al. 2003). Furthermore, *neph1* interacts with the nephrin-Nck signaling pathway involved in actin polymerization (Garg, Verma et al. 2007), which could explain the cause of the renal phenotype similar to that observed in nephrin and podocin deficiencies.

Fat1

Fat1 is a large transmembrane protein of the cadherin superfamily characterized by multiple repeats of the cadherin motif and cell adhesion properties (Suzuki 1996; Inoue, Yaoita et al. 2001). Fat1 has been localized to the SD and deletion of *Fat1* in mice results in lack of SD formation, foot process effacement, and perinatal lethality, indicating an important role in SD development and maintaining the structure of the filtration barrier (Cai, Liu et al. 2000). These mice also show defective eye and brain development. Fat1 has been shown to interact with ZO-1, a tight junction protein associated with the slit diaphragm (Inoue, Yaoita et al. 2001). Fat1 has been reported to regulate cell-cell adhesion by controlling actin dynamics (Tanoue and Takeichi 2004).

TRPC6

Transient receptor cation channel 6 (TRPC6) belongs to the TRP superfamily of ion-channel forming proteins involved in Ca^{2+} transport into the cell (Schlondorff and Pollak 2006). In the kidney, TRPC6 has shown to have a broad expression pattern wherein the glomerulus, expression is found in all three cell types; mesangial and endothelial cells, as well as in podocytes as a component of the SD, and major processes (Reiser, Polu et al. 2005; Winn, Conlon et al. 2005) (Schlondorff and Pollak 2006). Mutations in *TRPC6* gene have been reported to cause autosomal dominant focal segmental glomerulosclerosis type 2 (FSGS2) characterized by proteinuria and glomerular lesions (Reiser, Polu et al. 2005; Winn, Conlon et al. 2005). The mutations in TRPC6 have shown gain-of-function properties leading to increased levels of Ca^{2+} influx and defective cytosolic signaling. TRPC6 has been associated with the podocyte actin cytoskeleton wherein overexpression of TRPC6 was reported to induce actin cytoskeleton rearrangement (Moller, Wei et al. 2007).

1.3.3.5 Foot processes: basal surface

Integrin

Podocyte foot processes are attached to the GBM through cell-matrix adhesion molecules such as integrins and dystroglycans (Mundel and Shankland 2002). Integrins are $\alpha\beta$ heterodimeric transmembrane proteins that mediate cell-cell and cell-matrix communication, and provide a link between extracellular matrix and the cytoskeleton (Lowell and Mayadas 2012). The major integrin at the sole of podocyte foot processes is $\alpha3\beta1$ heterodimer. $\alpha3\beta1$ integrins mediate interaction with the GBM through laminin, type IV collagen and fibronectin (Adler 1992). $\alpha3\beta1$ also provide a link between the intracellular cytoskeleton and the GBM via paxillin, talin, vinculin, α -actinin and filamin (Faul, Asanuma et al. 2007).

The important role of integrins in filtration barrier function has been reported in several studies. For example, deletion of $\alpha3$ in mice has been reported to cause abnormal kidney development including defective glomerular capillaries, thickening and fragmentation of the GBM, lack of foot processes in podocytes, and perinatal death (Kreidberg, Donovan et al. 1996). Furthermore, podocyte-specific $\alpha3$ -deficient mice develop massive proteinuria accompanied by partially sclerosed glomeruli,

disorganized GBM and foot process effacement (Sachs, Kreft et al. 2006). In addition, podocyte-specific $\beta 1$ -deficient mice exhibit a severe glomerular phenotype including GBM abnormalities, foot process effacement, massive proteinuria, and neonatal death (Kanasaki, Kanda et al. 2008; Pozzi, Jarad et al. 2008).

Integrin-linked kinase (ILK) is an intracellular protein kinase that provides a link between the GBM and podocytes. Podocyte-specific ILK deficiency in mice has been reported to cause progressive FSGS, proteinuria, foot process effacement, GBM abnormalities, and renal failure, suggesting the important role of molecules mediating GBM-podocyte adhesion in glomerular filtration function (Dai, Stolz et al. 2006; El-Aouni, Herbach et al. 2006).

Dystroglycan

Dystroglycan, another class of $\alpha\beta$ heterodimeric cell-adhesion transmembrane molecule, has also been localized at the sole of podocyte foot processes. Dystroglycan has been reported to interact to the GBM through laminin, agrin and perlecan, and to the intracellular cytoskeleton, through utrophin (Raats, van den Born et al. 2000; Regele, Fillipovic et al. 2000). The role of dystroglycans in podocyte and the glomerular filtration barrier function remains elusive.

CD151

The cell surface protein, CD151, is a member of tetraspanin superfamily. CD151 is ubiquitously expressed and associated with integrins (Rubinstein 2011). In the glomerulus, CD151 has been localized at the basal surface of podocyte foot processes binding to $\alpha 3\beta 1$ integrin (Sterk, Geuijen et al. 2002). CD151-deficient patients show end-stage renal disease with focal thickening, and irregularities of the GBM, as well as skin lesions and hearing impairment (Karamatic Crew, Burton et al. 2004). CD151-null mice also show GBM fragmentation and thickening, focal glomerulosclerosis, proteinuria, and foot process effacement, suggesting the important role of CD15- $\alpha 3\beta 1$ integrin interaction in podocyte foot processes (Sachs, Kreft et al. 2006).

1.3.3.6 Foot processes: actin cytoskeleton

The plasma membrane of foot processes is subdivided into three aspects, which are in contact via the cytoskeleton (Nishibori, Katayama et al. 2011). The cytoskeletal machinery plays a central role in maintaining the structural and functional integrity of the filtration barrier. The reorganization of the cytoskeleton is associated with foot process effacement, a compromised filtration barrier, and proteinuria (Faul, Asanuma et al. 2007). This is not a surprising phenomenon since the podocyte cytoskeletal machinery is central to podocyte morphology, which in turn is key to proper function. In fact, many SD components are either directly or indirectly linked to the actin cytoskeleton (Faul, Asanuma et al. 2007). Hence, proteins regulating or stabilizing actin dynamics and the podocyte cytoarchitecture are highly important for the integrity of the glomerular filter.

α -actinin-4

α -actinin is a cytoskeletal actin-binding protein that cross links and bundles actin filaments. It interacts with a variety of proteins connecting the actin cytoskeleton to the cell membrane and regulates the activity of a variety of receptors (Otey and Carpen 2004). α -actinin-1 and α -actinin-4 are widely expressed and are the non-muscle (calcium sensitive) isoforms commonly associated with focal contacts and stress fibers (Sjoblom, Salmazo et al. 2008).

α -actinin-4 is abundantly expressed in podocyte foot processes and plays an important role in maintaining the glomerular filtration barrier function. Mutations in the *ACTN4* gene cause autosomal dominant FSGS, proteinuria, and progressing renal failure (Kaplan, Kim et al. 2000). Disease associated gene mutations have shown to cause, in part, a gain-of-function property resulting in a higher affinity to F-actin filaments (Kaplan, Kim et al. 2000; Weins, Kenlan et al. 2005), which could explain the podocytopathy and proteinuria observed in these patients. Furthermore, α -actinin-4 knockout mice develop severe glomerular lesions, foot process effacement, and proteinuria (Kos, Le et al. 2003).

Synaptopodin

The actin-associated protein synaptopodin is exclusively present in two highly dynamic structures; the dendritic spines of neurons and podocyte foot processes (Deller, Merten et al. 2000). Synaptopodin gene expression in podocytes has been shown to be differentiation dependent and correlated with the formation of cell processes (Mundel, Heid et al. 1997). Synaptopodin interacts with α -actinin and regulates its actin-bundling activity (Asanuma, Kim et al. 2005). In addition, synaptopodin interacts to the cytoplasmic adaptor protein CD2AP, which in turn directly interacts with the SD proteins, nephrin and podocin (Schwarz, Simons et al. 2001; Shih, Li et al. 2001; Huber, Kwoh et al. 2006).

Synaptopodin deficient mice exhibit spine and synaptic abnormalities but no renal phenotype (Asanuma, Kim et al. 2005). However, these mice were unable to recover from induced nephrotic syndrome and foot process effacement. Similarly, gene silencing of synaptopodin in cultured podocytes showed impaired actin filament reformation (Asanuma, Kim et al. 2005). Synaptopodin has also been shown to be an upstream regulator of RhoA, inducing the formation of stress fibers and cell motility in cultured podocytes (Asanuma, Yanagida-Asanuma et al. 2006). RhoA belongs to the family of small GTPases involved in various cell signaling pathways and regulation of actin dynamics (Jaffe and Hall 2005).

CD2AP

The adaptor protein CD2AP was first identified as a ligand interacting with the T-cell-adhesion protein CD2 (Wolf and Stahl 2003). In the kidney, CD2AP is primarily expressed in podocyte foot processes (Shih, Li et al. 1999). CD2AP knockout mice show a severe renal phenotype manifested as foot process effacement, proteinuria, and progressive glomerulosclerosis leading to kidney failure and death, suggesting a critical role in the glomerular filtration barrier function (Shih, Li et al. 1999; Li, Ruotsalainen et al. 2000). CD2AP heterozygous mice have an increased susceptibility to glomerular injury (Kim, Wu et al. 2003). In addition, CD2AP haploinsufficiency has been identified in patients with FSGS nephrotic syndrome (Kim, Wu et al. 2003). TGF- β -induced podocyte apoptosis has been associated with CD2AP insufficiency in mice, which could explain the pathomechanism and loss of podocytes observed in these animals (Schiffer, Mundel et al. 2004). CD2AP has been reported to interact with other

critical foot process proteins including nephrin, podocin and synaptopodin (Schwarz, Simons et al. 2001; Shih, Li et al. 2001; Huber, Kwoh et al. 2006). Thus, serving as a link between podocyte SD and foot process cytoskeleton.

Nck

Nck1 and nck2 are SH2/SH3 containing adaptor proteins implicated in tyrosine kinase-mediated signal transduction events that for instance involves regulation of actin dynamics (McCarty 1998). Nck1 or nck2 show overlapping and mutually compensatory functions, as mice lacking either nck1 or nck2 are viable without any renal phenotype (Bladt, Aippersbach et al. 2003). However, deletion of both nck genes was shown to cause embryonic lethality (Bladt, Aippersbach et al. 2003). In podocytes, nck proteins are localized in the cytoplasmic face of foot processes. Podocyte-specific nck1 and nck2 knockout mice displayed a similar renal phenotype observed in nephrin deficient mice, which include failure to form foot processes, proteinuria, glomerular lesions, as well as growth abnormalities (Jones, Blasutig et al. 2006). Nck has been reported to interact with the cytoplasmic tyrosine-containing domain of nephrin (Jones, Blasutig et al. 2006). Nephrin is tyrosine phosphorylated by Fyn kinase and subsequent interaction with nck stimulating actin polymerization. This could explain the renal phenotype similar to nephrin deletion (Tryggvason, Pikkarainen et al. 2006; Verma, Kovari et al. 2006; Jones, New et al. 2009).

1.4 GLOMERULOPATHY

A large amount of renal disorders are caused by injury to the glomerulus. Glomerulopathy may be due to primary renal disease where pathology is observed only in the glomerulus i.e., Immunoglobulin (Ig) A glomerulopathy (Lai 2012), or it may be secondary to a systemic disorder where multiple organs are affected as in diabetic nephropathy and lupus glomerulonephritis (Stitt-Cavanagh, MacLeod et al. 2009; Tsokos 2011). Glomerulopathies are broadly classified as nephritic or nephrotic syndrome. Nephritic syndrome is caused by inflammatory effects that are often associated with hematuria, mild proteinuria, hypertension, and decreased glomerular filtration rate (Moller, Pollak et al. 2006). On the other hand, nephrotic syndrome is

associated with massive proteinuria (>3.5 g protein/24 hours), hypoalbuminemia, edema, lipidemia and lipiduria (Jefferson, Nelson et al. 2011).

Proteinuria is the hallmark of glomerular disease often observed with foot process effacement, indicating a compromised filtration barrier (Patrakka and Tryggvason 2009). In a healthy individual only a small amount of albumin is lost in the final urine (Haraldsson and Sorensson 2004). The increased level of excreted albumin in the range of 30-300 mg/24 hours reflects microalbuminuria, whereas proteinuria is confirmed when urine excretes more than 300 mg of protein per day (Haraldsson, Nystrom et al. 2008). Proteinuria is caused by a variety of glomerular diseases; although most proteinuria syndromes are acquired, it is well known that genetic defects in the molecular components of podocyte foot processes or SD play a major role in many human hereditary proteinuria syndromes (Tryggvason, Patrakka et al. 2006). The identification and characterization of rare monogenic glomerular kidney diseases have provided valuable information in understanding the physiopathology of the glomerular filtration process (Mundel and Shankland 2002).

1.4.1 Idiopathic nephrotic syndrome

Three distinct histological variants of primary idiopathic nephrotic syndrome exist: membranous nephropathy (MN), minimal change nephrotic syndrome (MCNS), and focal segmental glomerulosclerosis (FSGS) (Wei and Reiser 2011). A common denominator in all three disease forms is injury to podocytes. Corticosteroid and immunosuppressive drug therapy is the main treatment in these patients, however, a significant proportion of patients do not respond to therapy (Eddy and Symons 2003).

1.4.1.1 Membranous nephropathy

MN is the most common cause of idiopathic nephrotic syndrome in adults, although in some patients it may be secondary to infection, drugs and toxic substances or other diseases such as autoimmune diseases, diabetes mellitus, and cancer (Ponticelli 2007). The disease exhibits a spectrum of severity and a majority of patients may have spontaneous remission or partial remission, whereas a considerable proportion with severe proteinuria progress toward end-stage renal disease (ESRD) within 5 to 15 years

(Nangaku, Shankland et al. 2005). MN is characterized by the *in situ* formation of immune-complex deposits located between the GBM and podocytes causing a membrane-like thickening of the glomerular capillary wall (Cybulsky 2011). The immune-complexes are formed locally due to a reaction between a circulating antibody and podocyte antigen (Ponticelli 2007). These immune-complexes trigger inflammation and complement activation and induce podocyte injury such as foot process effacement (Nangaku, Shankland et al. 2005).

1.4.1.2 Minimal change nephrotic syndrome

MCNS is the most common cause of idiopathic nephrotic syndrome in children and is clinically characterized by rapid onset of proteinuria (Eddy and Symons 2003). The usual presenting complaint in these patients is facial edema and abdominal distension (Tune and Mendoza 1997). The histology of glomeruli appear normal on light microscope despite the presence of massive proteinuria, however, electron microscopy of kidney biopsies show extensive foot process effacement (Chugh, Clement et al. 2012). The morphology of the GBM remains unchanged although reduced anionic charge has been reported (Chugh, Clement et al. 2012). The majority of MCNS patients respond to therapy, however, a high rate of relapse is present (Eddy and Symons 2003). Renal biopsy in these patients is only required if they do not respond to steroids, and in that case, one of the likely histological diagnosis is FSGS (Mathieson 2007).

1.4.1.3 Focal segmental glomerulosclerosis

FSGS is a major cause of ESRD (Daskalakis and Winn 2006). The initial clinical presentation may overlap to that observed in MCNS. FSGS is a subtype of idiopathic nephrotic syndrome (Meyrier 2005), however, deficiencies of several podocyte proteins (mentioned earlier) including nephrin, podocin, α -actinin-4, TRPC6 and CD2AP cause hereditary forms of FSGS and nephrotic syndrome. FSGS is not a disease but a pathological lesion observed first in the glomerulus that progress to other parts of the renal tissue (Meyrier 2005). The pathological diagnosis is characterized by the presence

of segmental collagenous sclerotic lesions in some glomeruli where only a part of the glomerulus is affected, hence the name focal segmental (Deegens, Steenbergen et al. 2008). Glomerular sclerosis is observed in a variety of kidney diseases and FSGS is considered to have a multifactorial etiology.

FSGS often presents a heterogeneous phenotype, however, proteinuria is detected in all FSGS patients indicating a compromised filtration barrier (Reidy and Kaskel 2007). Nephrotic FSGS is considered to be a disease associated with podocyte injury leading to decreased number of podocytes (Crosson 2007). The main treatment therapy in FSGS patients is corticosteroid and immunosuppressive drugs (Meyrier 2005). However, the majority of FSGS patients do not respond to therapy and can progress to chronic renal insufficiency (Wei and Reiser 2011). Recurrence of nephrotic syndrome and glomerular lesions is present in about 30% of renal transplants (Meyrier 2004).

1.4.2 The podocyte is a clear candidate for therapy in proteinuria

Glomerulopathies are a major cause of chronic kidney disease (CKD) that can progress to end-stage renal disease (ESRD). ESRD, with an increasing prevalence, is a significant cause of morbidity and mortality worldwide (Ruggenenti, Schieppati et al. 2001; Daskalakis and Winn 2006). ESRD can only be treated with continuous dialysis or renal transplantation (Leeuwis, Nguyen et al. 2010). In addition to poor clinical outcome, CKD and ESRD patient-care is a tremendous burden on healthcare budgets (Rastogi, Linden et al. 2008).

Considering the magnitude of the problems associated with CKD and ESRD it is essential to establish novel therapeutics aimed at the cellular level. As podocyte dysfunction has a crucial role in the initiation and progression of glomerulopathies, it clearly is a candidate for therapy in proteinuria (Patrakka and Tryggvason 2009; Mathieson 2012).

2 AIMS OF THE STUDY

This study is based on a previous genome-wide approach aimed to characterize the transcriptome of the renal glomerulus through large-scale sequencing and microarray profiling (Takemoto, He et al. 2006). More than 300 highly glomerulus-enriched transcripts were identified. In the thesis several of these transcripts were further characterized. The specific aims of the project are:

Paper I: To characterize the role of novel podocyte proteins **sult1b1** and **ankrd25** in the glomerular filtration barrier function

Paper II: To characterize the role of novel podocyte protein **pdlim2** in the glomerular filtration barrier function

Paper III: To characterize the role of novel podocyte proteins **hip1**, **nfasc** and **olfml2a** in the glomerular filtration barrier function

Paper IV: To characterize the role of novel podocyte protein **gprc5b** in the glomerular filtration barrier function

3 RESULTS AND DISCUSSION

It is known that several podocyte proteins essential for the glomerular filtration barrier function, such as nephrin, podocin and synaptopodin, show a very restricted expression pattern outside of the podocyte (Patrakka and Tryggvason 2010). With this in mind, we were primarily interested in identifying podocyte-associated proteins, which had not been previously reported in the glomerulus, and displayed a similar restricted expression pattern in the kidney and outside of the glomerulus. The table below includes the list of proteins identified in this study. In the thesis the proteins will be referred to by the abbreviated version.

Table I List of proteins described in the papers

| Name | Abbreviation |
|--|--------------|
| Sulfotransferase family 1B, member 1 | sult1b1 |
| KN motif and ankyrin repeat domains 2 | ankrd25 |
| PDZ and LIM domain 2 | pdlim2 |
| Huntingtin interacting protein 1 | hip1 |
| Neurofascin | nfasc |
| Olfactomedin-like 2A | olfml2a |
| G protein-coupled receptor family C, group 5, member B | gprc5b |

3.1 PAPER I: EXPRESSION OF NOVEL PODOCYTE-ASSOCIATED PROTEINS SULT1B1 AND ANKRD25

3.1.1 Expression of sult1b1 in the glomerulus

Sult1b1 is a member of the cytosolic sulfotransferase (SULT) protein family (Hildebrandt, Carrington et al. 2007). These enzymes transfer sulfate groups to specific molecules. Sulfotransferases are classified in two families: 1) the cytosolic SULTs involved in metabolism of drugs, steroids, bile acids and neurotransmitters, and 2) membrane-bound SULTs found in the Golgi apparatus which are involved in

sulfonation of peptides, proteins, lipids and glycosaminoglycans (Gamage, Barnett et al. 2006). Previous studies have reported the expression of sult1b1 in the liver, intestine and leukocytes (Dombrovski, Dong et al. 2006).

In this study, the expression of sult1b1 transcript in various mouse tissues was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Sult1b1 expression was observed in the liver, the brain and the glomerulus, whereas no expression was observed in the rest of the kidney fraction devoid of glomeruli. By using the polyclonal antibodies produced against the corresponding human protein we could further analyse the expression in the kidney. Using immunofluorescence staining in normal adult human kidney sections, sult1b1 was detected in the glomerular tuft, and no obvious positive signal was detected in the extraglomerular areas.

In Western blotting of human kidney lysates, several bands (~120, ~65, ~55, and ~35) were observed in the glomerular fraction, whereas no reactivity was observed in the kidney fraction lacking glomeruli. The predicted size of sult1b1 protein is ~35 kDa (www.ensembl.org) corresponding to the smallest protein recognized by our antibody. The subcellular distribution of sult1b1 protein was analyzed by double staining with Golgi marker, GM130. Colocalization revealed sult1b1 as a novel component of podocyte Golgi apparatus. Based on the restricted expression pattern of sult1b1, we speculate that this enzyme has a dedicated role in the podocytes; it could be involved in sulfonation of podocyte proteins such as podocalyxin, a highly sulfated and abundantly expressed protein in the podocytes. Podocalyxin is, as mentioned earlier, essential for maintaining the architectural integrity of foot processes and filtration barrier (Takeda, Go et al. 2000).

3.1.2 Expression of ankrd25 in the glomerulus

Ankrd25, also known as kank2, is member of a family of proteins (kank1-4) that contain N-terminal coiled-coil motifs and C-terminal ankyrin repeats (Sarkar, Roy et al. 2002). Ankyrin-repeats are highly conserved motifs mediating protein-protein interactions (Sedgwick and Smerdon 1999). The kank protein family has been shown to be involved in the regulation of actin polymerization and cell motility, and they have been associated with cancer, neuronal and developmental disorders (Kakinuma, Zhu et al. 2009).

In a previous report, the expression of *ankrd25* was observed in all human tissues that were analyzed, in which the detected expression levels were highest in cervix, colon, heart, kidney and lung tissues (Zhu, Kakinuma et al. 2008). The ubiquitous expression pattern of the *ankrd25* was also observed in our RT-PCR results. Concurrent with our microarray data (Takemoto, He et al. 2006), in Western blotting of human kidney lysate, a band of the size ~120 kDa was detected in the glomerular fraction. However, a very weak signal was detected in the rest of the kidney fraction.

Using immunofluorescence staining, a closer examination of *ankrd25* expression pattern in the kidney showed expression in podocytes, mesangial and vascular smooth muscle cells. In the podocyte, *ankrd25* was shown to partially colocalize with nephrin, indicating localization in the foot processes. In light of the fact that, the cytoskeletal machinery of podocytes play a key role in glomerular filtration barrier integrity, and the previously reported role of *ankrd25* in the regulation of actin dynamics, we speculate that *ankrd25* may play a role in regulating actin dynamics in podocyte foot processes.

3.2 PAPER II: PDLIM2 IS A NOVEL ACTIN-REGULATING PROTEIN OF PODOCYTE FOOT PROCESSES

3.2.1 Expression of *pdlim2* in the glomerulus

Pdlim2, also known as *mystique*, is a member of the actinin-associated LIM protein (ALP) family, which contain one N-terminal PDZ domain and one C-terminal LIM domain (Te Velthuis, Isogai et al. 2007). PDZ-LIM containing proteins are highly conserved scaffold proteins that mediate protein-protein interactions. These proteins are involved in a wide range of important biological processes including: cytoskeletal organization, organogenesis, and neuronal signaling (te Velthuis and Bagowski 2007).

In this study, we identified *pdlim2* as a novel molecular component of podocyte foot processes. The expression of *pdlim2* transcript in the kidney glomerulus in comparison to the rest of the kidney was confirmed by RT-PCR. At the protein level, the expression was confirmed with Western blotting of human kidney lysates and immunostainings. In Western blotting, a band of the size ~40 kDa was detected in the glomerular fraction, while no band was observed in the rest of the kidney fraction. The subcellular

localization of pdlim2 in podocytes was shown with immunofluorescence staining and immunoelectron microscopy.

In immunofluorescence staining of adult human kidney sections, a strong positive signal was detected in the glomerular tuft, whereas no signal was observed outside the glomeruli. Double staining with synaptopodin (foot process protein) and phalloidin (detecting actin) showed colocalization in foot processes; however, no colocalization was observed when double staining with vimentin (a marker for major processes). Localization of pdlim2 in the glomerulus by immunoelectron microscopy was observed in the cytosol of foot processes. Gold particles were mainly found in the cytoskeleton, observed as electron-dense areas. In addition, double labelling of pdlim2 and synaptopodin showed a close localization of these two proteins in the foot processes.

3.2.1.1 The role of pdlim2 in actin dynamics

Previously, pdlim2 has been suggested to act as an adaptor allowing for the interaction of various proteins involved in actin cytoskeleton (Torrado, Senatorov et al. 2004). In line with this, we suggest that pdlim2 is a novel actin-regulating molecule of podocyte foot processes. This was shown in cultured podocytes where pdlim2 was associated with stress fibers and cortical actin. The association of pdlim2 with stress fibers was investigated by transfecting differentiated human podocytes (Saleem, O'Hare et al. 2002) with full-length pdlim2, and treating them with latrunculin A at various concentrations. Latrunculin A is a drug that disrupts the actin cytoskeleton by preventing the repolymerization of actin monomers into filaments (Morton, Ayscough et al. 2000). Upon latrunculin A treatment, the pdlim2-positive cells displayed intact stress fibers in comparison to pdlim2-negative cells. This observation was better visible with an increasing concentration of latrunculin A. The loss of stress fibers caused significant changes in cell morphology; thus, pdlim2 stabilized actin structures in podocytes and promoted the maintenance of intact cell morphology under pathological stimulus.

To characterize the functional role of pdlim2 in actin dynamics and uncover interacting partners we performed the yeast two-hybrid screen. Two proteins associated with actin dynamics and identified in pdlim2 yeast two-hybrid screen, α -actinin-4 (Kaplan, Kim et al. 2000) and angiomin-like 1 (amotl1) (Gagne, Moreau et al. 2009), were further investigated. Double staining experiments in cultured podocytes revealed

colocalization of *pdlim2* with α -actinin-4 and *amotl1* in stress fibers. Double staining of adult human kidney sections with *amotl1* and α -actinin-4 showed colocalization with *pdlim2*. Furthermore, *pdlim2* interaction with α -actinin-4 and *amotl1* was confirmed with co-immunoprecipitation experiments in double-transfected HEK293 cells. As mentioned earlier, mutations in the α -actinin-4 gene has been associated with foot process effacement and proteinuria (Kaplan, Kim et al. 2000).

We speculate that *pdlim2* is likely to be linked in these disease processes as it directly interacts with α -actinin-4 and regulates actin dynamics in podocytes. Similarly, *Amotl1* may be involved in the regulation of actin dynamics in podocyte foot processes. Of note, we initially analyzed the renal phenotype of a *pdlim2*-deficient mouse line, and the animals did not show any clear glomerular abnormalities (unpublished data).

3.2.1.2 Differential expression of *pdlim2* in disease

The expression of *pdlim2* in biopsies of human proteinuric diseases including minimal change nephrotic syndrome (MCNS), membranous nephropathy (MN), and focal segmental glomerulosclerosis (FSGS) was examined by semi-quantitative immunoelectron microscopy. The expression of *pdlim2* in FSGS patients was unchanged compared to controls, although extensive foot process effacement was present. On the other hand, *pdlim2* expression was significantly decreased in MN and MCNS patient biopsies. Thus, *pdlim2* may be a useful diagnostic marker for MCNS as the differential diagnosis between FSGS and MCNS can be difficult in the clinic due to the overlapping histopathological features observed in both diseases (Eddy and Symons 2003).

3.3 PAPER III: HUNTINGTIN INTERACTING PROTEIN 1, NEUROFASCIN AND OLFACTOMEDIN-LIKE 2A ARE NOVEL COMPONENTS OF PODOCYTE MAJOR PROCESSES AND DEMONSTRATE THE PRESENCE OF PODOCYTES IN GLOMERULAR CRESCENTS

3.3.1 Expression of hip1 in the glomerulus

Hip1 has previously been studied mostly in the brain. It is known to be an endocytic adaptor protein involved in clathrin-mediated vesicle trafficking with the ability to bind to actin (Legendre-Guillemain, Wasiaik et al. 2004; Wilbur, Chen et al. 2008). In addition, hip1 is present in a complex with glutamate receptors, and studies in knockout animals have shown that hip1 facilitates glutamate signaling (Metzler, Li et al. 2003). We have identified hip1 as a novel component of podocyte major processes. The expression of the transcript was confirmed with RT-PCR in the glomerulus as opposed to the rest of the kidney. At the protein level, the expression was confirmed with Western blotting of human kidney tissue samples and immunostainings.

In Western blotting, a band of the size ~115 kDa was detected in the glomerular fraction, while no band was observed in the rest of the kidney fraction. In immunofluorescence staining of adult human kidney sections, a strong positive signal was detected in the glomerular tuft and no signal was detected in the extraglomerular areas. Overlapping double staining with vimentin, a marker for major processes suggests the localization of hip1 to major processes. In line with this, the majority of gold particles (55%) observed in immunoelectron microscopy experiments were located in the major processes.

To further analyze the expression of hip1 in the glomerulus and the association to major processes in development we used immunofluorescence staining in developing human kidney sections. Hip1 expression was observed in the capillary stage glomerulus, whereas no expression was observed in the earlier developmental stages, vesicle and S-shape glomerulus. Throughout the development of podocytes, the staining for hip1 colocalized with the major process marker vimentin. The capillary stage glomerulus is, in fact, the time at which the formation of major processes starts, which supports the idea that hip1 is associated with major processes. Previously, glutamate signaling has been reported to have a crucial role in podocytes (Giardino, Armelloni et al. 2009), and hip1 could be linked to the glutamate signaling machinery. However, our findings are conflicting with the fact that: 1) glutamate signaling is

associated with the podocyte slit diaphragm, and not major processes, and 2) the expression of other glutamate signaling proteins have so far not been observed in immature podocytes (Giardino, Armelloni et al. 2009). In this respect, we speculate that hip1 may have a role, separate from that of glutamate signaling pathway in podocytes. Hip1 may be linked to the prominent cytoskeletal machinery that governs the podocyte integrity. Of note, we initially analyzed the renal phenotype of hip1 knockout mice (Metzler, Li et al. 2003), and the animals did not show any overt renal phenotype (unpublished data).

3.3.2 Expression of nfasc in the glomerulus

Nfasc has previously been thought to be specific for the neuronal tissues where three major isoforms: NF155, NF166, and NF186 have been identified (Sherman, Tait et al. 2005). Nfasc is a member of L1 cell adhesion transmembrane molecules (L1-CAMs) (Herron, Hill et al. 2009). The cytoplasmic portion of these proteins has the ability to bind to the cytoskeletal protein ankyrin (Herron, Hill et al. 2009). In addition, the cytoplasmic tail of nfasc has been shown to bind to doublecortin, a microtubule binding/stabilizing protein (Kizhatil, Wu et al. 2002). Notably, mutation in the drosophila ortholog of nfasc impairs the assembly of microtubules in neurons (Godenschwege, Kristiansen et al. 2006). Consistent with this, nfasc deficient mice die soon after birth and display severe defects in the formation of axons (Sherman, Tait et al. 2005).

We have identified nfasc as a novel component of podocyte major processes. The expression of the transcript was confirmed with RT-PCR in the glomerulus as opposed to the rest of the kidney. At the protein level, the expression was confirmed with Western blotting of human kidney tissue samples and immunostainings. In Western blotting, a band of the size ~185 kDa was detected in the glomerular fraction, while no band was observed in the rest of the kidney fraction.

In immunofluorescence staining of adult human kidney sections, a strong positive signal was detected in the glomerular tuft and no signal was detected in the extraglomerular areas. Overlapping staining with vimentin, a marker for major processes, suggest the localization of hip1 to major processes. In immunofluorescence staining of developing kidney sections, nfasc expression was first observed at the capillary loop stage glomerulus wherein it colocalized with vimentin. No signal was

observed in the earlier stages, vesicle and S-shape glomerulus. These findings support the idea that nfasc associates with major processes, and could play a role in the microtubular dynamics of these cellular projections, similarly to that seen in drosophila (Godenschwege, Kristiansen et al. 2006). Of note, we have identified a nfasc-binding protein, doublecortin, in major processes (unpublished data), and at present, we are generating a podocyte-specific knockout mouse line to elucidate the functional role of nfasc in the kidney.

3.3.3 Expression of olfml2a in the glomerulus

Olfml2a is a member of olfactomedin domain containing proteins predicted to be a secreted protein associated with the plasma membrane (Tomarev and Nakaya 2009). We have identified olfml2a as a novel component of podocyte major processes. The expression of the transcript was confirmed with RT-PCR in the glomerulus as opposed to the rest of the kidney. At the protein level, the expression was confirmed with Western blotting of human kidney tissue samples and immunostainings. In Western blotting, a band of the size ~110 kDa was detected in the glomerular fraction, while no band was observed in the rest of the kidney fraction.

In immunofluorescence staining of adult human kidney sections, a strong positive signal was detected in the glomerular tuft while no signal was detected in the extraglomerular areas. Overlapping double staining with vimentin, a marker for major processes suggests the localization of olfml2a to major processes. Consistent with this, in immunoelectron microscopy experiments, the majority of gold particles (85%) were found in major processes. In immunofluorescence staining of developing kidney sections, olfml2a expression was not detected at the early vesicle stage glomerulus. At S-shape glomerulus, olfml2a was detected in the invading endothelial cells; however, olfml2a was not observed at a later stage in maturing glomerular endothelial cells. Olfml2a was first detected in the podocytes of the developing capillary loop stage, where it colocalized with vimentin. In accordance with this, overlapping expression of vimentin and olfml2a at the maturing stage glomerulus was detected at the apical aspects of podocytes. The above findings support the idea that olfml2a is associated with major processes both in developing and mature podocytes.

The molecular function of olfml2a is poorly understood. In a previous study, the olfactomedin domain of gliomedin has been shown to have the ability to bind to

neurofascin in Nodes of Ranvier of neurons, and contributing to the stabilization of these structures (Eshed, Feinberg et al. 2005). As olfm12a contains one olfactomedin domain, it is possible that this sort of interaction and functional similarities occurs in podocytes. Olfm12a, neurofascin and doublecortin may form a protein complex in major processes and stabilizing microtubular structures. Similarly to nfasc, a limitation to conduct molecular functional studies of olfm12a is the lack of its expression in immortalized podocyte cell lines.

3.3.4 Expression of novel podocyte markers in glomerular crescents

Crescentic lesions are often observed in inflammatory diseases affecting the glomerulus and are in general indicative of severe injury (Singh, Jeansson et al. 2011). These are characterized as a heterogenous composition of cells and matrix including: leukocytes, renal cells, and fibrin. The cellular composition of crescents has been unclear due to the presence and role of podocytes in these lesions (Singh, Jeansson et al. 2011). While some studies have reported the presence of podocytes in glomerular crescents (Moeller, Soofi et al. 2004; Thorner, Ho et al. 2008), other studies have reported the contrary (Nitta, Horita et al. 1999; Patrakka, Ruotsalainen et al. 2001; Usui, Kanemoto et al. 2003).

To gain further insight into this question, we used these novel podocyte markers in immunohistochemistry of crescentic human renal biopsies. Hip1 (14/26 crescents) and olfm12a (21/31 crescents) were detected in most cellular crescents. Of note, no reliable signal was detected with two different anti-nfasc antibodies. Previously, we reported pdlim2 as a novel component of podocyte foot processes (ref. paper II). Since pdlim2 is expressed earlier than other well-characterized foot process markers such as nephrin and synaptopodin, we used pdlim2 as a positive marker. Pdlim2 expression was observed in 30/43 cellular crescents identified. Thus, in this report, the glomerular crescents expressed three podocyte-specific markers providing further evidence for the presence of podocytes in these lesions.

3.4 PAPER IV: GPRC5B IS REQUIRED FOR THE INTEGRITY OF GLOMERULAR FILTRATION BARRIER

3.4.1 Expression of *gprc5b* in the glomerulus

Gprc5b is a member of the mammalian superfamily of G-protein-coupled receptors (GPCRs) (Brauner-Osborne and Krogsgaard-Larsen 2000). G-protein signaling is involved in many well-characterized cellular pathways; hence, molecules that affect GPCR pathways are widely used in drug therapy (Wess 1998). Podocytes express numerous GPCR, including receptors that are critical in the biology and disease of podocytes (Pavenstadt, Kriz et al. 2003).

Previously, the pathogenic role of G-protein signaling in podocytes has been reported using genetically modified mouse model strategies (Asanuma, Kim et al. 2005; Chen, Overcash et al. 2011; Boucher, Yu et al. 2012). In this report, we have identified a novel highly podocyte-specific GPCR. The expression of *gprc5b* transcript in mouse and zebrafish glomerulus was confirmed using RT-PCR. No signal was detected in the rest of the kidney devoid of glomeruli. At the protein level, the expression of *gprc5b* was confirmed with Western blotting of human kidney tissue samples and immunostainings.

In Western blotting, two bands of the sizes ~45 kDa and ~50 kDa were detected in the glomerular fraction, while no band was observed in the rest of the kidney fraction. However, the signal for the band of the size ~45 kDa was significantly stronger. In immunofluorescence staining of adult human kidney sections, a strong positive signal was detected in the glomerular tuft, while no signal was detected in the extraglomerular areas. Overlapping double staining with nephrin suggests the localization of *gprc5b* to foot processes; though, some signal was detected at the apical aspects of podocyte foot processes. Concurrent with immunofluorescence stainings, in immunoelectron microscopy experiments, the majority of gold particles (~90%) were localized to podocytes. In podocytes, gold particles were predominantly found within the apical membrane and the SD.

3.4.1.1 *The role of gprc5b in the zebrafish pronephron*

The zebrafish model was used to study the functional role of *gprc5b* in the glomerulus. Using RT-PCR, two *gprc5b* orthologs (*sgprc5b* and *ptgprc5b*) were detected in the zebrafish pronephros. However, *sgprc5b* showed a “pronephros-specific” expression pattern, similar to that observed for *gprc5b* in human glomerulus. The expression of *sgprc5b* in pronephros was similar to the expression of nephrin in the glomerulus, whereas, *ptgprc5b* expression was detected in both of the tissue samples, pronephros and the rest of fish devoid of pronephros. At the protein level, the expression of *gprc5b* was confirmed in Western blotting of zebrafish tissue samples. A band of the size ~35 kDa was detected in the pronephros fraction, while no band was observed in the control lane.

The role of *gprc5b* in the glomerulus was investigated by knocking down the expression of *sgprc5b* in zebrafish pronephros with antisense morpholino (MO) oligos. Severe cardiac edema was observed in 49% of the embryos that were injected with the morpholino (MO). This phenotype was first observed 72 h post-fertilization, the time point at which pronephros is functional. Pericardial edema in embryos injected with control MO was observed in only 6% of cases. Rescue experiments in zebrafish by co-injecting *sgprc5b* MO along with synthetic human *gprc5b* mRNA showed normal morphology, without any pericardial edema in 68% of cases as opposed to 51% observed in cases injected with only MO. A closer examination of the histology of the pronephros in *gprc5b* morphants showed expanded Bowman’s space. In electron microscopic analysis, similar expansion of the urinary space was detected. In addition, the podocyte foot processes were highly disorganized and seemingly infiltrating the basement membrane.

To further evaluate the effect of *gprc5b* knockdown in podocytes, we used a transgenic zebrafish line that expresses green fluorescence protein (GFP) from the zebrafish *podocin* promoter (He, Ebarasi et al. 2011). GFP expression in these fish is normally detected in 86% of the cases; however, injection of *sgprc5b* MO in these fish resulted in a reduced GFP expression to only 47% of the cases, indicating the occurrence of injury and/or the reduction of podocytes.

Pericardial edema observed in *sgprc5b* morphants has previously been reported in nephrin and podocin morphants, which in zebrafish is a sign of a compromised glomerular function (Kramer-Zucker, Wiessner et al. 2005). In addition, the expansion of Bowman’s capsule is also a pathological feature found in nephrin and podocin

zebrafish morphants, as well as in many human proteinuric diseases. Podocytes express numerous GPCRs that are important in biology and disease (Pavenstadt, Kriz et al. 2003). Several studies using genetically manipulated mouse models have provided evidence on the pathogenic role of G-protein signaling in podocytes (Asanuma, Kim et al. 2005; Xiao, He et al. 2011; Boucher, Yu et al. 2012).

Interestingly, studies on knockout mice line *gprc5a*, which is a member of the same protein family as *gprc5b*, have shown that the deletion of this receptor cause the activation of NF- κ B and STAT3 signaling pathway resulting in the formation of lung tumors (Chen, Deng et al. 2010; Deng, Fujimoto et al. 2010). Previous studies in transgenic mouse models have provided evidence that increased activation of NF- κ B and STAT3 signaling in podocytes is associated with podocyte damage and progressive renal diseases (Chuang and He 2010). For example, the progression of diabetic nephropathy in mice heterozygous for STAT3 null allele is significantly slower than in wild type animals (Hussain, Romio et al. 2009; Lu, Wang et al. 2009; Wiggins, Patel et al. 2010).

In this report, *gprc5b* knockdown in zebrafish exhibited classical features associated with loss of glomerular function. We hypothesize that *gprc5b* exerts an inhibitory effect on the NF- κ B and STAT3 pathways in podocytes, and the podocyte injury observed in *gprc5b* morphants may be caused by the increased activation of these pathways. Thus, *gprc5b* is a potential target candidate for pharmacological intervention in proteinuric renal diseases as it provides the opportunity to manipulate these signaling pathways specifically in podocytes.

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