Molecular mechanisms regulating glucose uptake into podocytes: The roles of ezrin, septin 7 and nonmuscle myosin IIA

Anita Agnieszka Wasik

Department of Pathology
Haartman Institute
University of Helsinki
Finland

ACADEMIC DISSERTATION

To be presented,
with permission of the Faculty of Medicine of the University of Helsinki,
for public examination
in the Lecture Hall 1 in the Haartman Institute, Haartmaninkatu 3, Helsinki,
on November 28th 2014, at 12 noon.

Helsinki 2014

Supervised by Docent, Associate professor Sanna Lehtonen
Department of Pathology
Haartman Institute
University of Helsinki
Helsinki, Finland

Reviewed by Docent Satu Kuure
Institute of Biotechnology
University of Helsinki
Helsinki, Finland

and

Professor Vesa Olkkonen
Minerva Institute for Medical Research
Helsinki, Finland

Official opponent Professor Karlhans Endlich
Institute of Anatomy and Cell Biology
University of Greifswald
Greifswald, Germany

Cover pictures: Immunofluorescence stainings of ezrin, septin 7 and nonmuscle myosin IIA in rat kidney and cultured podocytes.
ISBN 978-951-51-0114-3 (paperback)
ISBN 978-951-51-0115-0 (PDF)
http://ethesis.helsinki.fi
Hansaprint Oy
Helsinki 2014

To my family

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS 8

ABBREVIATIONS 9

ABSTRACT 10

1 INTRODUCTION 12

2 REVIEW OF THE LITERATURE 15

2.1 Overview of kidney structure and function 15

2.2 Glomerular filtration barrier 16

2.2.1 Endothelium 17

2.2.2 Glomerular basement membrane 17

2.2.3 Podocytes 17

2.2.4 Mesangium 18

2.3 Podocyte biology 18

2.3.1 Slit diaphragm 18

2.3.2 Basal domain of podocytes 19

2.3.3 Apical domain of podocytes 20

2.3.4 Podocyte cytoskeleton 20

2.3.4.1 Actin cytoskeleton assembly and reorganization 20

2.3.4.2 Components of the podocyte cytoskeleton 21

2.3.4.3 Podocyte proteins that regulate actin 22

2.4 Diabetic nephropathy 24

2.4.1 Definition and clinical features of diabetic nephropathy 24

2.4.2 Pathological features of diabetic nephropathy 25

2.4.2.1 Glomerular changes 25

2.4.2.2 Tubular changes 26

2.4.2.3 Interstitial changes 26

2.4.2.4 Vascular changes 27

2.4.3 Mechanisms associated with the development of diabetic nephropathy 27

2.5 Animal models of diabetic kidney disease 29

2.5.1 Streptozotocin-induced diabetic rats 30

2.5.2 Zucker rats 30

2.6 Podocytes in diabetes 30
2.6.1 Podocyte loss 31
2.6.2 Podocyte foot process effacement 31
2.7 Role of kidneys in maintaining glucose homeostasis 32
2.7.1 Role of kidneys in the regulation of glucose homeostasis 32
2.7.2 Glucose homeostasis in the kidney in diabetes mellitus 33
2.8 Molecular mechanisms regulating glucose transport into podocytes 33
2.8.1 Insulin signaling pathways 33
2.8.1.1 Insulin receptor and its substrates 34
2.8.1.2 The phosphatidylinositol 3-kinase signaling pathway 34
2.8.1.3 The cGMP-dependent protein kinase G signaling pathway 34
2.8.2 Glucose transporters in the kidney 35
2.8.3 Mechanisms of glucose uptake into podocytes 35
2.9 Role of insulin resistance in the pathogenesis of diabetic nephropathy 37
2.9.1 Mechanisms of insulin resistance 38
2.9.1.1 Hyperglycemia 38
2.9.1.2 Free fatty acids 38
2.9.1.3 Inflammation 38
2.9.2 Insulin resistance in kidney dysfunction 39
2.9.2.1 The correlation of insulin resistance and diabetic nephropathy 39
2.9.2.2 Podocytes and insulin resistance 39
2.9.3 Glucose transporters in diabetic nephropathy 40

3 AIMS OF THE STUDY 42

4 MATERIALS AND METHODS 43
4.1 Experimental animals 43
4.1.1 Sprague-Dawley rats 43
4.1.2 Streptozotocin rats 43
4.1.3 Zucker rats 43
4.2 Human study subjects 43
4.2.1 Human kidney samples 43
4.2.2 Human sera 44
4.3 Cell culture 44
4.4 Antibodies 45

4.5 Protein studies ... 47
4.5.1 Preparation of glomerular, tubular and cell lysates ... 47
4.5.2 Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) ... 48
4.5.3 Identification of proteins by LC-MS/MS ... 48
4.5.4 Immunoblotting ... 48
4.5.5 2-Deoxy-d-glucose uptake assay ... 49
4.5.6 Protein interaction studies ... 49
4.5.6.1 Co-immunoprecipitation assay ... 49
4.5.6.2 Production of GST-fusion proteins and pull-down assays ... 49
4.5.6.3 Duolink In Situ ... 50
4.5.7 Subcellular fractionation ... 50
4.6 Electron microscopy ... 50
4.7 Immunofluorescence microscopy ... 51
4.7.1 Immunofluorescence microscopy of kidney tissue ... 51
4.7.2 Immunofluorescence microscopy of cultured cells ... 51
4.7.3 Surface staining ... 51
4.8 Immunohistochemistry ... 52
4.9 Statistical analyses ... 52

5 RESULTS ... 53
5.1 Ezrin is downregulated in diabetic kidney glomeruli and regulates actin reorganization and glucose uptake in cultured podocytes (Study I) ... 53
5.1.1 Streptozotocin-induced diabetes leads to differential expression of glomerular proteins ... 53
5.1.2 Knockdown of ezrin induces dynamic remodelling of the actin cytoskeleton in podocytes ... 54
5.1.3 Knockdown of ezrin increases glucose uptake of podocytes and GLUT1 translocation to the plasma membrane ... 55
5.1.4 Cofilin-1 is involved in ezrin-mediated cortical actin remodelling ... 56
5.1.5 Phosphorylation of cofilin-1 is regulated by insulin in cultured podocytes and increases in glomeruli of diabetic rats ... 57
5.1.6 Expression of ezrin is reduced in glomeruli of obese Zucker rats and in glomeruli of human patients with Type 2 diabetes ... 57

5.2 Septin 7 and nonmuscle myosin IIA regulate glucose uptake into podocytes and compete for binding to the target membrane SNARE complex (Studies II and III) 58
5.2.1 Septin 7 is a novel interaction partner of CD2AP, nephrin and nonmuscle myosin IIA 58
5.2.2 Localization of septin 7 depends on CD2AP and an intact actin cytoskeleton 60
5.2.3 Depletion of septin 7 and nonmuscle myosin IIA affect glucose uptake in cultured podocytes 62
5.2.4 Septin 7 and nonmuscle myosin IIA interact with proteins involved in GSV trafficking 63
5.2.5 Phosphorylated myosin RLC competes with septin 7 for binding to the SNARE complex 63
5.2.6 Insulin regulates the association of septin 7 and phosphorylated myosin RLC with SNAP23 64
5.2.7 Septin 7 and NMHC-IIA affect SNARE complex formation 65
5.2.8 Phosphorylated myosin RLC is upregulated in glomeruli in diabetic rats and in cultured human podocytes exposed to sera from Type 1 diabetic patients with macroalbuminuria 66

6 DISCUSSION 67
6.1 Diabetes-induced changes in the protein expression profile of glomeruli isolated from rats with streptozotocin-induced diabetes 67
6.2 Role of ezrin in the regulation of actin reorganization and glucose uptake in cultured podocytes 67
6.3 Septin 7 and nonmuscle myosin IIA regulate glucose uptake into podocytes . . 69
6.4 Does increased insulin sensitivity protect podocytes from injury? 73

7 CONCLUSIONS AND FUTURE PERSPECTIVES 74

8 ACKNOWLEDGEMENTS 76

9 REFERENCES 79

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-III).

I **Anita A. Wasik**, Susanna Koskelainen, Mervi E. Hyvönen, Luca Musante, Eero Lehtonen, Kerttu Koskenniemi, Jukka Tienari, Antti Vaheri, Csaba Szala, Csaba Révész, Pekka Varmanen, Tuula A. Nyman, Peter Hamar, Harry Holthöfer, Sanna Lehtonen. Ezrin is down-regulated in diabetic kidney glomeruli and regulates actin reorganization and glucose uptake via GLUT1 in cultured podocytes. *Am J Pathol.* 184:1727-1739, 2014.

II **Anita A. Wasik**, Zydrune Polianskyte-Prause, Meng-Qiu Dong, Andrey S. Shaw, John R. Yates 3rd, Marilyn G. Farquhar and Sanna Lehtonen. Septin 7 forms a complex with CD2AP and nephrin and regulates glucose transporter trafficking. *Mol Biol Cell*. 23:3370-3379, 2012.

III **Anita A. Wasik**, Vincent Dumont, Jukka Tienari, Tuula A. Nyman, Christopher L. Fogarty**,** Markku Lehto, Per-Henrik Groop, Sanna Lehtonen. Septin 7 and nonmuscle myosin IIA compete for binding to the SNARE complex to regulate glucose uptake into podocytes. Submitted.

These articles are reproduced with the kind permission of their copyright holder.

In addition, some unpublished material is presented.

ABBREVIATIONS

AER	albumin excretion rate
CD2AP	CD2-associated protein
DKD	diabetic kidney disease
DN	diabetic nephropathy
ECM	extracellular matrix
ERM	ezrin/radixin/moesin family
ESRD	end-stage renal disease
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
GFB	glomerular filtration barrier
GFR	glomerular filtration rate
GLUT1	glucose transporter 1
GLUT4	glucose transporter 4
GSV	GLUT4 storage vesicle
IR	insulin receptor
IRS-1	insulin receptor substrate-1
MAPK	mitogen-activated protein kinase
NHERF2	Na^+/H^+ exchange regulatory factor 2
NM-II	nonmuscle myosin II
NMHC-IIA	nonmuscle myosin heavy chain IIA
PI3K	phosphoinositide 3-kinase
p-MHC	phosphorylated myosin IIA heavy chain
pp-RLC	phosphorylated myosin regulatory light chain
SD	slit diaphragm
SNAP23	synaptosome-associated protein, 23 kDa
SNARE	*N*-ethylmaleimide–sensitive fusion protein attachment protein receptor
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VAMP2	vesicle-associated membrane protein 2

ABSTRACT

Background. Diabetic nephropathy (DN) is a major microvascular complication of diabetes and a common cause of end-stage renal disease (ESRD) worldwide. DN first manifests as a small amount of albumin in urine (microalbuminuria) that develops into overt albuminuria as nephropathy progresses. The pathological features of DN include glomerular and tubular basement membrane thickening, mesangial extracellular matrix expansion, and glomerular visceral epithelial cell (podocyte) loss and podocyte foot process effacement. Podocytes are part of the glomerular filtration barrier and play an important role in the development of DN, as hyperglycemia may activate pathways that lead to their injury and loss. Interestingly, insulin signaling in podocytes is essential for maintaining normal kidney function as deficiency in insulin receptor (IR) signaling in podocytes can induce a disease state reminiscent of DN. Insulin resistance is also a risk factor for DN, and has been reported to be associated with microalbuminuria in both patients with Type 1 and Type 2 diabetes. At the cellular level, the mechanisms leading to the development of insulin resistance include mutations in the IR itself, as well as impairments in the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway or glucose transporter trafficking that regulate the uptake of glucose into cells. In podocytes, insulin rapidly induces remodelling of the actin cytoskeleton and leads to glucose uptake via glucose transporters 1 (GLUT1) and glucose transporters 4 (GLUT4). This process requires nephrin, which facilitates GLUT4 storage vesicle (GSV) fusion with the plasma membrane. However, the precise mechanisms regulating GSV trafficking and glucose uptake into podocytes are largely uncharacterized.

Results. To characterize the early pathophysiological mechanisms leading to podocyte injury in DN, we performed quantitative proteomic profiling of glomeruli isolated from control rats as well as those with streptozotocin-induced diabetes. Ezrin was found to be downregulated in diabetic glomeruli. In cultured podocytes, depletion of ezrin increased glucose uptake apparently by increasing translocation of glucose transporter GLUT1 to the plasma membrane. Loss of ezrin also induced actin remodelling under basal conditions, but reduced insulin-stimulated actin reorganization. Ezrin-dependent actin remodelling involved cofilin-1. Phosphorylated, inactive cofilin-1 was upregulated in diabetic glomeruli, suggesting altered actin dynamics. Furthermore, reduced expression of ezrin was found in the podocytes of human patients with diabetes.

Adaptor protein CD2-associated protein (CD2AP) interacts with nephrin and is essential for glomerular ultrafiltration. We found that the filament-forming GTPase septin 7 is also part of the CD2AP-nephrin protein complex. Septin 7 negatively regulates GSV trafficking by forming a physical barrier between the vesicles and the plasma membrane. To further characterize the function of septin 7 in regulating GSV trafficking, we searched for interaction partners of septin 7 and identified nonmuscle myosin heavy chain IIA (NMHC-IIA). Nonmuscle myosin IIA (NM-IIA) consists of heavy chains and essential and regulatory light chains. We observed that depletion of NMHC-IIA decreases insulin-stimulated glucose uptake into podocytes. Thus, septin 7 and NMHC-IIA play opposite roles in glucose transport. Septin 7 and phosphorylated myosin regulatory light chain (pp-RLC) compete for binding to SNAP23 (synaptosome-associated protein, 23 kDa), a plasma membrane SNARE (*N*-ethylmaleimide–sensitive fusion protein attachment protein receptor) protein involved in GSV exocytosis. Furthermore, insulin regulates the association of septin 7 and pp-RLC with SNAP23. Depletion of NMHC-IIA inhibits the association of VAMP2 (vesicle-associated membrane protein 2) with SNAP23 indicating that SNARE complex formation is reduced in the absence of NMHC-IIA. pp-RLC is upregulated in diabetic glomeruli and cultured human podocytes exposed to macroalbuminuric sera from patients with Type 1 diabetes. The data indicate that pp-RLC facilitates glucose uptake into podocytes. Activation of NM-IIA by phosphorylation of its RLC in glomeruli in diabetes suggests that NM-IIA may play a role in the development of DN.

Conclusions. Our findings indicate that ezrin, septin 7 and NMHC-IIA regulate glucose uptake into podocytes and may play a role in the development of renal complications in diabetes by regulating glucose transport and organization of the actin cytoskeleton in podocytes.

1 INTRODUCTION

Diabetes mellitus is a rapidly-growing global problem with social, health, and economic consequences. It is a chronic disease that occurs when the pancreas is no longer able to produce insulin (Type 1 diabetes mellitus, T1DM; insulin-dependent diabetes mellitus, IDDM), or when the cells fail to use insulin properly (Type 2 diabetes mellitus, T2DM; non insulin-dependent diabetes mellitus, NIDDM). Approximately 382 million people suffer from diabetes and the number is estimated to increase up to 592 million by 2035 (International Diabetes Federation, 2013). All forms of diabetes increase the risk of long-term micro- and macrovascular complications. The microvascular complications refer to diabetic nephropathy, retinopathy and neuropathy, and the macrovascular complications include cardiovascular disease, stroke and peripheral vascular disease. Diabetes is now the most common cause of ESRD (Zimmet et al., 2001).

Diabetic nephropathy (DN) affects around 40% of patients with diabetes, and the proportion of patients with ESRD caused by diabetes has progressively increased during the last few decades. DN is associated with increased risk of cardiovascular disease and mortality (Nathan et al., 2005; Groop et al., 2009) and with metabolic syndrome (Thorn et al., 2005). Four major histologic pathological features are observed in DN: (I) mesangial expansion, (II) glomerular basement membrane (GBM) thickening, (III) podocyte foot process effacement and podocyte apoptosis, and (IV) hyalinosis of afferent and efferent arterioles.

Hemodynamic (glomerular hypertension) and metabolic (hyperglycemia) factors contribute to the development and progression of diabetic kidney disease (DKD). Hemodynamic pathways include elevations of systemic and intraglomerular pressure and activation of various vasoactive hormone pathways, including the renin-angiotensin aldosterone system (RAAS), endothelin, and urotensin. Hyperglycemia results in accumulation of advanced glycated end-products (AGEs) and reactive oxygen species (ROS), and activation of the polyol and hexosamine pathways. This promotes activation of intracellular secondary messengers including protein kinases, transcription factors and growth factors which lead to modulation of the immune system and activation of the enzymes involved in glucose metabolism. Collectively, these factors increase albumin excretion rate (AER), extracellular matrix accumulation, cell hypertrophy and apoptosis (reviewed in Brownlee et al., 2001; reviewed in Soldatos and Cooper, 2008). Excess glucose may therefore activate several pathways that lead to podocyte injury (Lewko and Stepinski, 2009).

Insulin resistance may play a role in the development of kidney injury, as both clinical and experimental data suggest that insulin sensitizers have a renoprotective role in diabetes patients (Miyazaki et al., 2007), as well as in animal models (Zhang et al., 2008). Insulin resistance is associated with an increased risk of chronic kidney disease, microalbuminuria and overt proteinuria (Kurella et al., 2005; Chen et al., 2008; Ryu et al., 2009; Thomas et al., 2011). Interestingly, podocytes are uniquely insulin sensitive cells in the glomerulus of the kidney (Coward et al., 2005) and podocyte insulin resistance has been first demonstrated in early diabetes in T2DM db/db mice (Tejada et al., 2008). Podocyte-specific IR knockout mice develop albuminuria and histological features of DN even in the setting of normoglycemia (Welsh et al., 2010). Interestingly, the podocyte protein nephrin is crucial for the insulin sensitivity of podocytes (Coward et al., 2007) and its expression was shown to be reduced in early DN (Patari et al., 2003; Doublier et al., 2003; Jim et al., 2012). The inability of podocytes to respond to insulin may also result from upregulation of C-jun N-terminal kinase (JNK), a negative regulator of insulin signaling, as observed in glomeruli of db/db mice (Ijaz et al., 2009). In cultured human podocytes, saturated fatty acids induce insulin resistance *in vitro* (Lennon et al., 2009). Also, the protein tyrosine phosphatase SHP-1 (Drapeau et al., 2013), lipid phosphatase SHIP2 (Hyvönen et al., 2010) and Synip (Yamada et al., 2014) regulate insulin sensitivity of podocytes. In addition, hyperinsulinemia also appears to induce kidney dysfunction by promoting glomerular hyperfiltration and increased vascular permeability (reviewed in De Cosmo et al., 2013).

It is clear that podocytes have a functional system for glucose uptake (Lewko et al., 2005). Glucose uptake is initiated by the activation of the IR by insulin, which leads to a cascade of signaling events that coordinate translocation of GSV to the plasma membrane. The PI3K and the cGMP-dependent protein kinase G signaling pathways are implicated in the regulation of GSV trafficking in podocytes. Glucose uptake into podocytes is achieved through the facilitative glucose transporters GLUT1 and GLUT4 and depends on the slit diaphragm (SD) protein nephrin (Coward et al., 2007). GLUT2 and GLUT8 have also been observed in podocytes (Lewko et al., 2005; Schiffer et al., 2005), but their role in glucose uptake in these cells remains uncharacterized. GSV translocation to the plasma membrane occurs in multiple stages including approaching, tethering, docking and fusion. Actin and microtubule-based cytoskeleton provide a route for the vesicles to approach the plasma membrane. The exocyst complex proteins help tether the GSVs to the plasma membrane, and formation of the

SNARE complex between vesicle SNARE (v-SNARE) and target SNAREs (t-SNARE) mediates fusion of the GSVs with the plasma membrane (reviewed in Stöckli et al., 2011).

The studies in this thesis were initiated by characterization of the early molecular changes associated with the development of diabetic kidney injury by a comparative proteomics approach. We found that ezrin, an actin-binding protein, increases the dynamic reorganization of actin in podocytes and regulates glucose uptake via GLUT1. Futhermore, cofilin-1 was found to be involved in ezrin-mediated cortical actin remodelling. We also identified septin 7 as a novel interaction partner of CD2AP and nephrin, and characterized the function of septin 7 in podocytes. Our data indicate that septin 7 hinders GSV trafficking by forming a physical barrier between the vesicles and the plasma membrane and negatively regulates glucose uptake into podocytes. Septin 7 also competes with active nonmuscle myosin IIA for binding to the SNARE complex to regulate glucose uptake into podocytes. These results suggest that the regulators of glucose transporter trafficking and actin reorganization may associate with the development of diabetic kidney complications and provide suitable targets to enhance insulin sensitivity of podocytes.

2 REVIEW OF THE LITERATURE

2.1 Overview of kidney structure and function

The kidneys perform several major functions in the body. They play a vital role in normal physiology by maintaining fluid and electrolyte balance, acid-base balance, and glucose balance via glucose reabsorption and/or gluconeogenesis. They also excrete metabolic waste products and foreign chemicals, and secrete hormones that regulate blood pressure and red blood cell formation.

The basic structural and functional unit of the kidney is the nephron, which spans the kidney cortex and medulla. Each human kidney has about 1 million nephrons. A nephron consists of a glomerulus and a tubulus. A glomerulus consists of a capillary tuft located inside the Bowman's capsule (Figure 1B). The glomeruli localize in the kidney cortex, and they are responsible for plasma filtration. The distal part of the nephron is the tubular system that passes from the cortex deep into the kidney medulla (Figure 1A) and which is further divided into the proximal and distal tubule and the Loop of Henle. After passing through the renal tubule, the filtrate continues to the single collecting duct (Figure 1B).

Every day, about 180 liters of primary urine is normally formed by the kidney glomeruli. The tubular system allows the reabsorption of water and electrolytes, such that the final daily excretion is normally only ~1–1.5 liters.

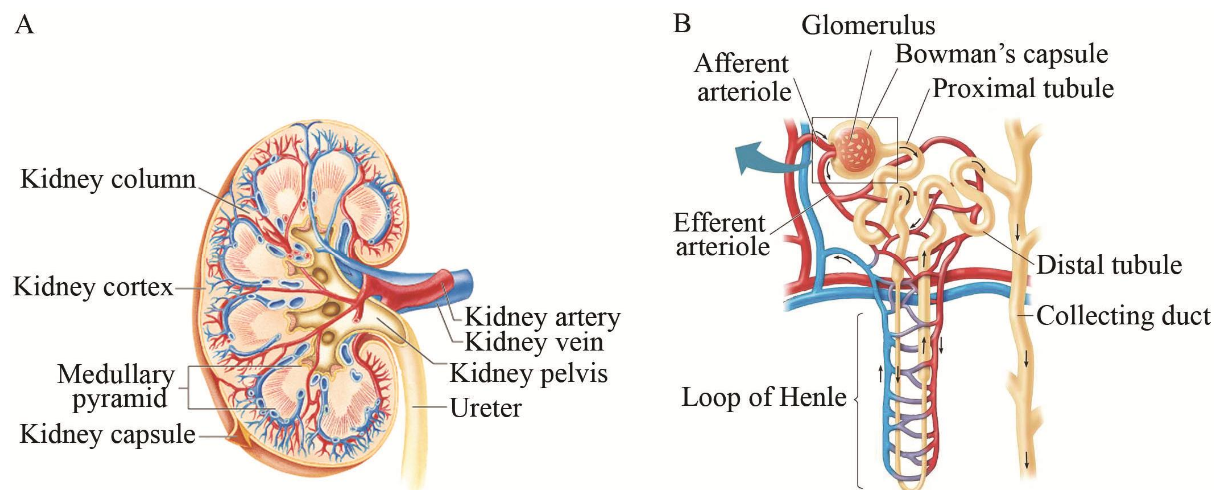

Figure 1. Schematic illustration of kidney (A) and nephron (B) anatomy. Modified from Marieb, 2001.

2.2 Glomerular filtration barrier

The size-, charge- and shape-selective ultrafiltration of plasma occurs in glomeruli (Figure 2A). Primary urine is filtrated from the plasma through the glomerular filtration barrier (GFB) to the Bowman's space and then passes to the tubular system. Water and small molecules pass the barrier, whereas molecules the size of albumin (~67 kDa) and larger are retained in the blood. The GFB consists of three distinct layers: the fenestrated endothelium, the glomerular basement membrane (GBM), and the podocytes with their slit diaphragms (SD) (Figure 2B).

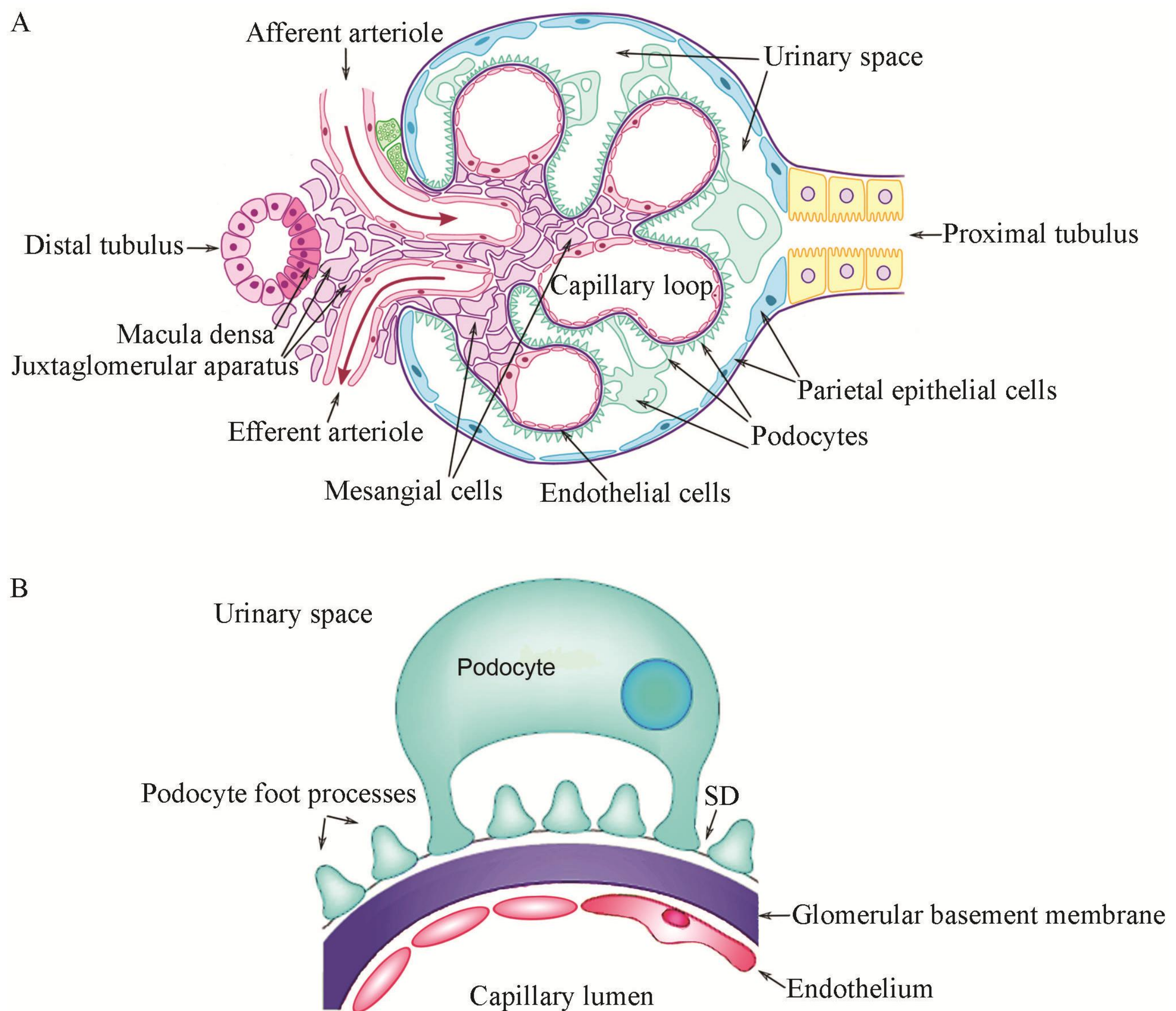

Figure 2. Schematic structure of the glomerulus (A) and the glomerular filtration barrier (B). SD: slit slit diaphragm. Modified from Komorniczak, 2009 (A) and Suh and Miner, 2013 (B).

2.2.1 Endothelium

The glomerular endothelium is the first part of the GFB that lines the interior surface of the glomerular capillaries. The presence of numerous trans-cellular holes (fenestrations) of 70–100 nm in diameter (in humans) provides the first structural barrier to albumin (reviewed in Haraldsson et al., 2008). The luminal side of the endothelial cells and the fenestrations are covered by an endothelial cell coat and a glycocalyx composed of proteoglycans, glycosaminoglycans, glycoproteins and glycolipids. Due to its negative charge, glycocalyx contributes to the charge-selective properties of the GFB (Jeansson and Haraldsson, 2006). The endothelium is involved also in the formation of the GBM by synthesizing laminin (St John and Abrahamson, 2001) and type IV collagen (Abrahamson et al., 2009). The endothelial cells communicate with the podocytes and the mesangial cells through a variety of signaling pathways in order to prevent proteinuria (Davis et al., 2007; Daehn et al., 2014).

2.2.2 Glomerular basement membrane

The GBM is an amorphous, 300- to 350-nm-thick extracellular structure that is surrounded and maintained by endothelial cells and podocytes. The GBM can be separated into three layers: lamina rara externa (outer, underneath the foot processes), lamina densa (central) and lamina rara interna (inner, subendothelial). The main components of the GBM include type IV collagen, laminin, nidogen (entactin) and heparan sulfate proteoglycans (HSPGs) which provide an anionic charge to the GBM and have been considered to contribute to the charge-selective filtration barrier (reviewed by Haraldsson et al., 2008).

2.2.3 Podocytes

Podocytes are polarized, highly specialized and terminally differentiated cells that surround the glomerular capillaries and form a key part of the glomerular filtration barrier. With respect to their cytoarchitecture, podocytes consist of three different segments. The cell body gives rise to major processes that branch into a network of interdigitating cellular extensions called foot processes. The foot processes of neighboring podocytes interact between the basal and apical sides of the podocytes with specialized 40-nm-wide cell-to-cell junctions called slit diaphragms (SD) (reviewed in Pavenstädt et al., 2003). The SD is highly permeable to water and small molecules, but not to larger proteins, like albumin. Podocyte

damage leads to retraction of the foot processes. Thus, during proteinuric diseases like diabetes mellitus, the foot processes lose their fine structure and collapse.

2.2.4 Mesangium

The mesangium consists of mesangial cells and their surrounding matrix and is located between the capillary loops of the glomerular tuft (reviewed by Haraldsson et al., 2008). The major role of the mesangium is to provide structural support for the glomerular capillaries. Mesangial cells resemble smooth muscle cells by having contractile properties; therefore they participate in the regulation of glomerular filtration (Becker, 1972; Ausiello et al., 1980). They also control the composition and turnover of the mesangial matrix (reviewed in Veis, 1993) and contribute to the removal of glomerular debris due to their phagocytic activity (Mauer et al., 1972; Elema et al., 1976).

2.3 Podocyte biology

Podocytes are polarized epithelial cells with apical, basal and junctional cell membrane domains (reviewed in Kerjaschki, 2001). The apical domain is facing the Bowman's space, and is characterized by the expression of podocalyxin. The basal domain is required to anchor the podocyte to the underlying GBM. The junctional domain contains a nephrin-based multimeric protein complex, which forms a SD, bridging the adjacent foot processes. The submembranous regions of all compartments are connected to the podocyte actin cytoskeleton (reviewed in Pavenstädt et al., 2003).

2.3.1 Slit diaphragm

The SD represents the only cell-cell contact between podocytes. The major component of the SD is nephrin (Ruotsalainen et al., 1999). Nephrin is a transmembrane protein of the immunoglobulin (Ig) superfamily and, together with the Neph-protein family (Neph 1-3), forms a framework of the SD (Ruotsalainen et al., 1999; Barletta et al., 2003; Gerke et al., 2003; Wartiovaara et al., 2004; Gerke et al., 2005). Based on its components, the SD is considered as a special cell adhesion structure with characteristics of both adherens and tight junctions (Schnabel et al., 1990; Reiser et al., 2000). Adherens junctions mediate cell to cell contact. The adherens junction proteins present in SD include cadherin family protein P-

cadherin (Reiser et al., 2000) and vascular endothelial (VE)-cadherin (Cohen et al., 2006), as well as catenins (Reiser et al., 2000; Lehtonen et al., 2004) and FAT1 (Inoue et al., 2001). Tight junctions act as cellular barriers for the transport of water, ions and proteins (Anderson, 2001), and function in maintaining cell polarity (Shin et al., 2006). The tight junction-associated proteins detected in the SD include Zonula occludens-1 (ZO-1) (Schnabel et al., 1990), membrane associated guanylate kinase inverted-1 (MAGI-1) (Hirabayashi et al., 2005), MAGI-2 (Lehtonen et al., 2005), calmodulin associated serin/threonine kinase (CASK) (Lehtonen et al., 2004), and junction adhesion molecule-1 (JAM-1) (Fukasawa et al., 2009). In addition, several other molecules, including cytoplasmic CD2AP (Shih et al., 1999) and podocin (Schwarz et al., 2001) have been shown to be connected to the SD.

The SD is a multifunctional protein complex which sends signals regulating cell polarity, cell survival and cytoskeleton organization. Phosphorylation of nephrin and Neph1 initiates signaling pathways in podocytes (reviewed in Benzing, 2004), and podocin contributes to intracellular signal transduction via interaction with nephrin (Boute et al., 2000; Huber et al., 2003; Li et al., 2004). CD2AP, together with nephrin, activates PI3K and stimulates serine/threonine kinase AKT-dependent signaling (Li et al., 2000; Huber et al., 2003). CD2AP and ZO-1 also appear to connect the SD to the actin cytoskeleton (Fanning et al., 2002; Lehtonen et al., 2002; Huber et al., 2003).

2.3.2 Basal domain of podocytes

The basal domain of podocyte foot processes is attached to the GBM via cell-matrix contacts. Two major podocyte adhesion receptors, α3β1-integrin complex and the dystroglycan complex, bind to GBM ligands such as collagen IV, laminin, and perlecan. Both integrins and dystroglycans are coupled to the actin cytoskeleton via adaptor molecules. Therefore, mechanical force can be transmitted from the GBM to the cytoskeleton of the foot processes and further to the podocyte cell body. The foot procesess are also involved in GBM turnover. They secrete matrix-modifying enzymes and participate in the synthesis and assembly of the GBM molecules (reviewed in Miner, 1999).

Studies showed reduced dystroglycan level in minimal change disease (Regele et al., 2000). During podocyte injury, altered interactions of dystroglycan with the GBM leads to podocyte effacement (Kojima et al., 2004), characterized by flattening of foot processes and reduction of the frequency of SD. β1 integrin–null mice die at birth and show effaced podocytes (Kreidberg et al., 1996).

2.3.3 Apical domain of podocytes

The apical membrane domain of podocytes is located on the luminal side, facing the urinary space. It has a negatively charged surface coat, consisting mainly of the anionic sialoprotein podocalyxin (Kerjaschki et al., 1984). This negative charge limits the passage of negatively charged proteins into the urinary space, prevents parietal cell adherence to podocytes, and keeps adjacent podocytes separated (Takeda et al., 2000). Podocalyxin is connected to the cortical actin cytoskeleton via the interaction with Na^+/H^+ exchange regulatory factor 2 (NHERF2) and activated ezrin (Takeda et al., 2001).

Studies have shown that the podocalyxin-rich apical membrane of podocytes is shed into the urine of patients with nephric syndrome and nephritis, indicating podocyte injury (Hara et al., 2005). Furthermore, disruption of podocalyxin or reduction of its negative charge results in dissociation of podocalyxin from the actin cytoskeleton, leading to foot process effacement (Takeda et al., 2001) and rearrangement of the cell-cell contacts between neighboring podocytes (Kurihara et al., 1992).

2.3.4 Podocyte cytoskeleton

The highly dynamic foot processes contain an actin-based cytoskeleton. The podocyte cytoskeleton is critical for structural integrity of the foot processes, coupling of the slit membrane complex with podocyte-GBM contacts, and maintaining of the mechanical strength and flexibility of the podocytes. The cytoskeleton of the major processes maintains contact with the metabolic machinery of the podocyte cell body and allows vesicular transport along the foot process.

2.3.4.1 Actin cytoskeleton assembly and reorganization

The precise organization and regulation of the actin cytoskeleton in podocytes are essential for the maintenance of the normal structure and function of these cells. Actin reorganization is a highly dynamic process, governed by continuous assembly and disassembly of the actin filaments. Signaling from all three podocyte domains is required for the regulation of the actin reorganization. Actin filaments can be organized in packed arrays or loosely associated in networks. The level of actin packing is defined by the actin cross-

linking molecule, α-actinin, and the associated motor proteins (like myosins), allowing contraction of the actin apparatus (Gordon et al., 2000).

Nck and its associated actin cytoskeleton regulatory proteins, including Wiskott-Aldrich syndrome protein (N-WASp) and actin-related protein (Arp2/3), are recruited to phosphorylated nephrin when rapid actin polymerization and cytoskeletal reorganization are required (Verma et al., 2006). The formation of new actin filaments is promoted by the Arp2/3 complex, which nucleates new filaments from the sides of preexisting filaments and thus induces the formation of a branched filament network (Mullins et al., 1998; Svitkina and Borisy, 1999).

The assembly of actin filaments is regulated by the small GTPases: RhoA, Rac1 and Cdc42 (Heasman and Ridley, 2008). RhoA directly initiates stress fiber assembly through its effectors, including Rho-associated protein kinase (ROCK) (Leung et al., 1996). ROCK in turn promotes stress fiber formation by inhibiting actin filament depolymerization through inactivation of actin depolymerizing factor/cofilins via LIM kinase and by inducing contractility through phosphorylation of myosin light chains. Phosphorylation of cofilin-1 on serine 3 leads to its inactivation resulting in reduced binding to actin and depolymerizing activity (Moriyama et al., 1996; Heyworth et al., 1997).

2.3.4.2 Components of the podocyte cytoskeleton

Podocyte function and architecture depend on the precise organization of three sets of elements: microfilaments (7-9 nm in diameter), intermediate filaments (10 nm in diameter) and microtubules (24 nm in diameter). The podocyte cell body and major processes contain microtubules and intermediate filaments like tubulin and vimentin (Drenckhahn and Franke, 1988; Cortes et al., 2000), whereas the foot processes contain dense actin filaments which are anchored to the GBM, the SD and the apical domain (Figure 3). There are two actin cytoskeletal networks in the foot processes: dense actin bundles above the level of the SD running parallel to the longitudinal axis, and a cortical actin network just below the plasma membrane of the foot processes (reviewed in Pavenstädt et al., 2003) (Figure 3).

The cytoskeleton maintains the shape of podocytes and enables continuous adapting. The cytoskeleton supports the glomerular capillary wall and opposes the high hydrostatic pressure necessary for glomerular filtration. To fulfill these functions, the actin network is modified by an assembly of linker and adaptor proteins.

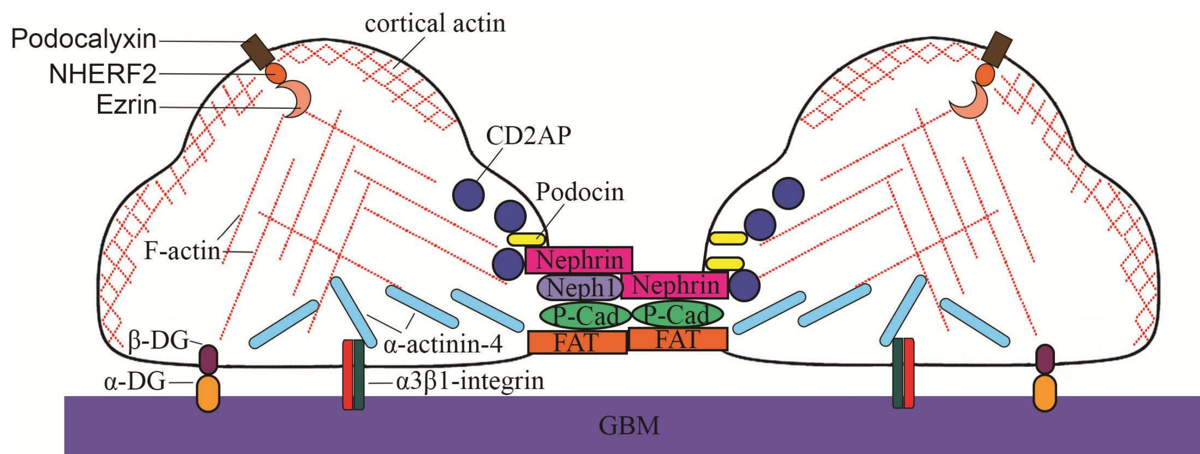

Figure 3. Molecular components of the podocyte foot processes actin cytoskeleton. CD2AP: CD2-associate protein; DG: dystroglycan; GBM: glomerular basement membrane; NHERF2: Na^+/H^+ exchange regulatory factor 2; P-Cad: P-cadherin. Modified from Mundel and Shankland, 2002.

2.3.4.3 Podocyte proteins that regulate actin

The assembly, maintenance, and disassembly of the actin cytoskeleton are mediated by a variety of actin-associated proteins with divergent molecular interactions and functional properties (reviewed in Zigmond, 1996) (Figure 3). Therefore, proteins regulating or stabilizing the actin cytoskeleton are of critical importance for sustained function of glomerular filtration (Shirato et al., 1996; Takeda et al., 2001; Smoyer and Randstom, 2002). Mutations in many of these proteins lead to kidney dysfunction.

α-actinin-4. α-actinin-4, an actin-binding protein which is localized to podocyte foot processes (Kaplan et al., 2000), has an important role in cross-linking of actin filaments into contractile bundles and in helping to form the anchoring complex for the ends of actin stress fibers. Mutations of ACTN4, encoding α-actinin, were found to cause a late-onset autosomal-dominant focal segmental glomerulosclerosis (FSGS) in humans (Kaplan et al., 2000) and podocyte-specific expression of mutant α-actinin 4 in mice leads to FSGS (Michaud et al., 2003). Actin filaments cross-linked by the mutant α-actinin-4 exhibit profound changes in structural and biomechanical properties (Weins et al., 2007), e.g. increased F-actin bundling and altered flexibility (Ward et al., 2008). Induction of proteinuria with puromycin in rats results in progressive podocyte damage due to an increase in α-actinin 4 expression (Shirato et al., 1996; Smoyer et al., 1997).

Ezrin. Ezrin, a member of of ezrin/radixin/moesin (ERM) family of actin-binding proteins (Arpin et al., 1994; Vaheri et al., 1997; Tsukita and Yonemura, 1999), is an important regulator of actin dynamics and links membrane proteins to the underlying actin cytoskeleton (Arpin et al., 1994; Vaheri et al., 1997). In glomeruli, ezrin is expressed in podocytes (Hugo et al., 1998), and together with NHERF2, connects the cell surface sialoprotein podocalyxin to actin (Takeda et al., 2001).

Cofilin-1. Cofilin-1 is an actin modulating protein that severs and depolymerizes F-actin and enhances dynamics of actin filaments (Nishida et al., 1984; Yonezawa et al., 1985; Theriot, 1997). Cofilin-1 plays a key role in regulating the actin cytoskeleton in podocytes (Garg et al., 2010; Ashworth et al., 2010). Knockdown or mutation of cofilin-1 disturbs the filtration barrier in zebrafish (Ashworth et al., 2010). Mice with podocyte-specific cofilin-1 deletion develop proteinuria by the age of 3 months (Garg et al., 2010).

CD2AP. CD2AP is cytoplasmic multifunctional adaptor protein that contains three SH3 (Src homology 3) domains at its N-terminus, proline-rich domains and a coiled-coil domain at the C-terminus. CD2AP localizes at the intracellular insertion site of the SD (Shih et al., 2001) and functions as a scaffolding protein connecting the actin cytoskeleton to plasma membrane proteins, such as nephrin and podocin (Shih et al., 2001). CD2AP interacts directly with actin (Lehtonen et al., 2002) and the actin-binding proteins CapZ (Hutchings et al., 2003) and cortactin (Lynch et al., 2003). Thus, CD2AP has a role in the regulation of the actin cytoskeleton. Mice lacking CD2AP develop severe nephrotic syndrome and foot process effacement at the age of 2-3 weeks and die of renal failure at age of 6-7 weeks (Shih et al., 1999). Mutations in CD2AP have also been reported in human patients with FSGS (Kim et al., 2003; Gigante et al., 2009). Moreover, CD2AP plays an important role in albumin overload-induced podocyte injury via disruption of the cytoskeleton (He et al., 2011).

Nephrin. Nephrin, a major structural protein of the SD, regulates actin dynamics in many ways. It interacts with the adaptor protein CD2AP and with IQGAP, an effector protein of small GTPases Rac1 and Cdc42 (Shih et al., 1999; Liu et al., 2004). Nephrin is known to be in complex with the Nck adaptor protein, which controls actin polymerization and is a potential regulator of actin cytoskeleton dynamics by recruiting the Arp2/3 complex (Jones et al., 2006). Furthermore, nephrin regulates the PI3K/protein kinase B pathway that is important for actin reorganization (Zhu et al., 2008). The human nephrin gene, *NPHS1*, is mutated in the congenital nephrotic syndrome of the Finnish type (CNF) (Kestilä et al.,

1998). In mice, inactivation of the nephrin gene results in a phenotype that resembles the findings in CNF patients (Putaala et al., 2001; Rantanen et al., 2002).

Nonmuscle myosin II. Nonmuscle myosin II (NM-II) is an actin-based motor protein that plays an essential role in actin cytoskeleton organization and cellular motility. It is composed of two heavy chains and two pairs of light chains. Nonmuscle myosin heavy chain IIA (NMHC-IIA) is a component of the actin cytoskeleton of podocyte foot processes (Endlich et al., 2001). Mutations in the *MYH9* gene, encoding NMHC-IIA, cause four autosomal dominant syndromes with variable degrees of nephropathy (Epstein et al., 1972; Peterson et al., 1985; Greinacher et al., 1990). NMHC-IIA has also been associated with FSGS and ESRD (Kopp et al., 2008). Mouse models with *Myh9*-related diseases manifest similar phenotypes as observed in humans (Zhang et al., 2012), and podocyte-specific deletion of Myh9 predisposes mice to glomerulopathy depending on the genetic background and the model of experimental injury (Johnstone et al., 2011; Zhang et al., 2012, Johnstone et al., 2013). Furthermore, knockdown of NMHC-IIA in zebrafish indicates that NMHC-IIA is essential for the formation and function of the glomerulus (Müller et al., 2011).

2.4 Diabetic nephropathy

DN is a microvascular complication of diabetic mellitus that affects around 40% of patients with diabetes. DN is the most common cause of renal replacement therapy worldwide (Zimmet et al., 2001).

2.4.1 Definition and clinical features of diabetic nephropathy

DN is categorized based on the albumin excretion rate (AER). The first sign of DN is microalbuminuria (incipient nephropathy), defined as AER 20-200 µg/min or 30-300 mg/24 h. Macroalbuminuria (AER >200 µg/min or >300 mg/24 h) or proteinuria (AER >500 mg/24 h), considered as overt nephropathy, occur over a period of years and subsequently lead to a decrease in estimated glomerular filtration rate (eGFR <60 ml/min/1.73 m^2) (Reutens and Atkins, 2011).

Microalbuminuria appears 5–15 years after the patient is diagnosed with diabetes mellitus. The AER values are predictors of DN (Viberti et al., 1982; Mogensen and Christensen, 1984), as AER increases with time. Microalbuminuria has been considered as a risk factor for macroalbuminuria (Caramori et al., 2000). Studies in 1980s showed that around 80% of

T1DM patients with microalbuminuria progress to overt proteinuria within 6-14 years (Viberti et al., 1982; Parving et al., 1982). However, this is not common in all patients. In some of the patients microalbuminuria may regress to normoalbuminuria (Perkins et al., 2003). Studies have shown that only 30-40% of those patients with microalbuminuria progress to macroalbuminuria within 10 years (Caramori et al., 2000) mostly due to the intensive glycemic and blood pressure control. The same is true for patients with T2DM (Adler et al., 2003).

Once microalbuminuria/macroalbuminuria presents, renal function progressively declines and ESRD develops. In early diabetes, increased GFR is commonly present (Wiseman et al., 1985; Caramori et al., 1999). As DN develops, GFR gradually decreases (Viberti et al., 1983; Skupien et al., 2012). Studies have reported that a subset of normoalbuminuric T1DM patients do not progress to macroalbuminuria despite reduced GFR and advanced renal lesions (Caramori et al., 2003; Molitch et al., 2010). This finding has been confirmed also for patients with T2DM (Kramer et al., 2003; MacIsaac et al., 2004).

2.4.2 Pathological features of diabetic nephropathy

DN is characterized by the presence of specific and pathological renal changes. The changes start early in the course of diabetes and progress as the disease develops. The pathology of the renal lesions is similar in T1DM and T2DM (Taft et al., 1994), although it has been suggested that there is more heterogeneity in T2DM (Chihara et al., 1986). In DN, the morphology of all four compartments in the kidney, glomeruli, tubuli, interstitium and vessels are affected.

2.4.2.1 Glomerular changes

The characteristic histological change in glomeruli in DN is mesangial matrix accumulation (Kimmelstiel and Wilson, 1936) due to increased production or reduced degradation of extracellular matrix (ECM) proteins (Schnaper et al., 1996). Mesangial expansion can be nodular (so-called Kimmelstein-Wilson nodules) and associates with reduced filtration surface area in glomeruli and, consequently, with reduced GFR and progressing albumin excretion (Mauer et al., 1984; Fioretto et al., 1995). The main ultrastructural and earliest detectable change in diabetic kidneys is the thickening of the GBM

due to increased accumulation of the extracellular matrix. GBM thickening may be detected as early as 1.5-2.5 years after the onset of T1DM (Osterby, 1973), even in normoalbuminuric patients (Perrin et al., 2006). Evidence for podocyte injury, including foot process widening and podocyte loss due to apoptosis has also been reported (Susztak et al., 2006) (Figure 4).

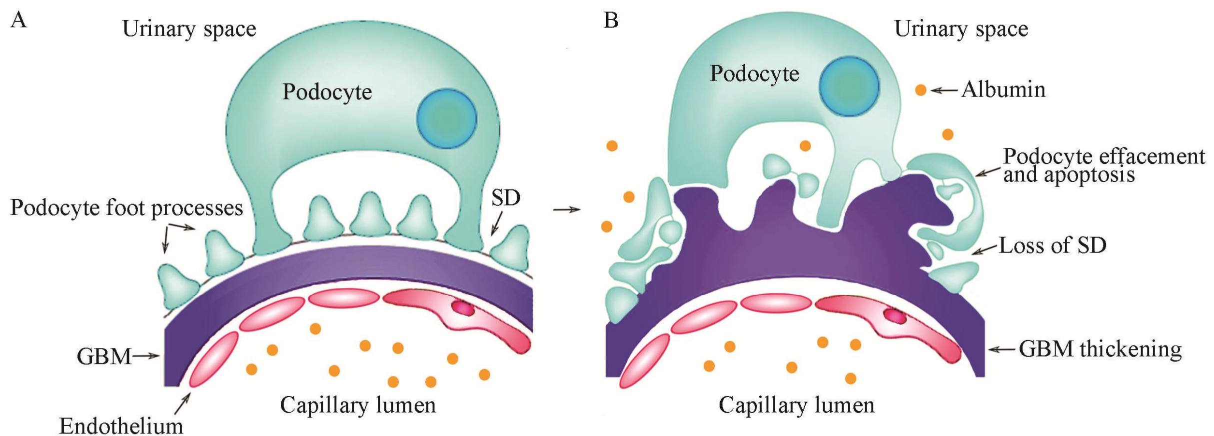

Figure 4. Characteristic glomerular filtration barrier changes during diabetic nephropathy. (A) Normal glomerular filtration barrier. (B) Diabetic kidney. GBM: glomerular basement membrane; SD: slit diaphragm. Modified from Suh and Miner, 2013.

2.4.2.2 Tubular changes

Development of progressive renal dysfunction in DN involves changes in the renal tubules. The changes usually develop before the onset of macroalbuminuria. Tubular hypertrophy, thickening of the tubular basement membrane, and tubular apoptosis may be present in DN (Brito et al., 1998).

2.4.2.3 Interstitial changes

The interstitium of the kidney comprises the extravascular intertubular spaces of the kidney and is bounded on all sides by tubular and vascular basement membranes. In diabetic kidneys, the interstitial fibrosis follows glomerular changes and is proportional to tubular atrophy (Mauer et al., 1984).

2.4.2.4 Vascular changes

Hyalinosis of afferent and efferent arterioles is also a typical lesion in DN. However, hyalinosis of efferent arterioles is specific for DN and distinct from other kidney diseases (Stout et al., 1994). Besides characteristic arteriolar hyalinosis, the presence of large vessels and arteriosclerosis may be observed in biopsy.

2.4.3 Mechanisms associated with the development of diabetic nephropathy

The factors involved in the pathogenesis of DN are multifaceted. Age, gender, age at onset of diabetes, poor glycemic control, smoking, dyslipidemia, and metabolic syndrome are risk factors of DN. Genetic susceptibility is also linked to this disease (reviewed in Harjutsalo and Groop, 2014). Two major factors, hemodynamic (glomerular hypertension) and metabolic (hyperglycemia), contribute to the development and progression of DKD. Hyperglycemia causes kidney damage through four major mechanisms. It leads to formation of advanced glycated end-products (AGEs) via irreversible nonenzymatic glycation of proteins and lipids that bind to a specific cell surface receptor for AGE (RAGE), leading to production of reactive oxygen species (ROS). Increase in protein kinase C (PKC) activation results in activation of growth factors and decreased production of endothelial nitric oxide (NO). Increased activity of polyol pathways leads to conversion of glucose to sorbitol by aldose reductase and further, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Hexosamine pathway activity, elevated in hyperglycemic conditions, increases the expression of growth factors at the mRNA level. Hemodynamic pathways include elevations of systemic and intraglomerular pressure and activation of various vasoactive hormone pathways, including the renin-angiotensin aldosterone system (RAAS), endothelin, and urotensin. RAAS is the major controller of blood pressure. Renin converts angiotensinogen to angiotensin I, which is further converted by angiotensin-converting enzyme to angiotensin II. The latter is the key promoter of vascular damage (reviewed in Brownlee et at., 2001; reviewed in Soldatos and Cooper, 2008). In patients with T1DM hypertension is associated with DN (Hasslacher et al., 1985) and aggressive treatment of hypertension improves kidney function (Parving et al., 1983).

The altered hemodynamic pathways act independently and in concert with metabolic pathways to activate intracellular secondary messengers such as PKC, mitogen-activated protein kinase (MAPK), nuclear transcription factors such as nuclear factor-κB (NF-κB) and

various growth factors such as the prosclerotic cytokines, transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF) and the angiogenic, permeability enhancing growth factor, vascular endothelial growth factor (VEGF) (reviewed in Brownlee et at., 2001; reviewed in Soldatos and Cooper, 2008). These growth factors have been implicated in modulation of the immune system and activation of enzymes involved in glucose metabolism.

Inflammation is an important factor implicated in the pathogenesis of DN. Inflammatory cytokines, mainly IL-1, IL-6, IL-18, as well as TNF-α are involved in this process. The renal cortical mRNA expression of IL-1, IL-6 and TNF-α were found to be upregulated in experimental diabetes (Navarro et al., 2006). In addition, IL-6 was shown to be elevated in plasma from T1DM and T2DM patients and has been associated with albuminuria (Myrup et al., 1996; Pickup et al., 2000; Saraheimo et al., 2003). Overexpression of IL-18 was reported in human tubular epithelial cells in patients with DN (Miyauchi et al., 2009). Elevated IL-18 levels in both serum and urine were also shown to be directly and strongly associated with the AER (Nakamura et al., 2005). Hyperglycemia promotes chronic inflammation also via NF-κB. Activation of NF-κB by PKC (Yerneni et al., 1999) and RAGE (Lander et al., 1997) was observed in diabetes (Bierhaus et al., 2001). C-reactive protein (CRP) was shown to be elevated in patients with T1DM and associates with the increased AER (Schalkwijk et al., 1999).

Dyslipidemia has also been pointed out as a pathophysiological factor in the development of DN, as it was found to parallel with DKD (Tolonen et al., 2008). In T1DM subjects, low high density cholesterol (HDL) and elevated triglycerides associate with obesity, which has also been linked to DN (Iseki et al., 2004). Hyperinsulinemia also plays a role by increasing glomerular capillary pressure and glomerular hyperfiltration, endothelial dysfunction and increased vascular permeability (reviewed in Groop et al., 2005). Furthermore, insulin resistance not only parallels but also precedes the development of DN in T1DM patients (Yip et al., 1993). In combination, all of these factors increase AER, extracellular matrix accumulation, cell hypertrophy, and apoptosis (Figure 5).

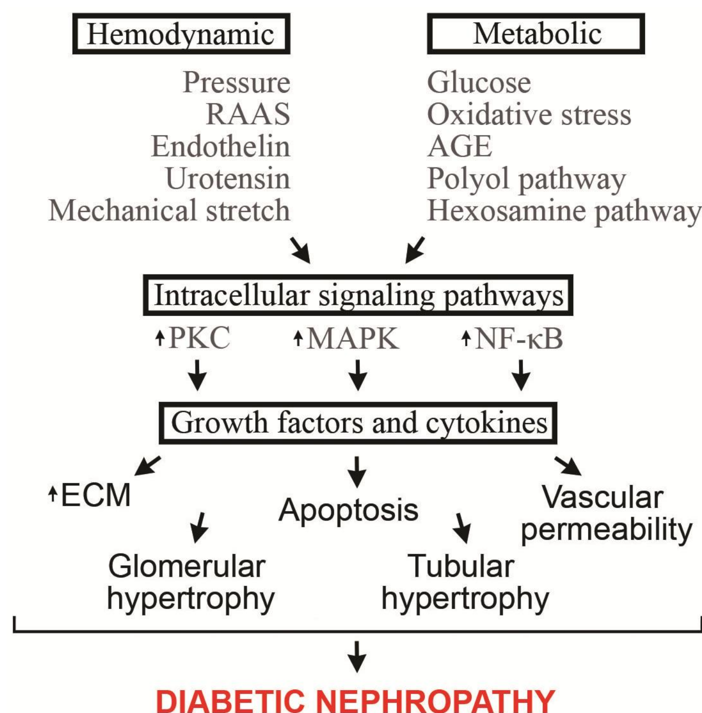

Figure 5. An overview of the pathogenic mechanisms involved in the development of diabetic nephropathy. AGE: advanced glycation end products; ECM: extracellular matrix; MAPK: mitogen-activated protein kinase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; PKC: protein kinase C; RAAS: renin-angiotensin-aldosterone system.

2.5 Animal models of diabetic kidney disease

Animal models of DKD are keys to understanding disease pathogenesis and to developing rational treatment strategies. There are, however, important anatomical and functional distinctions between animals and humans. Experimental models of diabetes only partially reproduce the pathophysiological and histological changes characteristic of human DN (Rees and Alcolado, 2005). Histological changes (glomerulosclerosis and Kimmelsteil–Wilson nodules) are generally much less severe in animal models than those seen in humans. The severity of glomerulosclerosis in rodent models varies considerably within species, suggesting that the genetic background independently affects the disease process. Declining glomerular filtration rate is often not seen. Perhaps the lack of some of these features in the models reflects the fact that human DN can take decades to develop, and it seems likely that their absence is due to the relatively short lifespan of the mice or rats (reviewed in King, 2012). However, rodent models remain the most popular species to approximate human disease. There has been a progressive increase in studies using mouse models, with a

corresponding decrease in the use of rats (Brosius et al., 2009). The large number of rodent models of DKD include artificially induced, spontaneously mutated and genetically engineered (knockout or transgenic) models.

2.5.1 Streptozotocin-induced diabetic rats

Streptozotocin (stz) model represents an artificially induced model for T1DM. Stz is a glucose analogue that enters insulin-producing β-cells in pancreas via glucose transporter 2 (GLUT2) and destroys the cells by acute necrosis or inflammatory reaction (Like and Rossini, 1976; Tesch and Allen, 2007), leading to T1DM. Stz is typically used to induce diabetes in mice, Sprague-Dawley or Wistar-Kyoto rats. Repeated doses are needed in mice to achieve diabetes (Siu et al., 2006). High doses of stz may result in a direct nephrotoxic effect and interfere with the hyperglycemia-induced kidney disease (Tay et al., 2005). Multiple low doses of stz are given to mice, which induces repetitive β-cell damage. However, with multiple low doses the level of albuminuria remains lower as a result of reduced direct nephrotoxicity of stz.

2.5.2 Zucker rats

Obese Zucker rats (fa/fa rats) are the spontaneous mutated model for T2DM. The rats have a missense mutation in the leptin receptor gene (fa/fa) (Chua et al., 1996). The rats exhibit physiological and metabolic similarities to human T2DM including insulin resistance, hyperlipidemia, hypertension and obesity. Hyperglycemia manifests by 12 weeks. Renal abnormalities, manifested as FSGS, mesangial matrix expansion and albuminuria develop late (Coimbra et al., 2000).

2.6 Podocytes in diabetes

Podocyte architecture and function are changed in both T1DM and T2DM (Bjorn et al., 1995; Pagtalunan et al., 1997; White et al., 2004). During disease progression, widening of the foot processes (Bjorn et al., 1995) and podocyte detachment and loss (Pagtalunan et al., 1997; White et al., 2004) become more marked and correlate with the level of proteinuria (Bjorn et al., 1995; White et al., 2002). Effacement is a well-described finding in DN and it

results from the disruption of the SD complex, changes in the foot process-GBM interaction and changes in the negatively charged apical domain of the podocytes. Effacement can also be caused by direct interference with the actin cytoskeleton (Smoyer et al., 1997; Ha, 2006).

2.6.1 Podocyte loss

The podocyte number is significantly reduced already early in diabetes (Pagtalunan et al., 1997; Steffes et al., 2001; White et al., 2002), and reduction in the number of podocytes correlates with increased AER and predicts progression of albuminuria (Meyer et al., 1999; White et al., 2002; White et al., 2004). Podocytes have been also reported in the urine of 53% of diabetic patients with microalbuminuria and in 80% of those with macroalbuminuria (Nakamura et al., 2000).

Hyperglycemia plays a role in podocyte loss through a number of diverse mechanisms. ROS were shown to induce podocyte apoptosis via activation of proapoptotic p38 MAPK and caspase-3 in cultured podocytes and in mouse models of diabetes (Susztak et al., 2006). In addition, TGF-β1 can induce MAPK mediated podocyte loss (Schiffer et al., 2001). Furthermore, high glucose activates RAAS and increased level of angiotensin II has been shown to promote podocyte apoptosis (Ding et al., 2002).

Activation of the PI3K/AKT via nephrin protects against podocyte apoptosis (Huber et al., 2003), and failure in AKT phosphorylation causes apoptosis in db/db mice (Tejada et al., 2008).

2.6.2 Podocyte foot process effacement

Podocyte foot process effacement could be caused by changes in SD-associated proteins, interference with the GBM or podocyte-GBM interaction, actin cytoskeleton abnormalities, or alterations in the negative apical membrane domain of podocytes (reviewed in Mundel and Shankland, 2002).

In T1DM and T2DM patients with micro- and macroalbuminuria, nephrin has been detected in urine (Patari et al., 2003). Loss of nephrin mRNA and protein in podocytes correlates with proteinuria and increased foot process widening (Langham et al., 2002; Koop et al., 2003; Benigni et al., 2004). Downregulation of nephrin expression has also been reported also in streptozotocin-induced diabetic rats and in the nonobese diabetic mouse

(Aaltonen et al., 2001; Forbes et al., 2002). Moreover, in streptozotocin-induced T1DM rats, nephrin protein was also found in the urine (Aaltonen et al., 2001).

In human and experimental models of diabetes expression of α3β1-integrin is decreased (Regoli and Bendayan, 1997; Chen et al., 2006). The suppression progresses with diabetes duration and may be involved in podocyte loss by detachment (Regoli and Bendayan, 1997; Chen et al., 2000).

Studies have also shown that the expression of podocalyxin is reduced in podocytes exposed to high glucose and in streptozotocin-induced T1DM rats (Qi et al., 2007; Fang et al., 2013).

2.7 Role of kidneys in maintaining glucose homeostasis

Despite daily fluctuations, plasma glucose is normally maintained within a narrow range (4-9 mM). The human kidney is involved in the regulation of glucose homeostasis. Under normal conditions, glucose filtered by glomeruli is completely reabsorbed, but under hyperglycemic conditions glucosuria may occur.

2.7.1 Role of kidneys in the regulation of glucose homeostasis

The mechanisms involved in the regulation of glucose homeostasis by the kidney include glucose release via gluconeogenesis, glucose uptake from the circulation and glucose reabsorption from the glomerular filtrate. About 10 to 15 % of the daily glucose intake that circulates in the body is used by the kidneys (Gerich, 1993). Glucose utilization is restricted mostly to the kidney medulla and glucose release occurs in the kidney cortex (Guder and Ross, 1984; Wirthensohn and Guder, 1986; Schoolwerth et al., 1988). Renal glucose release is stimulated by catecholamines (Stumvoll et al., 1995) and suppressed by insulin (Meyer et al., 1998a). In the postabsorptive state, after overnight fasting, the kidneys contribute to around 20% of the overall glucose release (Stumvoll et al., 1995; Ekberg et al., 1999; Moller et al., 2001). Surprisingly, in the postprandial state, kidney gluconeogenesis increases by twofold (Meyer et al., 2002).

2.7.2 Glucose homeostasis in the kidney in diabetes mellitus

Patients with T2DM dispaly increased renal glucose release in postabsorptive and postprandial states compared to healthy individuals (Meyer et al., 1998b; Meyer et al., 2004). As insulin resistance is present in diabetic patients, the process of renal glucose release is resistant to suppression by insulin. Furthermore, in T2DM subjects receiving overnight insulin infusions, renal glucose release remains increased as in patients with diabetes without infusion (Woerle et al., 2002).

Glucose uptake by the kidney is also greater in both postabsorptive and postprandial states in patients with T2DM (Meyer et al., 1998b; Meyer et al., 2004). Renal glucose reabsorption from the glomerular filtrate is also higher in those subjects (Mogensen, 1971). The kidney therefore contributes to the fasting and postprandial hyperglycemia in T2DM patients.

2.8 Molecular mechanisms regulating glucose transport into podocytes

Activation of the IR by insulin leads to a cascade of signaling events that coordinate translocation of the GSV to the plasma membrane and glucose uptake into podocytes. Glucose uptake is achieved through the facilitative glucose transporters GLUT1 and GLUT4. The PI3K and the cGMP-dependent protein kinase G signaling pathways are implicated in the regulation of the GSV trafficking in podocytes. GSV translocation occurs in multiple stages including approaching, tethering, docking and fusion.

2.8.1 Insulin signaling pathways

Podocytes are insulin-responsive cells (Coward et al., 2005). Similar to adipocytes and muscle cells, they respond to insulin by activating the PI3K and MAPK signaling pathways. Insulin can also activate CAP (Cbl-associated protein)/c-Cbl pathway in adipocytes but not in podocytes or muscle cells (Welsh et al., 2010). PI3K is implicated in glucose uptake by inducing the translocation of glucose transporters to the plasma membrane. Another signaling pathway that appears to stimulate glucose uptake in podocytes is the cGMP-dependent protein kinase G (PKG) pathway (Piwkowska et al., 2014). The MAPK signaling pathway does not mediate the metabolic action of insulin in this cell type, but is involved in proliferation, transcriptional regulation of various target genes, and production of cytokines (reviewed in Tian et al., 2000) (Figure 6).

2.8.1.1 Insulin receptor and its substrates

The IR is a heterotetrameric complex, consisting of two extracellular α subunits that bind insulin, and two transmembrane β subunits with tyrosine kinase activity (Lee and Pilch, 1994). The IR exists in two forms, A and B. IR-A is ubiquitously expressed and IR-B is expressed in insulin-sensitive tissues such as liver, muscle, adipose tissue, and kidney. Binding of insulin to IR leads to autophosphorylation of its tyrosine residues. This results in increased catalytic activity of the tyrosine kinase, which further phosphorylates tyrosine residues on the insulin receptor substrate-1 (IRS-1) (Ullrich et al., 1985). Upon phosphorylation, IRS-1 activates PI3K and MAPK pathways.

2.8.1.2 The phosphatidylinositol 3-kinase signaling pathway

Upon tyrosine phosphorylation, IRS proteins interact with the p85 regulatory subunit of the PI3K, which leads to its activation and translocation to the plasma membrane. The enzyme catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form phosphatidylinositol 3,4,5-trisphosphate (PIP_3). Insulin-stimulated increases in PIP_3 result in the phosphorylation and activation of AKT. The negative regulators of PI3K include protein phosphatases that dephosphorylate IRS-1 (Elchebly et al., 1999), as well as lipid phosphatases SHIP2 (SH2-domain-containing inositol polyphosphate-5 phosphatase 2) and phosphatase and tensin homolog (PTEN) (Vinciguerra and Foti, 2006).

2.8.1.3 The cGMP-dependent protein kinase G signaling pathway

cGMP-dependent protein kinase G type I (PKG-I) is a homodimer comprised of two identical subunits, which are found as two isoforms, Iα and Iβ (Hofmann et al., 2009). Dimerization of two PKG-I subunits increases its catalytic activity, and consequently its biological action. The PKG-Iα isoform is expressed in cultured rat podocytes (Piwkowska et al., 2012; Piwkowska et al., 2013), in which PKG-I activators, including insulin and hydrogen peroxide, regulate insulin signaling pathways via increased phosphorylation of IR and AKT. This enhances GLUT4 translocation to the plasma membrane and glucose uptake (Piwkowska et al., 2014).

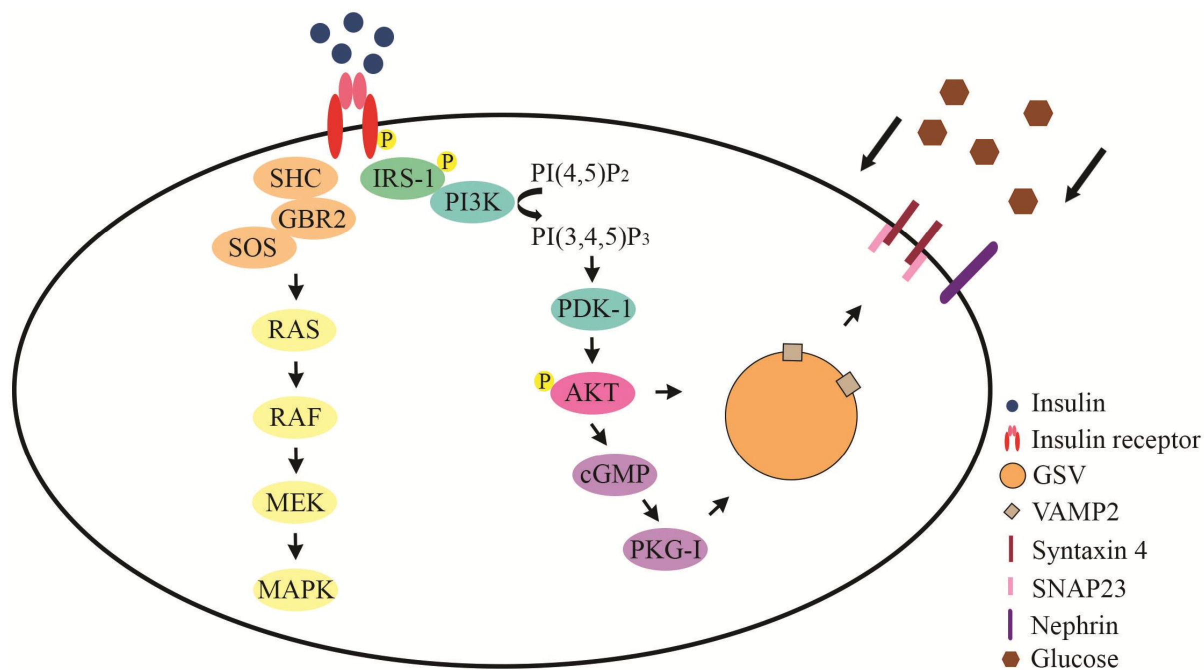

Figure 6. Schematic structure of the insulin signaling pathway in podocytes. GSV: GLUT4 storage vesicle; IRS-1: insulin receptor substrate-1; MAPK: mitogen-activated protein kinase; PDK-1: phosphoinositide-dependent kinase 1; PI3K: phosphatidylinositol 3-kinase; PKG-I: cGMP-dependent protein kinase G type I.

2.8.2 Glucose transporters in the kidney

The facilitative glucose transporter family includes 13 members (Devaskar and Mueckler, 1992), most of which catalyse the transport of glucose down its concentration gradient across the plasma membrane. In the kidney, GLUT1, GLUT3, and GLUT4 are expressed in mesangial cells and podocytes (Brosius et al., 1992; Heilig et al., 1995; Coward et al., 2005; Lewko and Stepinski, 2009). GLUT1 appears to be the predominant transporter in cultured mesangial cells (Li et al., 2001). GLUT2 is known to be expressed in podocytes (Lewko and Stepinski, 2009) and GLUT8 is present in podocytes and tubular cells (Schiffer et al., 2005).

2.8.3 Mechanisms of glucose uptake into podocytes

GLUT4 is the major insulin-inducible glucose transporter. In the basal state, GLUT4 is preferentially located in perinuclear depots and to a lesser extent in cytosolic vesicles and the plasma membrane. However, after insulin stimulation, it is mainly found in vesicles (GSVs) that translocate to the plasma membrane (reviewed in Stöckli et al., 2011). GSV trafficking

consists of several steps. The first step involves the actin and microtubule-based cytoskeleton in the approach of GSVs to the plasma membrane. The second step includes two processes: tethering and docking. The exocyst complex proteins help tether the GSVs to the plasma membrane, and docking is mediated by assembly of the SNARE complex. The last step of GSV trafficking to the cell surface is fusion, in which a specific interaction between v-SNARE and t-SNARE proteins allows merging of the lipid bilayers of the GSVs and the plasma membrane (Figure 7).

Mechanisms that regulate GSV trafficking and glucose uptake into podocytes are not well defined. GLUT1 and GLUT4 are known to mediate insulin-stimulated glucose uptake into cultured human podocytes, and GLUT1 also affects basal glucose uptake (Coward et al., 2005). GLUT2 and GLUT4 were described to mediate glucose uptake in cultured rat podocytes (Lewko et al., 2005). In human podocytes, GLUT4 translocation was shown to be dependent on the actin cytoskeleton (Coward et al., 2005). Furthermore, nephrin is important for the insulin-stimulated fusion of GLUT1 and GLUT4 vesicles with the plasma membrane through its interaction with VAMP2 (Coward et al., 2007). A recent study showed that phosphorylation of synip is required for its dissociation from syntaxin 4 and insulin-stimulated GLUT4 translocation and glucose uptake into podocytes (Yamada et al., 2014).

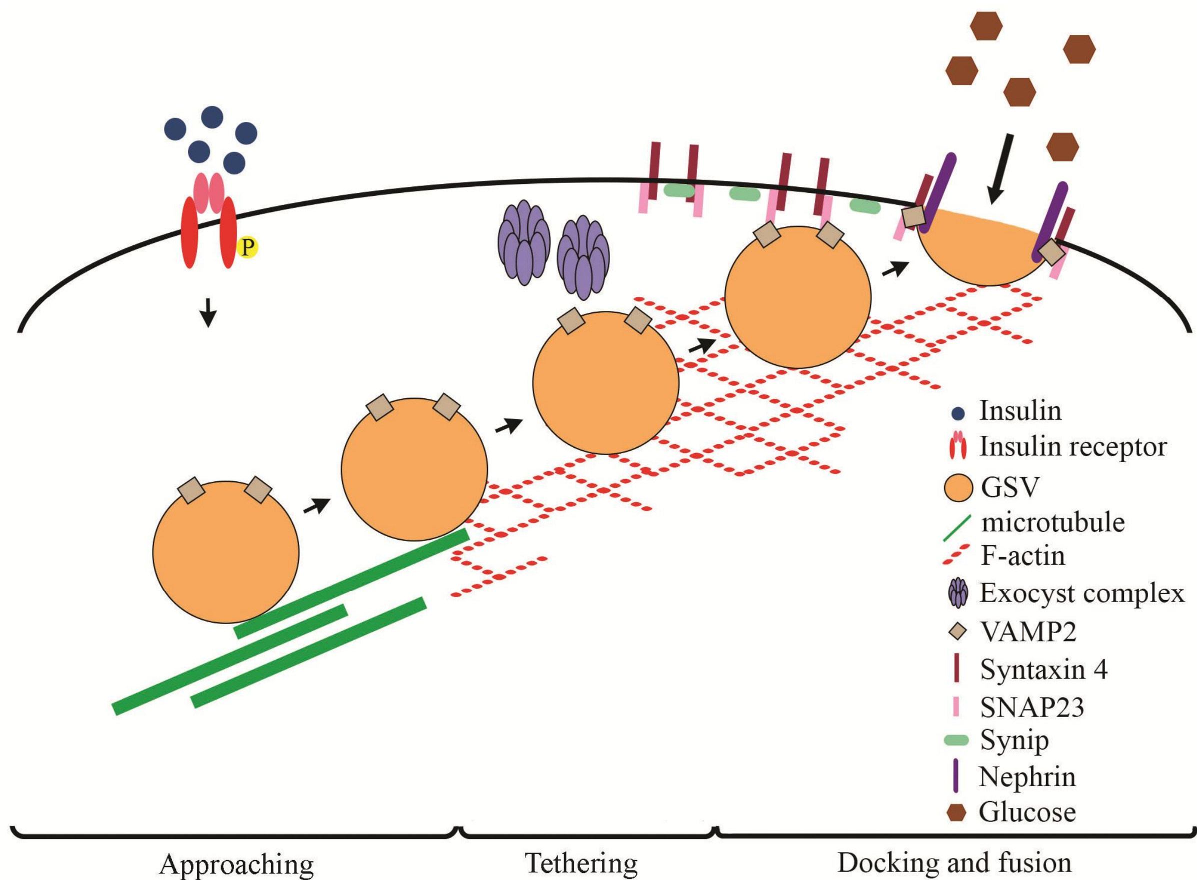

Figure 7. Overview of the steps involved in GLUT4 storage vesicle trafficking to the plasma membrane. Insulin-stimulated translocation of GLUT4 storage vesicles (GSV) consists of multiple steps. The first step involves approaching of GSVs from an intracellular location close to the cell surface after insulin stimulation. The second step, tethering, involves the exocyst complex which plays a role in the low-affinity interaction of GSVs with the plasma membrane. The last steps are docking, a high-affinity interaction of GSVs with the plasma membrane, and fusion, a merging of the lipid bilayers of the GSVs and the plasma membrane. The docking and fusion require formation of the ternary complex between v-SNARE, VAMP2, on the GSV and t-SNAREs, syntaxin-4 and SNAP23, on the plasma membrane.

2.9 Role of insulin resistance in the pathogenesis of diabetic nephropathy

Insulin resistance of podocytes may be an important initiator for many of the pathological processes observed in DN.

2.9.1 Mechanisms of insulin resistance

2.9.1.1 Hyperglycemia

Excess intracellular glucose induces AGE production, which in turn impairs PI3K-dependent insulin signaling (Miele et al., 2003). Moreover, hyperglycemia results in insulin resistance via mechanisms involving oxidative stress and ROS production by NADPH oxidase (reviewed in Brownlee, 2001). Studies have shown that activation of the AMP-activated protein kinase (AMPK) increases glucose transport and could have a beneficial effect in preventing insulin resistance (Tomas et al., 2002).

2.9.1.2 Free fatty acids

Similar to high glucose, free fatty acids (FFA) also stimulate ROS production through PKC-dependent activation of NADPH oxidase (Inoguchi et al., 2000). FFA were shown to be released from adipose tissue and their concentration is elevated in the insulin resistant state (Golay et al., 1986). Increased FFA inhibits insulin signaling and glucose uptake in muscle (Roden et al., 1996) and activates the PKC and NF-κB pathways (Itani et al., 2002).

2.9.1.3 Inflammation

Inflammation correlates with insulin resistance and adiposity in human patients with T2DM (Leinonen et al., 2003). Adipokines, secreted by adipose tissue, have both stimulating and inhibitory roles on insulin sensitivity. Proinflammatory cytokines including TNF-α and IL-6 are secreted by adipocytes and further promote peripheral insulin resistance. Infusion of TNF-α results in the development of insulin resistance in the skeletal muscle of healthy humans by leading to impaired AKT phosphorylation and dysfunction in glucose uptake (Plomgaard et al., 2005). Furthermore, TNF-α stimulates FFA release (Plomgaard et al., 2008). IL-6 also inhibits insulin signaling by interfering with the IR and IRS-1 (Senn et al., 2002). In contrast, leptin and adiponectin mediate insulin-sensitizing actions in peripheral tissues (reviewed in Beale, 2013).

2.9.2 Insulin resistance in kidney dysfunction

Several pieces of evidence suggest that reduced insulin action can affect kidney function. Insulin resistance plays a significant role in the pathogenesis of DN. A large number of epidemiological studies have reported the association between insulin resistance and kidney dysfunction in both non-diabetic and diabetic subjects. In fact, the podocyte IR knockout mouse develops podocyte apoptosis, effacement of foot processes, thickening of the GBM, and increased glomerulosclerosis and albuminuria.

2.9.2.1 The correlation of insulin resistance and diabetic nephropathy

Insulin resistance correlates with microalbuminuria in non-diabetic (Mykkanen et al., 1998), T1DM (Yip et al., 1993) and T2DM subjects (Groop et al., 1993; Forsblom et al., 1995; Parvanova et al., 2006), independent of blood pressure (Groop et al., 1993). This suggests that insulin resistance alone can lead to the development of microalbuminuria. Based on the data from patients with diabetes (Miyazaki et al., 2007) and from animal models (Zhang et al., 2008), insulin sensitizers have a renoprotective effect. This further supports the hypothesis of a pathogenic role of insulin resistance in DN.

2.9.2.2 Podocytes and insulin resistance

Insulin resistance of podocytes was first demonstrated in early diabetes in T2DM db/db mice (Tejada et al., 2008). In the same model, IR and PI3K signaling were lost in podocytes (Tejada et al., 2008). A similar loss of insulin signaling via PI3K was found in the glomeruli of T1DM streptozotocin rats and T2DM Zucker rats (Mima et al., 2011). Podocyte-specific IR knockout mice develop albuminuria and histological features of DN even in the setting of normoglycemia (Welsh et al., 2010). Interestingly, the podocyte protein nephrin is crucial for the insulin sensitivity of podocytes as measured by glucose uptake (Coward et al., 2007), and its expression was shown to be reduced in early DN (Doublier et al., 2003; Patari et al., 2003; Jim et al., 2012).

The role of adiponectin and cathepsin L in the development of podocyte insulin resistance may also be considered. Adiponectin may have a protective effect on podocytes via stimulation of the AMPK pathway and reduced oxidative stress, as well as modulation of glucose uptake into podocytes. Podocytes isolated from db/db mice showed decreased

expression of adiponectin receptor 1 and 2 (Kadowaki et al., 2006; Sharma et al., 2008). Further, cathepsin L, which is associated with the degradation of the IR subunit β, is upregulated in diabetic glomeruli (Yang et al., 2007; Sever et al., 2007).

Studies have shown that in glomeruli of db/db mice, upregulation of JNK, a negative regulator of insulin signaling, may result in the inability of podocytes to respond to insulin (Ijaz et al., 2009). In cultured human podocytes, the free fatty acid palmitate induces insulin resistance via reduced glucose uptake and reduced phosphorylation of IRS-1 and AKT (Lennon et al., 2009). Lipid phosphatase SHIP2 was shown to downregulate insulin response by reducing AKT activation in cultured human podocytes. Further, SHIP2 expression was found to be elevated in glomeruli of insulin resistant obese Zucker rats (Hyvönen et al., 2010).

2.9.3 Glucose transporters in diabetic nephropathy

Individual glucose transporters may affect podocyte function. GLUT1 expression was shown to be elevated in cultured human podocytes exposed to high glucose (Moutzouris et al., 2007). Similar changes were observed in mesangial cells exposed to high glucose *in vitro* and was associated with increased glucose uptake (Heilig et al., 1997) and stimulated production of extracellular matrix proteins (Heilig et al., 1995). Upregulation of GLUT1 was also described in glomeruli of streptozotocin–induced T1DM rats (D'Agora et al., 2001) and in T2DM db/db mice (Chen et al., 2003). Overexpression of GLUT1 in glomerular mesangial cells in mice mimics typical features of diabetic glomerular disease, without diabetes or hypertension (Wang et al., 2010). The data suggest that GLUT1 may play an important role in the development of DKD. However, the role of GLUT1 appears to vary depending on the cell type, as podocyte-specific overexpression of GLUT1 in diabetic mice reduces mesangial expansion (Zhang et al., 2010).

Chronic exposure of cultured human podocytes to high glucose reduced GLUT4 expression (Moutzouris et al., 2007). GLUT4 expression was also shown to be upregulated, and GLUT1 downregulated, in the glomeruli of normoalbuminuric T1DM patients and db/db mice (Guzman et al., 2014). However, glomeruli from T1DM patients with microalbuminuria and microalbuminuric db/db mice presented decreased GLUT4 and increased GLUT1 expression (Guzman et al., 2014). Furthermore, podocyte-specific GLUT4-deficient mice are protected from diabetes-induced podocyte hypertrophy, mesangial expansion, and

albuminuria (Guzman et al., 2014). This suggests that decreasing GLUT4 expression or attenuating its function may be beneficial in proteinuric kidney diseases.

In addition, GLUT3 was shown to be upregulated in human podocytes exposed to high glucose *in vitro* (Moutzouris et al., 2007), and GLUT8 expression levels was higher in podocytes of kidneys of diabetic db/db mice compared with nondiabetic mice (Schiffer et al., 2005). More studies will be required to elucidate the mechanisms of glucose transporter modulation in podocytes to provide further insight into their role in the pathogenesis of DN.

3 AIMS OF THE STUDY

The mechanisms leading to the development of DN are not fully understood, but podocyte damage is involved. Therefore, a more detailed characterization of the early pathophysiological changes in glomeruli is essential to deepen our knowledge of podocyte injury and the mechanisms behind the development of DN. Interestingly, with respect to glucose uptake, podocytes are uniquely insulin sensitive cells in the glomerulus and are able to absorb glucose via translocation of glucose transporters to the plasma membrane. Furthermore, recent data show that insulin signaling is essential for normal kidney function.

Defects in the trafficking of the glucose transporters may affect the insulin sensitivity of podocytes. Therefore, regulators of glucose transporter trafficking may provide suitable targets to enhance the insulin sensitivity of podocytes and prevent the development and progression of DN. The molecular mechanisms that regulate glucose uptake into podocytes are poorly characterized, but it has been shown that insulin-stimulated glucose uptake into podocytes depends on the podocyte protein nephrin. This thesis sought to characterize the early pathophysiological mechanisms leading to glomerular podocyte injury in DN, and to obtain a better understanding of the mechanisms of how glucose transporter trafficking, docking, and fusion are regulated in podocytes.

The specific objectives of this thesis work were the following:

I. To identify changes in the expression of glomerular proteins at an early stage of DN by comparative proteomics and to investigate the role of ezrin in regulating glucose transporter trafficking in podocytes.

II. To investigate the role of septin 7, a novel CD2AP and nephrin interaction partner, in podocytes.

III. To characterize the role of septin 7 and its interaction partner, nonmuscle myosin heavy chain IIA, in the regulation of glucose transporter trafficking in podocytes.

4 MATERIALS AND METHODS

4.1 Experimental animals

4.1.1 Sprague-Dawley rats

The kidneys of Sprague-Dawley rats were used to investigate the expression, localization, and interactions of proteins (Studies I-III).

4.1.2 Streptozotocin rats

Male Sprague-Dawley rats (Toxi-coop, Dunakeszi, Hungary) were injected intraperitoneally with streptozotocin (stz) (Sigma-Aldrich, St. Louis, MO, USA). Development of diabetes was confirmed by an oral glucose tolerance test one week after stz injection and at sacrifice four weeks after stz injection. Urine was collected and urinary albumin was measured by a rat albumin-specific ELISA kit (Immunology Consultants Laboratory Inc, Portland, OR, USA). Animal procedures were approved by the Animal Care and Use Committee of the Semmelweis University (Study I).

4.1.3 Zucker rats

Obese (fa/fa) and lean (fa/+) Zucker rats (Crl:ZUC-Leprfa) were purchased from Charles River Laboratories (Sulzfeld, Germany). Blood glucose, urinary albumin, and creatinine measurements have been described in (Hyvönen et al., 2010). Animal work was approved by the national Animal Experimental Board (Studies I and III).

4.2 Human study subjects

4.2.1 Human kidney samples

Kidney samples of renal cancer patients with or without T2DM were obtained from surgical nephrectomies performed for diagnostic purposes at Helsinki and Uusimaa Hospital district, and were from the nonmalignant part of the kidney. Albuminuria, the clinical sign of

DN, was determined from the medical records. The use of human material was approved by the local Ethics Committee (Studies I and III).

4.2.2 Human sera

Serum samples were obtained from 4 healthy controls and 5 patients with T2DM from the Finnish Diabetic Nephropathy Study (FinnDiane). Urinary AER was defined as normal (<30 mg/24 h), microalbuminuria (≥30, <300 mg/24 h), and macroalbuminuria (≥300 mg/24 h). Fasting glucose values were measured using a Hemocue device (Hemocue, Helsinki, Finland), and serum lipids were determined with a Konelab analyzer (Thermo Scientific, Vantaa, Finland). Other biochemical analyses were performed in an accredited hospital laboratory (HUSLAB, Helsinki, Finland). The use of human material was approved by the local Ethics Committee (Study III).

4.3 Cell culture

Several cell lines were used in this thesis work to investigate the expression, protein-protein interactions, and functions of ezrin, septin 7, and nonmuscle myosin heavy chain IIA. The cell lines used and their culture media are shown in Table 1. Mouse podocytes were maintained in 5% CO_2 at 33°C. Human podocytes were cultured in 5% CO_2 at 33°C (undifferentiated podocytes) and shifted to 37°C for 14 days in 5% CO_2 for differentiation. HIRc (rat fibroblasts stably overexpressing human insulin receptor), HEK293, HEK293T and HEK293FT cells were maintained in 5% CO_2 at 37°C (Studies I-III). Cells were transfected with ON-TARGET plus SMARTpool or siCONTROL Non-Targeting Pool#2 siRNAs (Dharmacon, Lafayette, CO, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) (Studies I-III). Mouse podocytes were transiently transfected with pcDNA 3.1-SEPT7 or pcDNA 3.1 as a control using Lipofectamine 2000 (Invitrogen) (Study III). HEK293T cells were co-transfected with pLNCX2-nephrin and packaging pKAT2 vectors using Lipofectamine 2000 (Invitrogen). Virus-containing medium was used to infect mouse podocytes (Study III). In some studies, human podocytes were treated with 5 µM cytochalasin D (Sigma-Aldrich) for 30 min (Study II), and HIRc cells and mouse podocytes were treated with 50 µM forchlorfenuron (FCF) (Sigma-Aldrich) for 4 h (Study II).

Cell line	Culture media	Reference / supplier	Study
Wild-type and CD2AP KO mouse podocytes	DMEM medium containing 4.5 g/L glucose supplemented with L-glutamine, 10% fetal calf serum, penicillin and streptomycin	Schiffer et al., 2004	I, III
Human podocytes	RPMI 1640 medium containing L-glutamine supplemented with 10% fetal calf serum and insulin, transferrin and selenite	Saleem et al., 2002	II
HIRc	DMEM medium containing 1 g/L glucose supplemented with L-glutamine, 10% fetal calf serum, penicillin and streptomycin	McClain et al., 1987	II
HEK293, HEK293T, HEK293FT	DMEM medium containing 4.5 g/L glucose supplemented with L-glutamine, 10% fetal calf serum, penicillin and streptomycin	ATCC	II, III

Table 1. Cell lines used in Studies I-III.

4.4 Antibodies

The primary and secondary antibodies used in Studies I-III are presented in Table 2 and Table 3.

Antibody	Description	Reference / supplier	Study
ezrin (3C12)	mouse monoclonal	Bohling et al., 1996	I
phosphorylated ezrin	rabbit polyclonal	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	I
NHERF2	rabbit polyclonal	kindly provided by Dr. Peijian He (Emory University, Atlanta, GA, USA)	I
moesin	rabbit polyclonal	AbD Serotec (Oxford, UK)	I
podocin	rabbit polyclonal	Sigma-Aldrich	I
podocalyxin	rabbit polyclonal	Miettinen et al., 1990	I
cofilin-1	rabbit polyclonal	Abcam (Cambridge, UK)	I
phosphorylated cofilin-1 (Ser3)	rabbit monoclonal	Cell Signaling Technology (Denvers, MA, USA)	I
septin 7 (N12)	goat polyclonal	Santa Cruz Biotechnology	II, III

septin 7 (H120)	rabbit polyclonal	Santa Cruz Biotechnology	II, III
septin 7 (C)	rabbit polyclonal	Immuno-Biological Laboratories Co., Ltd. (Gumma, Japan)	II, III
septin 9	rabbit polyclonal	kindly provided by Koh-ichi Nagata (Aichi Human Service Center, Japan)	II
septin 11	rabbit polyclonal	kindly provided by Koh-ichi Nagata	II
CD2AP 209	rabbit polyclonal	Lehtonen et al., 2008	II
CD2AP 211	rabbit polyclonal	Lehtonen et al., 2008	II
CD2AP 1774	rabbit polyclonal	Lehtonen et al., 2000	II
nephrin #1034	rabbit polyclonal	Ahola et al., 2003	II, III
nephrin	guinea pig polyclonal	PROGEN Biotechnik (Heidelberg, Germany)	II, III
nephrin	mouse polyclonal	Topham et al., 1999	III
Pan AKT	mouse monoclonal	R&D Systems (Minneapolis, MN, USA)	II
phosphorylated AKT (Ser473)	rabbit polyclonal	Cell Signaling Technology	II
nonmuscle myosin IIA heavy chain	rabbit polyclonal	Biomedical Technologies Ins. (Stoughton, MA, USA)	III
phosphorylated myosin IIA heavy chain (Ser1943)	rabbit polyclonal	ECM Bioscience (Versailles, KY, USA)	III
phosphorylated myosin light chain 2 (Thr18/Ser19)	rabbit polyclonal	Cell Signaling Technology	III
GLUT1	rabbit polyclonal	Millipore (Billerica, MA, USA)	I
GLUT4	rabbit polyclonal	Abcam	I, II
SNAP23	rabbit polyclonal	Synaptic System (Goettingen, Germany)	III
SNAP23	mouse monoclonal	Sigma-Aldrich	III
syntaxin 4	rabbit polyclonal	Sigma-Aldrich	II, III
VAMP2	mouse monoclonal	Synaptic System	II, III
actin	mouse monoclonal	Sigma-Aldrich	I-III
tubulin	mouse monoclonal	Sigma-Aldrich	I-III

Table 2. Primary antibodies used in Studies I-III.

Antibody	Description	Supplier	Study
AlexaFluor 488	donkey anti-mouse	Molecular Probes (Life Technologies, Carlsbad, CA, USA)	I
AlexaFluor 488	donkey anti-goat	Molecular Probes	II
AlexaFluor 488	donkey anti-rabbit	Molecular Probes	II, III
AlexaFluor 555	donkey anti-mouse	Molecular Probes	I, III
AlexaFluor 555	donkey anti-rabbit	Molecular Probes	I, II
AlexaFluor 680	donkey anti-rabbit	Invitrogen	II
IRDye 680	donkey anti-mouse	LI-COR (Lincoln, NE, USA)	I-III
IRDye 800	donkey anti-rabbit	LI-COR	I-III
TrueBlot®	HRP-conjugated anti-mouse	eBioscience (San Diego, CA, USA)	II, III
TrueBlot®	HRP-conjugated anti-rabbit	eBioscience	II, III
TRITC-phalloidin	F-actin staining	Molecular Probes	I, II

Table 3. Secondary antibodies used in Studies I-III.

4.5 Protein studies

4.5.1 Preparation of glomerular, tubular and cell lysates

Glomeruli and tubules were isolated from rat kidney cortex using a graded sieving method (sieves of decreasing pore sizes: 250 µm, 150 µm and 75 µm) (Orlando et al., 2001). Tubules were collected from the 150 µm sieve and glomeruli from the 75 µm sieve. Glomeruli and tubules were lysed in 1% NP-40, 20 mM HEPES, pH 7.5, 150 mM NaCl. Cells were lysed in 1% NP-40, 20 mM HEPES, pH 7.5, 150 mM NaCl or 50mM HEPES, pH 7.6, 0.5% Triton X-100, 0.5% CHAPS or 0.5% NP-40, 100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA by rotation at 4°C for 30 min. All buffers were supplemented with 1x Complete EDTA-free proteinase inhibitor cocktail (Roche, Basel, Switzerland), 50 mM sodium fluoride and 1 mM sodium orthovanadate. Detergent-insoluble material was removed by centrifugation (16 000 x g at 4°C for 15 min), and the protein concentrations were measured by using a Bradford assay (Studies I-III).

4.5.2 Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)

Rat glomeruli were isolated by graded sieving (Orlando et al., 2001), lysed in 7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]- 1-propanesulfonate, 30 mmol/L Tris-HCl, pH 8.0, and 0.2% SDS, and sonicated. Protein concentrations were measured using 2D Quant Kit (GE Healthcare, Chalfont St. Giles, UK). Glomerular lysates from rats with streptozotocin-induced diabetes or controls, and an internal standard (a pool of all samples) were labelled using CyDye DIGE Fluor minimal labeling kit (GE Healthcare). Isoelectric focusing was performed with Immobiline™ DryStrips (GE Healthcare). Proteins were resolved in polyacrylamide gels and imaged with Typhoon™ 9400 (GE Healthcare). Spot detection, matching, and intensity-based quantitation were performed using DeCyder™ 2D 7.0 software (GE Healthcare). Spots with at least 1.5-fold difference in expression between control and streptozotocin-induced diabetic groups were considered differentially expressed and significant (Study I).

4.5.3 Identification of proteins by LC-MS/MS

Proteins were detected from silver-stained 2D SDS-PAGE gel (Study I) or from a GelCode Blue (Pierce Chemical Co, Rockford, IL, USA) stained gel (Study III). Protein spots of interest were in-gel digested with trypsin. The resulting peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described previously (Ohman et al., 2010). Database searches were done with Mascot (Matrix Science Ltd, London, UK) through ProteinPilot 2.0 (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) interface against the SwissProt database via UniProt (http://www.uniprot.org). The search criteria were: rodent-specific taxonomy (Study I) and human-specific taxonomy (Study III). All the reported protein identification scores were statistically significant ($p<0.05$) (Studies I and III).

4.5.4 Immunoblotting

Proteins were separated by SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore), which were blocked with 5% skimmed milk or with Odyssey blocking buffer (LI-COR). Membranes were incubated with specific primary antibodies, washed, and the bound antibodies were detected using secondary antibodies conjugated with Alexa Fluor 680

(Invitrogen) or IRDye 680 or 800 (LI-COR). Blots were scanned and quantified using an Odyssey Infrared Imaging System (LI-COR) (Studies I-III).

4.5.5 2-Deoxy-d-glucose uptake assay

Mouse podocytes were serum-starved for 20 h and treated with or without 20 nM insulin for 15 min in Krebs Ringer phosphate buffer (128 mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl, 10 mM Na_2HPO_4, pH 7.4). HIRc cells were serum-starved for 16 h and treated with or without 200 nM insulin for 15 min in Krebs Ringer phosphate buffer. Cells were exposed to 50 μmol/l (1 μCi/ml) 2-deoxy-d-[(1, 2-3H(N)]-glucose (Perkin Elmer-Cetus, Boston, MA, USA) for 5 min at 33°C (mouse podocytes) or 37°C (HIRc), washed with ice-cold PBS, and solubilized in 1% Triton X-100 in PBS. β-Emission was measured by Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter (Perkin Elmer-Cetus) (Studies I-III).

4.5.6 Protein interaction studies

4.5.6.1 Co-immunoprecipitation assay

Rat glomeruli, human podocytes, or mouse podocytes were lysed as previously described. Lysates were precleared with protein A or G Sepharose beads (Invitrogen or Zymed, South San Francisco, CA, USA) or TrueBlot® anti-rabbit or anti-mouse Ig IP beads (eBiosciences) and incubated overnight at 4°C with primary antibodies or purified rabbit or mouse IgGs serving as controls. The antibody-protein complexes were captured to beads, and unbound proteins were removed by washing with lysis buffer. Samples were boiled in Laemmli sample buffer, separated by SDS-PAGE and immunoblotted (Studies II and III).

4.5.6.2 Production of GST-fusion proteins and pull-down assays

GST, GST-CD2AP SH3 domains (Palmen et al., 2002), GST-nephrin (Lehtonen et al., 2004) and GST-septin 7 fusion proteins covering different regions of the protein (Nagata et al., 2004) were produced in *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA, USA). The fusion proteins were purified with glutathione-Sepharose beads (Invitrogen) and analyzed by SDS-PAGE and Coomassie staining. Rat glomerular lysates were precleared with glutathione-

Sepharose beads and incubated with GST-fusion proteins or GST on beads at 4°C for 4 h. Beads were washed with lysis buffer, boiled in Laemmli buffer, separated by SDS-PAGE and immunoblotted (Study II).

4.5.6.3 Duolink In Situ

Interactions of SNAP23 with septin 7, nonmuscle myosin IIA, phosphorylated myosin heavy chain, and phosphorylated myosin regulatory light chain were detected by the proximity ligation assay kit Duolink: PLA probe anti-rabbit plus, PLA probe anti-mouse minus (Olink Bioscience, Uppsala, Sweden). The samples were processed following the manufacturer's instructions and the fluorescence images were captured using a Zeiss Axioplan2 microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Quantification of the detected PLA signals was performed using the DuoLink Image Tool (Olink Bioscience) (Study III).

4.5.7 Subcellular fractionation

Mouse podocytes were homogenized by passage through a 25G needle and the resulting homogenate was centrifuged at 1000 x g, 4°C for 5 min to obtain post-nuclear supernatant (PNS). The PNS was ultracentrifuged 100 000 x g at 4°C for 1 h using a SW55 Ti rotor to obtain cytosol (S100) and membrane (P100) fractions. Equal volumes of the fractions were separated in SDS-PAGE for immunoblotting (Study II).

4.6 Electron microscopy

Kidney samples were fixed in 1.5% glutaraldehyde, 3% paraformaldehyde, 5% sucrose in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 2 h, postfixed in 1% osmium tetroxide (OsO_4) in the same buffer for 1 h, stained *en-bloc* in 1% uranyl acetate in 10% ethanol for 1 h, dehydrated in ethanol and embedded in Epon. Sections were stained with uranyl acetate and lead citrate and examined in a JEM-1400 Transmission Electron Microscope (Jeol) equipped with TEM CCD camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany) (Study I).

4.7 Immunofluorescence microscopy

4.7.1 Immunofluorescence microscopy of kidney tissue

Rat kidney cryosections were fixed with PFA (followed by permeabilization with Triton X-100) or acetone, washed with PBS, blocked with CAS-BLOCK (Invitrogen) and incubated with primary antibodies diluted in ChemMate™ (DakoCytomation, Glostrup, Denmark) at 4°C overnight. The following day, sections were washed with PBS, incubated with AlexaFluor secondary antibodies (Molecular Probes) and Hoechst 33342 (Sigma-Aldrich) for nuclear staining. Sections were washed with PBS, mounted with Mowiol and examined with a Zeiss Axioplan2 microscope (Carl Zeiss Microscopy) (Studies I and II).

4.7.2 Immunofluorescence microscopy of cultured cells

Cells on glass coverslips were fixed with PFA, followed by permeabilization with Triton X-100. After washing with PBS, cells were blocked in 2% FCS, 2% bovine serum albumin, 0.2% fish skin gelatin or CAS-BLOCK and incubated with primary antibodies at room temperature for 1 h. After washing with PBS, cells were incubated with AlexaFluor secondary antibodies. Actin stress fibers were visualized with TRITC-phalloidin (Molecular Probes) and nuclei with Hoechst 33342 (Sigma-Aldrich). Cells were washed with PBS and mounted in Mowiol. Samples were examined with a Zeiss Axioplan2 microscope (Carl Zeiss Microscopy), a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) or with a Leica SP8 confocal microscope (Leica Microsystems) (Studies I-III).

4.7.3 Surface staining

Mouse podocytes stably expressing nephrin were grown on coverslips. Cells were incubated on ice for 15 min with primary antibody diluted in ice-cold PBS and 5% FBS. After washes with PBS, cells were fixed with PFA, washed with PBS and blocked with 5% FBS, followed by incubation with AlexaFluor secondary antibody (Molecular Probes). Cells were washed with PBS and mounted in Mowiol. Samples were examined with a Zeiss Axioplan2 microscope (Carl Zeiss Microscopy) (Study III).

4.8 Immunohistochemistry

Human and mouse kidney samples were fixed with 10% formalin, dehydrated, and embedded in paraffin. Immunoperoxidase staining was performed with a VectaStain Elite kit (Vector Laboratories, Burlingame, CA, USA). Sections were deparaffinized, antigen retrieval was performed by boiling for 15 min in a microwave oven in 10 mM citric acid, pH 6.0 (Studies I-III) or in 10 mM Tris, 1 mM EDTA and 0.05% Tween 20, pH 9.0 (Study III), and endogenous peroxidase was inactivated by incubation in hydrogen peroxide in methanol for 30 min. Sections were blocked with CAS-block (Invitrogen) and incubated with primary antibodies diluted in ChemMate™ (DakoCytomation) and with biotinylated secondary antibodies followed by incubation with ABC-reagent and AEC (Sigma-Aldrich) for colour development. Sections were counterstained with hematoxylin, mounted with Shandon Immu-Mount (Thermo Scientific, Waltham, MA, USA) and examined by light microscope (Studies I-III).

4.9 Statistical analysis

In all experiments, the differences between the groups were evaluated with Student's t-test. *P*-values less than 0.05 were considered statistically significant. Sex frequencies were compared between cases and controls with the $\chi 2$ test. Normalized intensities of matched spots were compared between the groups, and spots with intensity changes >1.5-fold with a CI >95% were considered differentially expressed and significant. Results are presented as means and STDEV. Analyses were performed using Excel (Microsoft, Redmond, WA, USA) or SPSS PASW Statistics (version 18) software (IBM, Armonk, NY, USA) (Studies I-III).

5 RESULTS

5.1 Ezrin is downregulated in diabetic kidney glomeruli and regulates actin reorganization and glucose uptake in cultured podocytes (Study I)

5.1.1 Streptozotocin-induced diabetes leads to differential expression of glomerular proteins

To identify differentially expressed proteins that could be associated with the development of diabetic kidney injury at an early stage, quantitative proteomic profiling of glomeruli isolated from rats with streptozotocin-induced diabetes and controls was performed using 2D-DIGE coupled with mass spectrometry. Analysis was performed 4 weeks after induction of diabetes. The streptozotocin-injected rats used for the analysis were albuminuric and had high blood glucose compared to controls (Study I, Figure 1A and B), but did not yet show ultrastructural changes in glomeruli (Study I, Figure 1C and D). DeCyder software analysis revealed 2274 spots, out of which 29 exhibited statistically significant differences (Student's t-test value ≤0.05) (Study I, Supplemental Figure S1; Study I, Supplemental Table S1). Mass spectrometry identified a single protein in 12 of these spots, including several actin binding and actin cytoskeleton organizing proteins. Among these were cytoskeletal linkers ezrin and NHERF2, which were downregulated in the diabetic rat glomeruli. Organization of the actin cytoskeleton in podocytes is altered in several types of glomerular diseases (Mathieson, 2012), but the regulation of actin dynamics in podocytes in diabetic nephropathy is not well characterized. We therefore chose ezrin and NHERF2 for further analysis.

Ezrin and NHERF2 showed a 2.1-fold and 1.94-fold decrease, respectively, in diabetic glomeruli (Study I, Figure 1E-H; Study I, Supplemental Table S1). Quantitative Western blotting confirmed that ezrin and NHERF2 were downregulated by 49% and 42%, respectively, in the glomeruli of the streptozotocin-induced diabetic rats compared with citrate-treated rat glomeruli (Study I, Figure 2A and B). We also observed that ezrin expression was downregulated in mouse podocytes cultured in high glucose media (25 mM) for two weeks (Wasik et al., unpublished data, Figure 8). Phosphorylation of ezrin (p-ezrin) at threonine 567 was also reduced by 49% (Study I, Figure 2A and B). Immunostaining revealed decreased expression of ezrin, NHERF2, and p-ezrin in the glomeruli of diabetic rats confirming the data obtained by quantitative immunoblotting (Study I, Figure 2C-E and G-I). Podocalyxin, which is linked to ezrin and the actin cytoskeleton via NHERF2 (Orlando et al.,

2001), was also downregulated by 35% in the glomeruli of streptozotocin-treated rats (Study I, Figure 2A and B). The expression of podocin remained unchanged (Study I, Figure 2A, B, F and J). Immunostaining further confirmed the 2D-DIGE and quantitative Western blotting results.

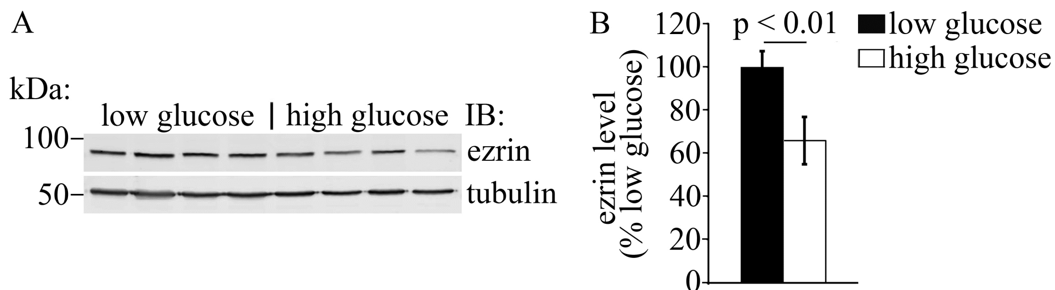

Figure 8. High glucose leads to downregulation of ezrin in mouse podocytes. (A) Ezrin is downregulated by 34% in mouse podocytes cultured in high glucose media for two weeks. Tubulin is included as a loading control. (B) Quantification of the ezrin level in (A). Mouse podocytes were grown in high glucose media (25 mM) for two weeks. Cell lysates were separated by SDS-PAGE and immunoblotted with anti-ezrin and anti-tubulin IgGs. Bars show the mean and error bars the SD of three independent experiments. Statistical analysis was carried out with Student's t-test.

5.1.2 Knockdown of ezrin induces dynamic remodelling of the actin cytoskeleton in podocytes

To study the role of ezrin in regulating podocyte function, we used siRNA-mediated knockdown of ezrin in cultured mouse podocytes (Study I, Figure 3). Transfection of podocytes with ezrin siRNA reduced the expression level of ezrin by 60% compared to the control siRNA-transfected cells (Study I, Figure 3A and B). Another member of the ERM family, moesin, was not affected by ezrin knockdown (Study I, Figure 3A and B).

As normal structure and function of podocytes are maintained by dynamic actin cytoskeleton and ezrin is an important modulator of F-actin (Arpin et al., 1994; Vaheri et al., 1997), we analyzed the effect of ezrin depletion on actin cytoskeleton organization in podocytes. In control siRNA-treated cells, ezrin was expressed in the cytoplasm (Study I, Figure 3C) while phalloidin, which visualizes filamentous actin, showed prominent actin stress fibers (Study I, Figure 3D). Knockdown of ezrin by siRNA (Study I, Figure 3E) led to

reduction of actin stress fibers and accumulation of actin in the cortical region of podocytes (Study I, Figure 3F).

Next, we searched for the mechanisms by which ezrin mediates actin remodelling. A previous study showed that phosphorylated ERM-proteins co-distribute with cofilin-1 and F-actin (Marsick et al., 2012). Cofilin-1 modulates actin dynamics via depolymerizing and severing actin filaments (Nishida et al., 1984; Yonezawa et al., 1985; Theriot, 1997) and cofilin-1 is an important regulator of actin cytoskeleton organization also in podocytes (Garg et al., 2010; Ashworth et al., 2010). We therefore investigated the localization of cofilin-1 and phosphorylated, inactive cofilin-1 (p-cofilin-1) in ezrin depleted and control podocytes. We found that cofilin-1 and phosphorylated cofilin-1 localize in the cytoplasm in control siRNA-transfected podocytes (Study I, Figure 3G and H), whereas both proteins were partially translocated to the cell periphery in ezrin-depleted podocytes (Study I, Figure 3I and J). This suggests that ezrin loss induces partial relocalization of cofilin-1 to the plasma membrane to mediate remodelling of the cortical actin cytoskeleton.

5.1.3 Knockdown of ezrin increases glucose uptake of podocytes and GLUT1 translocation to the plasma membrane

Dynamic remodelling of the actin cytoskeleton has been implicated in the translocation and fusion of the GSVs with the plasma membrane and thus, in insulin-mediated glucose uptake (Tong et al., 2001). An active role of cortical actin in this process has been proposed previously (Omata et al., 2000). Ezrin increases the dynamic reorganization of the actin cytoskeleton (Study I, Figure 3) and functions in the release of insulin granules by regulating exocytosis in β-cells (Lopez et al., 2010). This suggests that ezrin could regulate the trafficking of glucose transporters and glucose uptake in podocytes. To study this, we measured 2-deoxy-D-glucose uptake in mouse podocytes transfected with ezrin siRNA. We found that under basal conditions and in serum-starved podocytes, glucose uptake was increased in ezrin-depleted podocytes by 53% and 31%, respectively, compared to control siRNA-transfected cells (Study I, Figure 5A and B). Insulin stimulation increased glucose uptake by 110% in control siRNA-transfected podocytes (Study I, Figure 5B). However, in ezrin siRNA-transfected podocytes insulin increased glucose uptake only by 76% compared to serum-starved, control siRNA-transfected cells (Study I, Figure 5B). These results indicate that loss of ezrin in podocytes increases glucose uptake, but insulin-stimulated glucose uptake is significantly inhibited by knockdown of ezrin.

We further investigated whether ezrin depletion would affect the trafficking of glucose transporters, GLUT1 and GLUT4, in podocytes. Introduction of ezrin siRNA into mouse podocytes induced translocation of GLUT1 to the plasma membrane under basal, starved and insulin-stimulated conditions (Study I, Figure 8A, B, E, F, I and J). GLUT4 remained in the cytoplasm of ezrin-depleted podocytes in basal and starved conditions (Study I, Figure 8C, D, G and H), but translocated to the plasma membrane after insulin stimulation (Study I, Figure 8K and L). This suggests that depletion of ezrin increases glucose uptake by enhancing translocation of GLUT1 to the plasma membrane.

5.1.4 Cofilin-1 is involved in ezrin-mediated cortical actin remodelling

As cofilin-1-mediated actin reorganization is involved in the translocation of GSVs to the cell surface in muscle cells (Chiu et al., 2010) and knockdown of ezrin induces dynamic remodelling of the actin cytoskeleton in podocytes, we analyzed both actin organization and cofilin-1/p-cofilin-1 localization in control and ezrin-depleted podocytes stimulated with insulin. In control siRNA-transfected podocytes insulin stimulation induced actin remodelling and increased cortical actin (Study I, Figure 6A and B). In starved, ezrin-depleted podocytes cortical actin was present (Study I, Figure 6C), but insulin stimulation reduced the cortical actin and increased stress fibers (Study I, Figure 6D). These data indicate that insulin stimulation increases cortical actin reorganization in control podocytes, and that ezrin depletion reduces insulin-induced cortical actin remodelling.

We also found that localization of cofilin-1 and p-cofilin-1 reflects the dynamic changes observed in the organization of the actin cytoskeleton upon ezrin knockdown and insulin stimulation. In control siRNA-transfected podocytes, insulin induced partial translocation of cofilin-1 and p-cofilin-1 from their cytoplasmic location (Study I, Figure 6E and I) to the cell periphery (Study I, Figure 6F and J). Introduction of ezrin siRNA into serum-starved podocytes resulted in cofilin-1 and p-cofilin-1 partial translocation to the periphery of podocytes (Study I, Figure 6G and K), but after insulin stimulation of ezrin-depleted podocytes, both proteins were found only in the cytoplasm (Study I, Figure 6H and L). The data suggest that cofilin-1 is involved in ezrin-mediated remodelling of cortical actin.

5.1.5 Phosphorylation of cofilin-1 is regulated by insulin in cultured podocytes and increases in glomeruli of diabetic rats

A previous study showed that short-term treatment of muscle cells with insulin leads to dephosphorylation of cofilin-1 (Chiu et al., 2010). Similarly, a reduced level of inactive, phosphorylated cofilin-1 was observed in podocytes stimulated with insulin for 15 minutes (Study I, Figure 7A-C). However, culturing podocytes in the presence of insulin for 48 hours increased the level of phosphorylated cofilin-1 (Study I, Figure 7D-F). The effect of insulin on the activity of cofilin-1 is thus determined by the acute or chronic nature of the exposure, either activating or inactivating it, respectively.

Furthermore, we speculated that the level of phosphorylated cofilin is altered in glomeruli of streptozotocin-treated rats. Quantitative Western blotting indicated that the level of total cofilin-1 remained unchanged, but phosphorylated cofilin-1 was increased in the glomeruli of the diabetic rats (Study I, Figure 9). This suggests that cofilin-1 is inactivated (phosphorylated) in glomeruli in experimental diabetes.

5.1.6 Expression of ezrin is reduced in glomeruli of obese Zucker rats and in glomeruli of human patients with Type 2 diabetes

Obese Zucker rats and their age-matched lean controls were used to investigate the expression level of ezrin. Quantitative Western blotting revealed that ezrin expression is decreased by 20% in the glomeruli of obese Zucker rats when compared to lean Zucker rats (Study I, Supplemental Figure S2).

The expression of ezrin was also studied in human kidney biopsy specimens obtained from patients with T2DM and controls. Interestingly, immunohistochemistry revealed significantly lower expression of ezrin in the glomeruli of patients with diabetes (Study I, Figure 10).

5.2 Septin 7 and nonmuscle myosin IIA regulate glucose uptake into podocytes and compete for binding to the target membrane SNARE complex (Studies II and III)

5.2.1 Septin 7 is a novel interaction partner of CD2AP, nephrin and nonmuscle myosin IIA

As the adaptor protein CD2AP is essential for glomerular ultrafiltration (Shih et al., 1999), we searched for novel interaction partners of CD2AP. A pull-down assay of glomerular lysates with the GST-3.SH3 domain of CD2AP followed by mass spectrometry identified the small cytoskeletal GTPase septin 7 as a novel interaction partner of CD2AP (Study II, Figure 1A). Septin 7 was originally discovered in yeast (Hartwell, 1997) and shown to be involved in cell division, polarity, and vesicle trafficking (Hsu et al., 1998; Beites et al., 1999; Kinoshita et al., 2002; Kremer et al., 2007). To analyze which domain(s) of CD2AP are required for the interaction with septin 7, we performed experiments to pull down endogenous septin 7 with the different SH3 domains of CD2AP and with GST as a negative control. The pull-downs, resolved by SDS-PAGE and immunoblotted with septin 7 antibody, showed that 2.SH3 and 3.SH3 domains of CD2AP bind to septin 7 (Study II, Figure 1B). Pull-down assays with different domains of septin 7 fused to GST were also performed. The COOH-terminus of septin 7 was found to be involved in the binding to CD2AP (Study II, Figure 1D). To confirm the physiological interaction between septin 7 and CD2AP, a co-immunoprecipitation was performed, showing that two different CD2AP antibodies co-immunoprecipitate septin 7 from HEK293 cells (Study II, Figure 1E). Furthermore, double staining with septin 7 and CD2AP in rat kidney sections revealed partial colocalization of the proteins, supporting their interation (Study II, Figure 2D-F).

Mammalian septins have been shown to form homo-oligomers *in vitro* and hetero-oligomeric polymers *in vitro* and *in vivo* (Joberty et al., 2001; Nagata et al., 2004). Using an immunoprecipitation approach, we found that septin 7 forms a complex with septin 9 and septin 11 in differentiated cultured human podocytes (Study II, Supplemental Figure S3A-C) We further found that depletion of septin 7 reduces the expression of the other members of the complex in human podocytes (Study II, Supplemental Figure S3D and E) and in HIRc cells (Study II, Supplemental Figure S4A and B).

In order to characterize the function of septin 7 in podocytes, we searched for its interaction partners. Co-immunoprecipitation analysis of human podocytes with septin 7 antibody, followed by mass spectrometry, identified NMHC-IIA as a novel interaction

partner of septin 7 (Study III, Figure 1A). NM-II is a major component of the actomyosin cytoskeleton (reviewed in Sellers, 2000). In human cells, three NMHCs (IIA, IIB, and IIC) are encoded by distinct genes (*MYH9*, *MYH10*, and *MYH14*, respectively) and constitute the NM-II isoforms, which are named NMHC-IIA, NMHC-IIB, and NMHC-IIC, respectively (Conti and Adelstein, 2008; Vicente-Manzanares et al., 2009). NM-IIs are ubiquitously expressed but differ with respect to their localization and expression level in the cells (Conti and Adelstein, 2008). NMHC-IIA is widely expressed in the kidney, mainly in glomerulus and peritubular vessels (Arrondel et al., 2002), whereas NMHC-IIB localizes in mesangial cells (Johnstone et al., 2011). NM-II is comprised of two heavy chains (MHCs) and two pairs of light chains: essential light chains (ELCs) and regulatory light chains (RLCs) (reviewed in Sellers, 2000). NM-IIA is activated and regulated by the reversible phosphorylation of the MHC (p-MHC) on its serine 1943 residue (S1943) and RLC (pp-RLC) on its threonine 18 (T18) and serine 19 (S19) residues (Scholey et al., 1980; Sellers et al., 1981). The physical interaction of septin 7 and NMHC-IIA was confirmed using co-immunoprecipitation and Western blotting in cultured human and mouse podocytes, and in rat glomeruli (Study III, Figure 1B). Further, we produced by retroviral infection mouse podocytes stably expressing nephrin (Study III, Figure 2A and B), and found that both septin 7 (Study II, Figure 1F-H) and NMHC-IIA (Study III, Figure 2H) bind to nephrin in rat glomeruli and nephrin-expressing mouse podocytes, respectively.

Expression of septin 7, CD2AP, and NMHC-IIA was confirmed by Western blotting in proliferating and differentiated cultured human podocytes (Study II, Figure 2B; Study III, Figure 1C). We also analyzed the localization of septin 7 and NMHC-IIA in cultured human podocytes. Septin 7 appears as filaments in proliferating and differentiated podocytes (Study II, Figure 2H and I). In proliferating podocytes, septin 7 also localizes in the midbodies (Study II, Figure 2G). We also investigated septin 7 localization in subcellular fractions of mouse podocytes and found that septin 7 is present in both the membrane and cytosolic fractions (Study II, Supplemental Figure S6A-C). NMHC-IIA shows a cytoplasmic filamentous pattern in human podocytes (Study III, Figure 1E).

To confirm the expression of septin 7 and NMHC-IIA in glomeruli, rat glomerular and tubular lysates were isolated by the sieving technique and immunoblotted with septin 7 and NMHC-IIA antibodies, showing that septin 7 and NMHC-IIA are expressed in both glomeruli and tubuli in the kidney (Study II, Figure 2A; Study III, Figure 1D). To more precisely localize septin 7, NMHC-IIA, p-MHC, and pp-RLC in glomeruli, immunopreroxidase staining of mouse and human kidney sections was performed. Septin 7,

NMHC-IIA, p-MHC and pp-RLC were shown to be expressed in podocytes (Wasik et al., unpublished data, Figure 9; Study II, Figure 2C; Study III, Figure 1F).

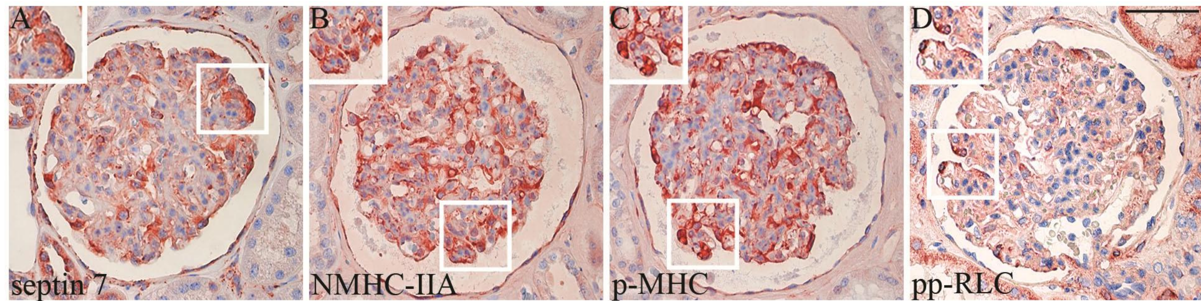

Figure 9. Septin 7, NMHC-IIA, phosphorylated MHC and phosphorylated RLC are expressed in human glomeruli, where they localize in podocytes. Human kidney samples were fixed with formaldehyde and embedded in paraffin. Deparaffinized sections were stained with anti-septin 7 (A), anti-NMHC-IIA (B), anti-phosphorylated MHC (p-MHC) (C) and anti-phosphorylated RLC (pp-RLC) (D) IgGs. VectaStain Elite ABC kit (Vector Laboratories) and AEC (DakoCytomation) were used for detection. Hematoxylin-counterstained slides were photographed using Nikon Eclipse 800 microscope. Higher magnifications of the boxed regions are shown in the insets. Scale bar, 50 μm.

5.2.2 Localization of septin 7 depends on CD2AP and intact actin cytoskeleton

In cultured mouse podocytes, septin 7 was shown to be expressed in a filamentous pattern along actin stress fibers (Wasik et al., modified Figure 10A-D and I-L; Study II, Supplemental Figure S1A-D and I-L). When actin organization was disrupted with cytochalasin D, the majority of septin 7 localized in rings formed by rolling up of linear septin 7 filaments (Wasik et al., modified Figure 10E-H and M-P; Study II, Supplemental Figures S1E-H and M-P). These rapid changes in localization reveal that septin 7 organization in podocytes is dynamic and depends on an intact actin cytoskeleton.

We also analysed whether septin 7 localization depends on CD2AP. In wild-type mouse podocytes, septin 7 is expressed as short filaments (Study II, Figure 3A-D), whereas in CD2AP -/- podocytes, septin 7 filaments are lost (Study II, Figure 3E-H) indicating that loss of CD2AP is associated with the disappearance of actin stress fibers and septin 7 filaments.

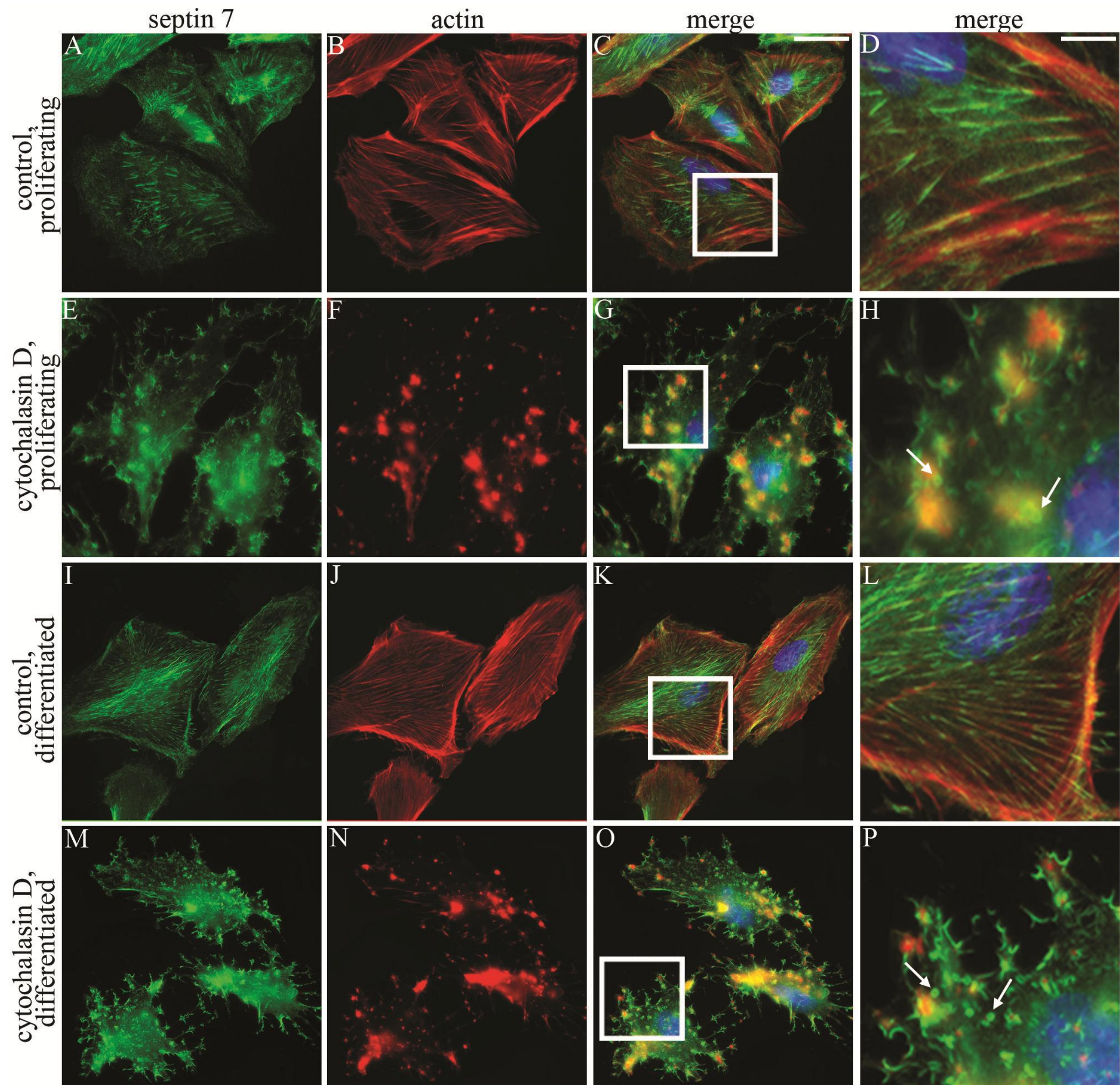

Figure 10. Localization of septin 7 depends on an intact actin cytoskeleton. Double labeling of proliferating (A-H) and differentiated (I-P) human podocytes for septin 7 (green) and actin (red). Nuclei are visualized with Hoechst (blue). In proliferating and differentiated human podocytes, septin 7 appears as filaments which occasionally co-localize with actin stress fibers (A-D, I-L). In podocytes treated with cytochalasin D, septin 7 curled and localized in rings (arrows) (E-H, M-P). Cultured human podocytes were treated or not with 5 µM cytochalasin D, fixed with PFA, labeled with septin 7 IgG and phalloidin, and examined by fluorescence microscopy. Scale bar, 20 µm; in D, H, L, P, 7 µM (Modified from Study II, Supplemental Figure S1).

5.2.3 Depletion of septin 7 and nonmuscle myosin IIA affect glucose uptake in cultured podocytes

Septins are known to be involved in exocytosis in nondividing neurons (Hsu et al., 1998; Beites et al., 1999). As podocytes are terminally differentiated cells, we hypothesised that septin 7 may affect exocytosis in these cells. Our hypothesis was supported by the fact that septin 7 interacts with nephrin (Study II, Figure 1F-H), a protein that is known to be involved in GSV fusion with the plasma membrane (Coward et al., 2007). Indeed, knockdown of septin 7 by siRNA in HIRc cells increased 2-deoxy-D-glucose uptake under basal, starved, and insulin-stimulated conditions compared to the control siRNA-treated cells (Study II, Figure 4A-C). Alteration of septin 7 assembly and dynamics by forchlorfenuron (FCF) (Hu et al., 2008) caused a similar effect as septin knockdown by siRNA in both HIRc cells (Study II, Figure 4D and E) and mouse podocytes (Study II, Figure 4F and G). FCF also changed septin 7 organization to bundle-like filaments that accumulate at the cell periphery, without affecting the actin cytoskeleton (Study II, Supplemental Figure S5).

We also measured glucose uptake in mouse podocytes overexpressing septin 7 (Study III, Supplemental Figure S1) and found that glucose uptake was decreased by 20% in septin 7-overexpressing podocytes compared to empty vector-transfected cells under basal conditions (Study III, Supplemental Figure S1F). In serum-starved podocytes, overexpression of septin 7 did not affect glucose uptake compared to serum-starved, empty vector-transfected cells (Study III, Supplemental Figure S1G). In insulin-stimulated septin 7-overexpressing podocytes, glucose uptake remained unchanged compared to serum-starved, empty vector-transfected cells (Study III, Supplemental Figure S1G). In contrast, in control podocytes insulin increased glucose uptake (Study III, Supplemental Figure S1G). This confirmed our previous data showing that septin 7 is a negative regulator of glucose uptake into podocytes.

We also further investigated the role of NMHC-IIA in the regulation of GSV trafficking in podocytes and found that siRNA-mediated depletion of NMHC-IIA in mouse podocytes (Study III, Figure 2C and D) decreased insulin-stimulated glucose uptake (Study III, Figure 2F) without affecting glucose uptake at basal (Study III, Figure 2E) and starved states (Study III, Figure 2F). This indicates that septin 7 and NMHC-IIA have opposite effects on glucose uptake into podocytes.

5.2.4 Septin 7 and nonmuscle myosin IIA interact with proteins involved in GSV trafficking

To search for the mechanism by which septin 7 regulates glucose uptake, we first determined if septin 7 regulates the insulin-activated PI3K signaling pathway. Phosphorylation of AKT was examined in septin 7 depleted and control siRNA-treated HIRc cells. The results indicate that depletion of septin 7 has no effect on the total level of AKT or on the insulin-stimulated phosphorylation of AKT, indicating that loss of septin 7 does not affect insulin signal transduction (Study II, Figure 5A-D). Previous studies have also shown that myosin IIA depletion does not inhibit insulin signal transduction (Chung et al., 2010).

To determine if septin 7 and NMHC-IIA could be involved in GSV trafficking, we investigated their interaction with the proteins involved in the process. GSV trafficking consists of several steps. The SNAREs complex, including v-SNARE, VAMP2, and t-SNAREs, such as syntaxin 4 and SNAP23, function in the docking and fusion steps of GSVs with the plasma membrane (Foster and Klip, 2000; reviewed in Sollner, 2003; Watson et al., 2004). Co-immunoprecipitation with septin 7 antibodies revealed that septin 7 interacts with VAMP2 in HIRc cells and the interaction is not dependent on insulin stimulation (Study II, Figure 5E and F). We also found that NMHC-IIA forms a complex with SNAP23 in mouse podocytes (Study III, Figure 2G) and that septin 7 is also a part of this complex (Study III, Figure 2H).

5.2.5 Phosphorylated myosin RLC competes with septin 7 for binding to the SNARE complex

Septin 7 and NMHC-IIA have opposite effects on glucose uptake into podocytes and both interact with the t-SNARE protein, SNAP23. Accordingly, we hypothesized that septin 7 and NMHC-IIA compete for binding to SNAP23 (Figure 11A). Indeed, co-immunoprecipitation and Duolink proximity ligation assay (PLA) studies on NMHC-IIA-depleted and control siRNA-treated mouse podocytes revealed increased complex formation between septin 7 and SNAP23 in NMHC-IIA-depleted podocytes (Figure 11B; Study III, Figure 3A-E).

We further knocked down septin 7 to investigate the effect of septin 7 loss on binding of NMHC-IIA, p-MHC, and pp-RLC to SNAP23. Although loss of septin 7 decreased the interaction of SNAP23 with NMHC-IIA (Study III, Figure 4A and B) and SNAP23 with p-MHC (Study III, Figure 4A and C), it increased complex formation between SNAP23 and pp-

RLC (Figure 11C; Study III, Figure 4A and D). The interactions were further confirmed by Duolink proximity ligation assay (Study III, Figure 4E-M).

To further confirm that septin 7 and pp-RLC compete for binding to SNAP23, we overexpressed septin 7. Increased expression of septin 7 facilitated complex formation of SNAP23 with NMHC-IIA (Study III, Supplemental Figure S2A and B) and SNAP23 with p-MHC (Study III, Supplemental Figure S2A and C), and, as expected, decreased the interaction of SNAP23 with pp-RLC (Study III, Supplemental Figure S2A and D). The interactions were confirmed by Duolink proximity ligation assays (Study III, Supplemental Figure S2E-M), which confirmed our hypothesis that septin 7 competes with pp-RLC for binding to SNAP23.

5.2.6 Insulin regulates the association of septin 7 and phosphorylated myosin RLC with SNAP23

As knockdown of NMHC-IIA reduced insulin-induced glucose uptake and septin 7 and pp-RLC competed for binding to SNAP23, we speculated that insulin may regulate the association between septin 7, pp-RLC and SNAP23. Using co-immunoprecipitation and Duolink proximity ligation assays, with and without insulin stimulation, we found that insulin stimulation reduces the association of septin 7 with SNAP23 (Study III, Figure 5A, B, D, E and I), but does not change the interaction of septin 7 with syntaxin 4 (Study III, Figure 5A and C). In line with this, insulin increases complex formation between pp-RLC and SNAP23 (Study III, Figure F, G and J). These data provide mechanistic insight to the regulatory role of insulin in glucose uptake in podocytes by affecting SNAP23 complex formation with septin 7 and pp-RLC (Figure 11B and C).

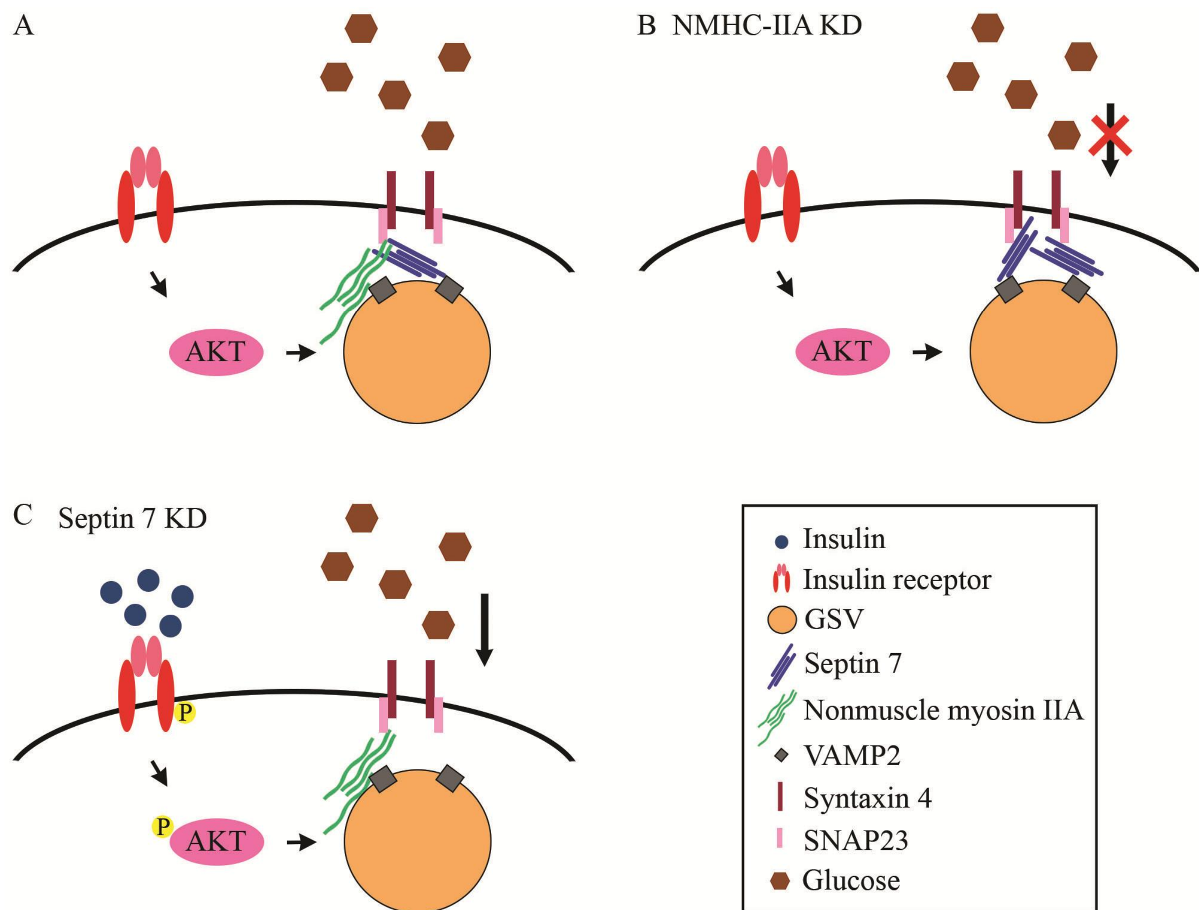

Figure 11. Schematic illustration of how septin 7 and phosphorylated myosin RLC compete for binding to SNAP23. (A) Both septin 7 and nonmuscle myosin IIA form a complex with SNAP23 in the basal state. (B) NMHC-IIA knockdown (NMHC-IIA KD) increases the interaction between septin 7 and SNAP23. (C) Loss of septin 7 (septin 7 KD) increases the interaction between phosphorylated myosin RLC and SNAP23.

5.2.7 Septin 7 and NMHC-IIA affect SNARE complex formation

The fact that septin 7 associates with both v-SNARE VAMP2 (Study II, Figure 5E and F) and t-SNARE SNAP23 (Study III, Figure 2H), as well as with nephrin (Study II, Figure 1F-H) led us to speculate that septin 7 forms a filamentous barrier between the GSVs and the plasma membrane. Previous studies have shown that GSV fusion with the plasma membrane is facilitated by the interaction of nephrin with VAMP2 (Coward et al., 2007). Accordingly, we investigated the association of nephrin and VAMP2 in septin 7-depleted HIRc cells which overexpress nephrin. Septin 7 knockdown increased the interaction between nephrin and VAMP2 (Study II, Figure 6B and C) and also between syntaxin 4 and VAMP2 (Study II,

Figure 6B and D), supporting the view that septin 7 forms a filamentous barrier between the GSVs and the plasma membrane.

Our data suggest that depletion of NMHC-IIA affects complex formation between v-SNAREs and t-SNARES. Indeed, we observed that siRNA-mediated knockdown of NMHC-IIA reduces the interaction of VAMP2 with SNAP23 (Study III, Figure 6A-E), whereas the interaction between VAMP2 and syntaxin 4 is not affected (Study III, data not shown). This suggests that knockdown of NMHC-IIA inhibits the association between the two key components of the SNARE complex, VAMP2 and SNAP23, and therefore reduces the final steps of GSV trafficking and fusion with the plasma membrane in podocytes, which leads to reduced glucose uptake.

5.2.8 Phosphorylated myosin RLC is upregulated in glomeruli in diabetic rats and in cultured human podocytes exposed to sera from Type 1 diabetic patients with macroalbuminuria

The expression of NMHC-IIA, p-MHC, and pp-RLC was studied in the glomeruli of 40-week old obese Zucker rats and age-matched lean controls. Obese Zucker rats are insulin resistant and slightly diabetic due to a mutation in the leptin receptor gene, and develop albuminuria by 40 weeks of age (Chua et al., 1996). Quantitative Western blotting revealed that NMHC-IIA and p-MHC were downregulated in the glomeruli of obese Zucker rats when compared to lean controls (Study III, Figure 7A-C), whereas pp-RLC was significantly upregulated in the glomeruli of obese rats (Study III, Figure 7A and D).

The expression of septin 7, NMHC-IIA, p-MHC, and pp-RLC was also studied by immunohistochemistry in human kidney samples obtained from patients with T2DM and from controls. The patients with T2DM did not have clinical nephropathy, and histopathological analysis revealed no diagnostic signs of DN. The expression of septin 7, NMHC-IIA and p-MHC did not differ between the groups, however, pp-RLC showed significantly higher expression in the glomeruli of patients with diabetes (Study III, 7E-G).

We next investigated the expression levels of septin 7, NMHC-IIA, p-MHC, and pp-RLC in human podocytes treated with sera of T1DM patients with normo- and macroalbuminuria. The expression of p-MHC, NMHC-IIA, and septin 7 did not differ between the groups (Study III, Figure 8A-D). Interestingly, the pp-RLC level was increased by 75% in human podocytes treated with sera from macroalbuminuric patients compared to normoalbuminuric controls (Study III, Figure 8A and E).

6 DISCUSSION

6.1 Diabetes-induced changes in the protein expression profile of glomeruli isolated from rats with streptozotocin-induced diabetes

To identify differentially expressed proteins that could be associated with the development of podocyte injury in diabetes, we performed quantitative proteomic profiling of glomeruli isolated from rats with streptozotocin-induced diabetes and controls. Major changes were observed in actin-binding proteins, including ezrin and NHERF2, apoptosis-associated proteins, regulators of oxidative tolerance, and DNA binding and repair proteins (Study I, Supplemental Table S1). The actin cytoskeleton integrity in podocytes is altered in several types of glomerular diseases. This raises great interest in proteins that function in the regulation of dynamic actin organization as potential therapeutic targets in the treatment of glomerular diseases, including DN. Consequently, we chose ezrin for further analysis. In podocytes, ezrin and its interaction partner, NHERF2, are important regulators of podocyte function as they link podocalyxin, the major sialoprotein of podocytes, to the actin cytoskeleton (Takeda et al., 2001). However, the exact role of ezrin protein complex in podocyte injury in DN has not been studied before.

6.2 Role of ezrin in the regulation of actin reorganization and glucose uptake in cultured podocytes

To fulfill its biological function, ezrin needs to be activated via phosphorylation on its threonine 567 residue (Fievet et al., 2004). We found that in glomeruli of streptozotocin-treated rats, the levels of both total and phosphorylated ezrin were reduced. We also found that the expression of ezrin is downregulated in glomeruli of obese Zucker rats and in podocytes of human patients with T2DM without clinical nephropathy or histopathological diagnostic signs of DN. Supporting our findings, phosphorylation of ezrin has been reported to be reduced in the skeletal muscle of obese patients with T2DM (Chun et al., 2011). Previously, reduction of ezrin was suggested as a marker for evaluating the prognosis of nephrotic syndrome in children (Ostalska-Nowicka et al., 2006). Our data indicate that downregulation of ezrin and/or its activity may be involved in the development of diabetic complications.

We further observed that depletion of ezrin induces dynamic remodelling of cortical actin in cultured podocytes under basal conditions, but diminishes the insulin-induced dynamics of cortical actin. In muscle cells, remodelling of cortical filamentous actin is required for GLUT4 translocation (Tong et al., 2001). In podocytes, glucose uptake and GLUT4 translocation also depend on the actin cytoskeleton (Coward et al., 2005). Using an siRNA approach, we found that depletion of ezrin in podocytes increased glucose uptake under basal conditions. After insulin stimulation, glucose uptake was still enhanced but remained lower in ezrin-depleted podocytes than in control siRNA-transfected podocytes. The increase in glucose uptake in ezrin knockdown podocytes was due to enhanced trafficking of the constitutive glucose transporter, GLUT1, to the plasma membrane. Despite reduced cortical actin remodelling, we observed translocation of GLUT4 to the plasma membrane after insulin stimulation in ezrin-depleted podocytes. However, this may be due to the fact that ezrin depletion achieved by siRNA was only partial.

Ezrin-dependent remodelling of actin involves the actin severing protein cofilin-1. Phosphorylation of cofilin-1 on serine 3 leads to its inactivation resulting in reduced binding to actin and depolymerizing activity (Moriyama et al., 1996; Heyworth et al., 1997). Cofilin-1 is essential for structural changes to the actin cytoskeleton during recovery from podocyte injury, as cofilin-1 mutant podocytes fail to recover following injury (Garg et al., 2010). Further, knockdown or mutation of cofilin-1 disturbs the filtration barrier in zebrafish (Ashworth et al., 2010), and mice with podocyte-specific deletion of cofilin-1 develop proteinuria (Garg et al., 2010). We observed phosphorylated cofilin-1 at the cell membrane of cultured podocytes after insulin stimulation. This may appear controversial, as one would expect the dynamic organization of cortical actin to require cofilin-1 in its active, dephosphorylated form. However, a similar observation was previously made in human epidermoid carcinoma KB cells, in which cofilin and p-cofilin were found at the plasma membrane after insulin stimulation (Arai and Atomi, 2003). The authors speculated that phosphorylated cofilin at the membrane could reflect a rapid phosphorylation/dephosphorylation cycle of cofilin or it could represent the residual pool of phosphorylated protein waiting to be dephosphorylated (Arai and Atomi, 2003).

In cultured podocytes, phosphorylation of cofilin-1 depends on the length of insulin stimulation. Similar to muscle cells (Chiu et al., 2010), short-term insulin treatment of

podocytes induced cofilin-1 dephosphorylation. However, long-term insulin treatment increased the level of phosphorylated cofilin-1. Inactivation of cofilin-1 by phosphorylation may lead to disturbed actin remodelling and disturbances in podocyte function. Further, cofilin-1 is phosphorylated in diabetic rat glomeruli and it was previously shown to be phosphorylated in human patients with glomerular diseases affecting podocytes (Ashworth et al., 2010). This would possibly reduce the ability of podocytes to respond to changes in glomerular pressure. Surprisingly, in puromycin aminonucleoside-induced nephrotic glomeruli, phosphorylation of cofilin-1 was shown to be reduced (Garg et al., 2010). This may be due to differences between species and diseases or disease models, or the stage of disease progression. They may also reflect constant recycling of cofilin-1 between its active and inactive forms. Nevertheless, our data indicate an important role for ezrin and cofilin-1 in maintaining the functional organization of the actin cytoskeleton in cultured podocytes.

6.3 Septin 7 and nonmuscle myosin IIA regulate glucose uptake into podocytes

In cultured human podocytes, septin 7 forms a complex with CD2AP, an 80-kDa cytoplasmic adapter protein that was originally found as an interaction partner of the T-cell adhesion molecule CD2 (Dustin et al., 1998) and shortly after was shown to be essential for kidney ultrafiltration function (Shih et al., 1999). The C-terminus of septin 7 (aa 179-418), which contains a coiled-coil domain and part of the GTP-binding domain, as well as the SH3 domains of CD2AP, participate in the formation of the complex. Septin 7 filaments partially overlap with actin stress fibers in podocytes, suggesting that the association of septin 7 and actin is mediated by other molecules. CD2AP may mediate binding of septin 7 to actin, as CD2AP binds actin directly (Lehtonen et al., 2002) and the localization of CD2AP depends on an intact actin architecture (Lehtonen et al., 2002). Septin 7 also associates with nephrin, which has previously been shown to form a complex with CD2AP (Shih et al., 2001; Palmen et al., 2002). Further, we found that septin 7 localization depends on CD2AP, as CD2AP-/- podocytes show loss of actin stress fibers (Yaddanapudi et al., 2011) and septin 7 filaments.

Septins are evolutionarily conserved and contain a GTP-binding domain and variable NH_2 and COOH termini (Kinoshita, 2003; Hall et al., 2005). To date, 13 septin genes have been reported in mammals. Many of them undergo alternative splicing, which means that the number of septin isoforms is even greater (Kinoshita, 2003; Hall et al., 2005). These GTP-binding proteins have several roles in cell division, cytoskeletal organization, and vesicle

trafficking (Hsu et al., 1998; Beites et al., 1999; Kinoshita et al., 2002; Kremer et al., 2007). One factor that is crucial for their functions is the ordered assembly of individual septins into homo- and heterooligomeric core complexes that form higher-order structures such as filaments or rings (Joberty et al., 2001; Nagata et al., 2004). Septin 7 forms a complex with septin 2, septin 6, septin 8, septin 9b, and septin 11 (Kinoshita, 2003; Nagata et al., 2004). We found that septin 7 associates with septin 9 and septin 11 in podocytes. The fact that septin 7 lacks the proline-rich domain (that binds SH3 domains) and its presence in septin 9 (Hall and Russell, 2004) suggest that the interaction between septin 7 and CD2AP could be mediated by septin 9.

Our data demonstrate that also NMHC-IIA interacts with septin 7. Previously found that septin 2 binds directly to NM-IIA (Joo et al., 2007). NMHC-IIA is expressed in podocytes, and has been suggested to function as a major component of the actin-myosin contractile apparatus, helping podocytes to respond to changes in glomerular pressure and contributing to foot process retraction (Arrondel et al., 2002). It may be that NMHC-IIA also participates in mediating the interaction of septin 7 with the actin filaments.

Septin 7 localizes in the midbody of proliferating podocytes, and therefore it apparently participates in regulating cell division in podocytes. However, mature podocytes *in vivo* are terminally differentiated and do not divide. Studies in post-mitotic neurons showed that septins regulate exocytosis (Hsu et al., 1998; Beites et al., 1999; Vega and Hsu, 2003). Furthermore, interaction of septin 7 with nephrin, a protein required for the fusion of the GSVs with the plasma membrane in podocytes (Coward et al., 2007), suggested that septin 7 could function in vesicle trafficking in these cells. We therefore investigated the role of septin 7 in GSV exocytosis. We found that septin 7 ablation does not affect the insulin-stimulated activation of the PI3K signaling pathway in podocytes, indicating that septin 7 rather regulates the trafficking of GSV. Indeed, depletion of septin 7 increases glucose uptake under basal conditions, but the increase is more pronounced after insulin stimulation, indicating that septin 7 negatively regulates GSV trafficking. The data also suggest that septin 7 affects both the constitutive glucose transporter GLUT1 and the insulin-stimulated glucose transporter GLUT4. The trafficking of GSVs involves a SNARE complex that facilitates the docking and fusion of GSVs with the plasma membrane (Foster and Klip, 2000; reviewed in Sollner, 2003; Watson et al., 2004). The SNARE complex includes a v-SNARE on GSVs, VAMP2, and t-SNAREs on the plasma membrane, such as syntaxin 4 and SNAP23 (Rothman, 1994; Weber et al., 1998). We found that septin 7 interacts with VAMP2 independently of insulin stimulation. Septin 7 also binds to the plasma membrane proteins nephrin and SNAP23.

Nephrin regulates glucose uptake in podocytes by interacting with VAMP2 and facilitates the fusion of GSVs with the plasma membrane (Coward et al., 2007). Binding of septin 7 to both vesicle and target SNAREs indicates that septin 7 forms a filamentous barrier and thereby restricts movement of the vesicles to the plasma membrane. Indeed, depletion of septin 7 increases the interaction between nephrin and VAMP2, and also between syntaxin 4 and VAMP2. Importantly, we found that insulin stimulation reduces the interaction between septin 7 and SNAP23. This allows formation of the SNARE complex and further supports a filametous barrier function of septin 7. The interaction of septin 7 with nephrin in glomeruli suggests that the two proteins may function together in regulating GSV trafficking in glomeruli *in vivo*.

Opposite to the negative regulatory role of septin 7 in glucose uptake, NMHC-IIA positively regulates insulin-stimulated glucose uptake into podocytes. The role of NMHC-IIA in regulating glucose uptake is supported by our finding that that NMHC-IIA interacts with SNAP23 and nephrin in podocytes. Previous studies in adipocytes have also shown also that pharmacological inhibition of myosin II activity (Steimle et al., 2005; Fulcher et al., 2008) or knockdown of myosin IIA (Chung et al., 2010) significantly reduced insulin-stimulated glucose uptake by inhibiting GLUT4 incorporation into the plasma membrane and GLUT4 intrinsic activity, but not by impairing insulin signal transduction and GLUT4 translocation (Chung et al., 2010).

NM-IIA is activated by phosphorylation of its regulatory light chain (RLC) (Scholey et al., 1980). The observation that septin 7 and NMHC-IIA have opposite effects on glucose uptake, and the fact that septin 7 and pp-RLC compete for binding to SNAP23, provide a mechanistic insight into how GSV trafficking in podocytes is regulated by septin 7 and NM-IIA, indicating an important role for pp-RLC. Phosphorylation of the RLC by a specific myosin light chain kinase (MLCK) has previously been shown to be essential for granule transport in chromaffin cells (Neco et al., 2004) and insulin-stimulated GLUT4 translocation in adipocytes (Choi et al., 2006; Fulcher et al., 2008). Insulin increases phosphorylation of the RLC and inhibition of its phosphorylation subsequently blocks glucose transport (Choi et al., 2006). We found that pp-RLC is upregulated in glomeruli of human patients with T2DM and in obese Zucker rats, as well as in human podocytes exposed to sera of T1DM patients with macroalbuminuria. This indicates that some factors in the macroalbuminuric sera activate RLC and may thereby increase GSV trafficking and glucose uptake into podocytes. A

previous study showed that septin 2-containing filaments act as a scaffold for NM-IIA and ROCK and CRIK kinases to fully activate NM-IIA to ensure progression of cytokinesis (Joo et al., 2007). Further studies are needed to define whether septin complexes scaffold NM-IIA and MLCK to control GSV trafficking and glucose uptake in insulin sensitive cells.

Depletion of NMHC-IIA inhibits v-SNARE/t-SNARE association in both podocytes (this study) and adipocytes (Chung et al., 2010). In adipocytes, knockdown of NMHC-IIA inhibits insulin-stimulated interaction between VAMP2 and syntaxin 4 (Chung et al., 2010). We did not observe any difference in the complex formation between VAMP2 and syntaxin 4 in podocytes under basal conditions, similarly as shown previously in adipocytes (Chung et al., 2010). However, we found that loss of NMHC-IIA in podocytes inhibits the interaction between VAMP2 and SNAP23. In addition, increased interaction of septin 7 and SNAP23 reduces the binding capacity of SNAP23 to VAMP2. Importantly, we found that insulin stimulation reduces the interaction between septin 7 and SNAP23, and increases the interaction between pp-RLC and SNAP23. We can therefore conclude that SNAP23 is an important t-SNARE in podocytes that mediates insulin-stimulated glucose uptake and is regulated by binding to septin 7 or pp-RLC.

It is possible that NMHC-IIA and septin 7 also regulate GSV trafficking via modulating actin dynamics, as both proteins bind either directly or indirectly to actin. In podocytes, glucose uptake depends on an intact actin cytoskeleton (Coward et al., 2005) and defective reorganization of actin filaments has been shown to impair GSV fusion with the plasma membrane in adipocytes (Lopez et al., 2009) and muscle cells (Tong et al., 2001). Active NM-IIA is required for F-actin localization at the plasma membrane in adipocytes (Stall et al., 2014), and depletion of NMHC-IIA in podocytes leads to loss of actin stress fiber organization (Hays et al., 2014).

Further studies are required to characterize the exact steps of GSV trafficking that septin 7 and NM-IIA regulate. Binding to the SNARE proteins and modulation of the SNARE complex formation by septin 7 and NM-IIA indicate their involvement in GSV tethering, docking or fusion with the plasma membrane. Whether increased glucose uptake by an increase in active NM-IIA in diabetes is protective or predisposes to DN requires further analysis.

6.4 Does increased insulin sensitivity protect podocytes from injury?

Several studies have shown the contribution of insulin resistance to the development of DKD. Insulin resistance correlates with microalbuminuria in both diabetic (Yip et al., 1993; Parvanova et al., 2006) and non-diabetic subjects (Palaniappan et al., 2003). Furthermore, insulin signaling is essential for kidney function (Welsh et al., 2010), and podocytes can develop insulin resistance (Tejada et al., 2008). The protective effect of increased insulin sensitivity on podocytes could be expected based on several clinical and experimental studies showing that many insulin sensitizers reduce albuminuria (Jauregui et al., 2009). Studies have also shown that differential regulation of glucose transporter expression may have a beneficial role in DN. Overexpression of GLUT1 in podocytes was reported to protect from DN (Zhang et al., 2010), while podocyte-specific depletion of GLUT4 protects from DN (Guzman et al., 2014). In this thesis work we identified ezrin, septin 7 and NMHC-IIA as a novel regulators of glucose uptake into podocytes. More data is needed to conclude how individual glucose transporters and regulators of insulin signaling and glucose transport machinery affect podocyte function *in vivo*, and whether they would provide suitable targets to enhance insulin sensitivity of the podocytes and to prevent the development or progression of DN.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis work provides new discoveries in podocyte biology, documents the involvement of new players, ezrin, septin 7 and nonmuscle myosin IIA in the regulation of glucose uptake into podocytes, and suggests mechanisms on how this regulation occurs.

Since podocyte structure and function depend on the regulation of the actin cytoskeleton and actin organization is altered in many glomerular diseases, proteins that regulate dynamic actin organization may be potential targets in the treatment of glomerular diseases, including DN. We found that depletion of ezrin induces reorganization of cortical actin in cultured podocytes under basal conditions, but diminishes the insulin-induced dynamics of cortical actin. We also found that ezrin-mediated cortical actin remodelling involves cofilin-1. It is well known that cortical filamentous actin is important for GLUT4 translocation. Accordingly, we discovered that knockdown of ezrin increases glucose uptake by podocytes. Further studies are required to clarify the precise role of ezrin in modulating glucose uptake activity *in vivo*, and its effects on the function of podocytes. More investigations are also required to define whether ezrin regulates the dynamics of cofilin-1 *in vivo*, and whether the effect is direct or via other proteins. The specific role of cofilin-1 in regulating the trafficking of glucose transporters and glucose uptake in podocytes both *in vitro* and *in vivo*, together with ezrin and/or other partners also needs further study.

Defects in glucose transporter machinery may be one reason for cellular insulin resistance. Therefore, regulators of glucose transporter trafficking may provide suitable targets to enhance insulin sensitivity of podocytes and to prevent the development and progression of DN. Here, we present opposite effects for the filamentous small GTPase septin 7 and NMHC-IIA on glucose uptake into podocytes, and show that septin 7 and phosphorylated myosin RLC compete for binding to the exocytotic t-SNAREs. However, it is unknown if septin 7 and NMHC-IIA affect insulin sensitivity in podocytes *in vivo*, and whether septin 7 and NMHC-IIA also regulate the final steps of GSV trafficking via modulating actin dynamics.

Collectively, altered expression of ezrin and phosphorylated myosin RLC in the glomeruli of human patients with diabetes suggests their possible association with the development of DN. Targeting of ezrin, septin 7 and nonmuscle myosin IIA could be a concept for the

treatment of podocyte insulin resistance, a strategy that may be prove useful in preventing or alleviating DN.

8 ACKNOWLEDGMENTS

This study was carried out at the Department of Pathology, Haartman Institute, University of Helsinki, during 2008-2014. First, I would like to thank the former and present directors of the Haartman Institute, Professor Seppo Meri and Professor Tom Böhling for providing me with a research-friendly, stimulating atmosphere and excellent research facilities.

I wish to express my sincere and deep gratitude to my supervisor Professor Sanna Lehtonen. I have been amazingly fortunate to have a supervisor who gave me the freedom to explore on my own, while at the same time patiently guided me through this project throughout these years. She taught me how to question thoughts and express ideas. Her impact in this work is tremendous. I highly treasure Sanna's kindness and understanding and admire her for never-ending enthusiasm for new projects and innovations. I appreciate that she always had time to discuss with me despite her busy schedule.

To the official reviewers of this thesis, Docent Satu Kuure and Professor Vesa Olkkonen, I am grateful for their time, interest and constructive comments that help to improve my thesis.

I want to thank the members of my thesis committee, Professor Päivi Miettinen and Professor Vesa Olkkonen for support and insightful advice on my work throughout these years.

I owe my gratitude to my all co-authors for their invaluable collaboration.

I wish to thank all the current and former lab members for their various forms of support during my thesis. Especially Niina Ruoho, Vincent Dumont, Pauliina Saurus, Tuomas Tolvanen, Sara Kuusela, Sonja Lindfors, Zydrune Polianskyte-Prause, Hong Wang, Mervi Hyvönen, Eija Heikkilä, Silvia Berra and Mervi Ristola are acknowledged for creating an excellent working atmosphere and for the scientific discussions that we had. I also thank them for the nice times shared outside the lab. Mervi Ristola and Pauliina Saurus are also warmly thanked for their nice company on conference trips. Niina Ruoho is acknowledged for her skillful technical help, and with whom I could talk about my personal matters. It was great to work with you all.

My special thanks go to Annakaisa Herrala, my friend and exchange student supervisor during Socrates program in Helsinki in 2007, who introduced me to the international world of science and to Finnish culture.

I am grateful for my Polish friends. Barbara Janus, Lidia Rychlińska, Katarzyna Leskinen and Magdalena Dobrowolska have listened to and supported me in the different phases of life and this PhD project. I owe my warm acknowledgments to my international friends: Silvia Berra, Noriko and Marko Falck, Riikka and Kimmo Ollikainen, Piritta and Konstra Sirvio, Ulla-Mari and Mika Hyötyläinen, Outi and Ville Kauppinen, Satu Ruuska, Timo Arola, Teemu Virrankoski and Mikko Lehtinen. Having you guys made my life in Finland so fun. Lidia, Silvia, Barbara, Katarzyna, Noriko, Riikka and Ulla-Mari are also thanked for amazing and unforgettable girl's nights.

I am grateful for the love and endless support of Ilkka Talvitie, the man who has taken care of me and listened through all my problems and worries. I thank him for making all of our trips so exciting and successful. I learned from them so much. Ilkka always encouraged me to do all the things that at first I thought were impossible. It has given me opportunities to experience the feelings of success and accomplishment. I wish to acknowledge also his parents, Liisa and Erkki Talvitie, for kindness, understanding and always warm hospitality at their place.

Finally, and most of all, I want to thank my fantastic family to whom I dedicate this thesis. They have always been there for me, no matter what. Without them I would not have been where I am now. The relationships and bonds that I have with my family hold an enormous amount of meaning to me. I admire them for all of their accomplishments in life, for all of the knowledge and wisdom that they have passed on to me over the years. Their endless support has allowed me to follow my dreams and live a life the way I wanted. My dear parents, Mirosława and Stefan Wąsikowie, are thanked for their love, constant care and unflinching belief in me. My grandparents, Marianna and Feliks Wiśniewscy, for giving me a second home, the magic place to which I could come any time and in which I feel always so relaxed. Thank You for your big love. I always fall short of words and feel it is impossible to describe their support. Special thanks go to my brother, Sebastian Wąsik, for being the best brother anyone could ever have and for being my best friend who is always by my side. Thank you for being optimistic and supportive for what I have been aiming at. My brother has a special

spirit which does not let me fall down and brings a smile to my face many times every day, even when we are far from each other. Since we were kids I have been following his paths, which eventually took me all the way to the academic world of natural sciences. I am proud to be his sister. Special thanks go to my goddaughter, Iga Wąsik, for cheering me up in a special way.

I have been financially supported by the Doctoral Programme in Biomedicine (formerly the Helsinki Biomedical Graduate Program), the Diabetes Research Foundation (Diabetestutkimussäätiö), the Kidney Foundation (Munuaissäätiö) and the Biomedicum Helsinki Foundation. All are sincerely acknowledged.

Helsinki, 2014

Anita Agnieszka Wasik

9 REFERENCES

Aaltonen, P., Luimula, P., Astrom, E., Palmen, T., Gronholm, T., Palojoki, E., Jaakkola, I., Ahola, H., Tikkanen, I. & Holthofer, H. 2001, "Changes in the expression of nephrin gene and protein in experimental diabetic nephropathy", *Laboratory Investigation; a Journal of Technical Methods and Pathology,* vol. 81, no. 9, pp. 1185-1190.

Abrahamson, D.R., Hudson, B.G., Stroganova, L., Borza, D.B. & St John, P.L. 2009, "Cellular origins of type IV collagen networks in developing glomeruli", *Journal of the American Society of Nephrology : JASN,* vol. 20, no. 7, pp. 1471-1479.

Adler, A.I., Stevens, R.J., Manley, S.E., Bilous, R.W., Cull, C.A., Holman, R.R. & UKPDS GROUP 2003, "Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64)", *Kidney International,* vol. 63, no. 1, pp. 225-232.

Ahola, H., Heikkilä, E., Aström, E., Inagaki, M., Izawa, I., Pavenstädt, H., Kerjaschki, D. & Holthöfer, H. 2003, "A novel protein, densin, expressed by glomerular podocytes", *Journal of the American Society* of *Nephrology*, vol. 14, no. 7, pp. 1731-1737.

Anderson, J.M. 2001, "Molecular structure of tight junctions and their role in epithelial transport", *News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society,* vol. 16, pp. 126-130.

Arai, H. & Atomi, Y. 2003, "Suppression of cofilin phosphorylation in insulin-stimulated ruffling membrane formation in KB cells", *Cell Structure and Function,* vol. 28, no. 1, pp. 41-48.

Arpin, M., Algrain, M. & Louvard, D. 1994, "Membrane-actin microfilament connections: an increasing diversity of players related to band 4.1", *Current Opinion in Cell Biology,* vol. 6, no. 1, pp. 136-141.

Arrondel, C., Vodovar, N., Knebelmann, B., Grunfeld, J.P., Gubler, M.C., Antignac, C. & Heidet, L. 2002, "Expression of the nonmuscle myosin heavy chain IIA in the human

kidney and screening for MYH9 mutations in Epstein and Fechtner syndromes", *Journal of the American Society of Nephrology : JASN,* vol. 13, no. 1, pp. 65-74.

Ashworth, S., Teng, B., Kaufeld, J., Miller, E., Tossidou, I., Englert, C., Bollig, F., Staggs, L., Roberts, I.S., Park, J.K., Haller, H. & Schiffer, M. 2010, "Cofilin-1 inactivation leads to proteinuria--studies in zebrafish, mice and humans", *PloS One,* vol. 5, no. 9, pp. e12626.

Ausiello, D.A., Kreisberg, J.I., Roy, C. & Karnovsky, M.J. 1980, "Contraction of cultured rat glomerular cells of apparent mesangial origin after stimulation with angiotensin II and arginine vasopressin", *The Journal of Clinical Investigation,* vol. 65, no. 3, pp. 754-760.

Barletta, G.M., Kovari, I.A., Verma, R.K., Kerjaschki, D. & Holzman, L.B. 2003, "Nephrin and Neph1 co-localize at the podocyte foot process intercellular junction and form cis hetero-oligomers", *The Journal of Biological Chemistry,* vol. 278, no. 21, pp. 19266-19271.

Beale, E.G. 2013, "Insulin signaling and insulin resistance", *Journal of investigative medicine the official publication of the American Federation for Clinical Research,* vol. 61, no. 1, pp. 11-14.

Becker, C.G. 1972, "Demonstration of actomyosin in mesangial cells of the renal glomerulus", *The American Journal of Pathology,* vol. 66, no. 1, pp. 97-110.

Beites, C.L., Xie, H., Bowser, R. & Trimble, W.S. 1999, "The septin CDCrel-1 binds syntaxin and inhibits exocytosis", *Nature Neuroscience,* vol. 2, no. 5, pp. 434-439.

Benigni, A., Gagliardini, E., Tomasoni, S., Abbate, M., Ruggenenti, P., Kalluri, R. & Remuzzi, G. 2004, "Selective impairment of gene expression and assembly of nephrin in human diabetic nephropathy", *Kidney International,* vol. 65, no. 6, pp. 2193-2200.

Benzing, T. 2004, "Signaling at the slit diaphragm", *Journal of the American Society of Nephrology : JASN,* vol. 15, no. 6, pp. 1382-1391.

Bierhaus, A., Schiekofer, S., Schwaninger, M., Andrassy, M., Humpert, P.M., Chen, J., Hong, M., Luther, T., Henle, T., Kloting, I., Morcos, M., Hofmann, M., Tritschler, H.,

Weigle, B., Kasper, M., Smith, M., Perry, G., Schmidt, A.M., Stern, D.M., Haring, H.U., Schleicher, E. & Nawroth, P.P. 2001, "Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB", *Diabetes,* vol. 50, no. 12, pp. 2792-2808.

Bjorn, S.F., Bangstad, H.J., Hanssen, K.F., Nyberg, G., Walker, J.D., Viberti, G.C. & Osterby, R. 1995, "Glomerular epithelial foot processes and filtration slits in IDDM patients", *Diabetologia,* vol. 38, no. 10, pp. 1197-1204.

Bohling, T., Turunen, O., Jaaskelainen, J., Carpen, O., Sainio, M., Wahlstrom, T., Vaheri, A. & Haltia, M. 1996, "Ezrin expression in stromal cells of capillary hemangioblastoma. An immunohistochemical survey of brain tumors", *The American Journal of Pathology,* vol. 148, no. 2, pp. 367-373.

Boute, N., Gribouval, O., Roselli, S., Benessy, F., Lee, H., Fuchshuber, A., Dahan, K., Gubler, M.C., Niaudet, P. & Antignac, C. 2000, "NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome", *Nature Genetics,* vol. 24, no. 4, pp. 349-354.

Brito, P.L., Fioretto, P., Drummond, K., Kim, Y., Steffes, M.W., Basgen, J.M., Sisson-Ross, S. & Mauer, M. 1998, "Proximal tubular basement membrane width in insulin-dependent diabetes mellitus", *Kidney International,* vol. 53, no. 3, pp. 754-761.

Brosius, F.C.,3rd, Alpers, C.E., Bottinger, E.P., Breyer, M.D., Coffman, T.M., Gurley, S.B., Harris, R.C., Kakoki, M., Kretzler, M., Leiter, E.H., Levi, M., McIndoe, R.A., Sharma, K., Smithies, O., Susztak, K., Takahashi, N., Takahashi, T. & Animal Models of Diabetic Complications Consortium 2009, "Mouse models of diabetic nephropathy", *Journal of the American Society of Nephrology : JASN,* vol. 20, no. 12, pp. 2503-2512.

Brosius, F.C.,3rd, Briggs, J.P., Marcus, R.G., Barac-Nieto, M. & Charron, M.J. 1992, "Insulin-responsive glucose transporter expression in renal microvessels and glomeruli", *Kidney International,* vol. 42, no. 5, pp. 1086-1092.

Brownlee, M. 2001, "Biochemistry and molecular cell biology of diabetic complications", *Nature,* vol. 414, no. 6865, pp. 813-820.

Caramori, M.L., Fioretto, P. & Mauer, M. 2003, "Low glomerular filtration rate in normoalbuminuric type 1 diabetic patients: an indicator of more advanced glomerular lesions", *Diabetes,* vol. 52, no. 4, pp. 1036-1040.

Caramori, M.L., Fioretto, P. & Mauer, M. 2000, "The need for early predictors of diabetic nephropathy risk: is albumin excretion rate sufficient?", *Diabetes,* vol. 49, no. 9, pp. 1399-1408.

Caramori, M.L., Gross, J.L., Pecis, M. & de Azevedo, M.J. 1999, "Glomerular filtration rate, urinary albumin excretion rate, and blood pressure changes in normoalbuminuric normotensive type 1 diabetic patients: an 8-year follow-up study", *Diabetes Care,* vol. 22, no. 9, pp. 1512-1516.

Chen, C.A., Hwang, J.C., Guh, J.Y., Chang, J.M., Lai, Y.H. & Chen, H.C. 2006, "Reduced podocyte expression of alpha3beta1 integrins and podocyte depletion in patients with primary focal segmental glomerulosclerosis and chronic PAN-treated rats", *The Journal of Laboratory and Clinical Medicine,* vol. 147, no. 2, pp. 74-82.

Chen, H., Liu, Z., Li, S., Chen, Y., Yang, B., Cai, J., Wang, Q. & Li, L. 2008, "The relationship between body fat distribution and renal damage in Chinese with obesity", *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association,* vol. 116, no. 2, pp. 99-103.

Chen, H.C., Chen, C.A., Guh, J.Y., Chang, J.M., Shin, S.J. & Lai, Y.H. 2000, "Altering expression of alpha3beta1 integrin on podocytes of human and rats with diabetes", *Life Sciences,* vol. 67, no. 19, pp. 2345-2353.

Chen, S., Heilig, K., Brosius, F.I. & Heilig, C. 2003, "Diabetes increases glomerular GLUT1 and antisense-GLUT1 protects against diabetic glomerulosclerosis", *Journal of the American Society* of *Nephrology,* vol. 14, pp. 46A.

Chihara, J., Takebayashi, S., Taguchi, T., Yokoyama, K., Harada, T. & Naito, S. 1986, "Glomerulonephritis in diabetic patients and its effect on the prognosis", *Nephron,* vol. 43, no. 1, pp. 45-49.

Chiu, T.T., Patel, N., Shaw, A.E., Bamburg, J.R. & Klip, A. 2010, "Arp2/3- and cofilin-coordinated actin dynamics is required for insulin-mediated GLUT4 translocation to the surface of muscle cells", *Molecular Biology of the Cell,* vol. 21, no. 20, pp. 3529-3539.

Choi, Y.O., Ryu, H.J., Kim, H.R., Song, Y.S., Kim, C., Lee, W., Choe, H., Leem, C.H. & Jang, Y.J. 2006, "Implication of phosphorylation of the myosin II regulatory light chain in insulin-stimulated GLUT4 translocation in 3T3-F442A adipocytes", *Experimental & Molecular Medicine,* vol. 38, no. 2, pp. 180-189.

Chua, S.C.,Jr, Chung, W.K., Wu-Peng, X.S., Zhang, Y., Liu, S.M., Tartaglia, L. & Leibel, R.L. 1996, "Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor", Science, vol. 271, no. 5251, pp. 994-996.

Chun, K.H., Choi, K.D., Lee, D.H., Jung, Y., Henry, R.R., Ciaraldi, T.P. & Kim, Y.B. 2011, "In vivo activation of ROCK1 by insulin is impaired in skeletal muscle of humans with type 2 diabetes", *American Journal of Physiology. Endocrinology and Metabolism,* vol. 300, no. 3, pp. 536-542.

Chung, T.K., Hosaka, T., Harada, N., Jambaldorj, B., Fukunaga, K., Nishiwaki, Y., Teshigawara, K., Sakai, T., Nakaya, Y. & Funaki, M. 2010, "Myosin IIA participates in docking of Glut4 storage vesicles with the plasma membrane in 3T3-L1 adipocytes", *Biochemical and Biophysical Research Communications,* vol. 391, no. 1, pp. 995-999.

Cohen, C.D., Klingenhoff, A., Boucherot, A., Nitsche, A., Henger, A., Brunner, B., Schmid, H., Merkle, M., Saleem, M.A., Koller, K.P., Werner, T., Grone, H.J., Nelson, P.J. & Kretzler, M. 2006, "Comparative promoter analysis allows de novo identification of specialized cell junction-associated proteins", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, no. 15, pp. 5682-5687.

Coimbra, T.M., Janssen, U., Grone, H.J., Ostendorf, T., Kunter, U., Schmidt, H., Brabant, G. & Floege, J. 2000, "Early events leading to renal injury in obese Zucker (fatty) rats with type II diabetes", *Kidney International,* vol. 57, no. 1, pp. 167-182.

Conti, M.A. & Adelstein, R.S. 2008, "Nonmuscle myosin II moves in new directions", *Journal of Cell Science,* vol. 121, no. Pt 1, pp. 11-18.

Cortes, P., Mendez, M., Riser, B.L., Guerin, C.J., Rodriguez-Barbero, A., Hassett, C. & Yee, J. 2000, "F-actin fiber distribution in glomerular cells: structural and functional implications", *Kidney International,* vol. 58, no. 6, pp. 2452-2461.

Coward, R.J., Welsh, G.I., Koziell, A., Hussain, S., Lennon, R., Ni, L., Tavare, J.M., Mathieson, P.W. & Saleem, M.A. 2007, "Nephrin is critical for the action of insulin on human glomerular podocytes", *Diabetes,* vol. 56, no. 4, pp. 1127-1135.

Coward, R.J., Welsh, G.I., Yang, J., Tasman, C., Lennon, R., Koziell, A., Satchell, S., Holman, G.D., Kerjaschki, D., Tavare, J.M., Mathieson, P.W. & Saleem, M.A. 2005, "The human glomerular podocyte is a novel target for insulin action", *Diabetes,* vol. 54, no. 11, pp. 3095-3102.

D'Agord Schaan B, Lacchini S, Bertoluci MC, Irigoyen MC, 2001, " Increased renal GLUT1 abundance and urinary TGF-beta 1 in streptozotocin-induced diabetic rats: implications for the development of nephropathy complicating diabetes", *Hormone and Metabolic Research,* vol. 33, pp. 664–669.

Daehn, I., Casalena, G., Zhang, T., Shi, S., Fenninger, F., Barasch, N., Yu, L., D'Agati, V., Schlondorff, D., Kriz, W., Haraldsson, B. & Bottinger, E.P. 2014, "Endothelial mitochondrial oxidative stress determines podocyte depletion in segmental glomerulosclerosis", *The Journal of Clinical Investigation,* vol. 124, no. 4, pp. 1608-1621.

Davis, B., Dei Cas, A., Long, D.A., White, K.E., Hayward, A., Ku, C.H., Woolf, A.S., Bilous, R., Viberti, G. & Gnudi, L. 2007, "Podocyte-specific expression of angiopoietin-2 causes proteinuria and apoptosis of glomerular endothelia", *Journal of the American Society of Nephrology : JASN,* vol. 18, no. 8, pp. 2320-2329.

De Cosmo, S., Menzaghi, C., Prudente, S. & Trischitta, V. 2013, "Role of insulin resistance in kidney dysfunction: insights into the mechanism and epidemiological evidence", *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association,* vol. 28, no. 1, pp. 29-36.

Devaskar, S.U. & Mueckler, M.M. 1992, "The mammalian glucose transporters", *Pediatric Research,* vol. 31, no. 1, pp. 1-13.

Ding, G., Reddy, K., Kapasi, A.A., Franki, N., Gibbons, N., Kasinath, B.S. & Singhal, P.C. 2002, "Angiotensin II induces apoptosis in rat glomerular epithelial cells". *American Journal* of *Physiology*. *Renal physiology*, vol. 83, no. 1, pp. 173-180.

Doublier, S., Salvidio, G., Lupia, E., Ruotsalainen, V., Verzola, D., Deferrari, G. & Camussi, G. 2003, "Nephrin expression is reduced in human diabetic nephropathy: evidence for a distinct role for glycated albumin and angiotensin II", *Diabetes,* vol. 52, no. 4, pp. 1023-1030.

Drapeau, N., Lizotte, F., Denhez, B., Guay, A., Kennedy, C.R. & Geraldes, P. 2013, "Expression of SHP-1 induced by hyperglycemia prevents insulin actions in podocytes", *American Journal of Physiology. Endocrinology and Metabolism,* vol. 304, no. 11, pp. 1188-1198.

Drenckhahn, D. & Franke, R.P. 1988, "Ultrastructural organization of contractile and cytoskeletal proteins in glomerular podocytes of chicken, rat, and man", *Laboratory Investigation; a Journal of Technical Methods and Pathology,* vol. 59, no. 5, pp. 673-682.

Dustin, M.L., Olszowy, M.W., Holdorf, A.D., Li, J., Bromley, S., Desai, N., Widder, P., Rosenberger, F., van der Merwe, P.A., Allen, P.M. & Shaw, A.S. 1998, "A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts", *Cell*, vol. 94, no. 5, pp. 667-677.

Ekberg, K., Landau, B.R., Wajngot, A., Chandramouli, V., Efendic, S., Brunengraber, H. & Wahren, J. 1999, "Contributions by kidney and liver to glucose production in the postabsorptive state and after 60 h of fasting", *Diabetes,* vol. 48, no. 2, pp. 292-298.

Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A.L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C.C., Ramachandran, C., Gresser, M.J., Tremblay, M.L. & Kennedy, B.P. 1999, "Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene", *Science,* vol. 283, no. 5407, pp. 1544-1548.

Elema, J.D., Hoyer, J.R. & Vernier, R.L. 1976, "The glomerular mesangium: uptake and transport of intravenously injected colloidal carbon in rats", *Kidney International,* vol. 9, no. 5, pp. 395-406.

Endlich, N., Kress, K.R., Reiser, J., Uttenweiler, D., Kriz, W., Mundel, P. & Endlich, K. 2001, "Podocytes respond to mechanical stress in vitro", *Journal of the American Society of Nephrology : JASN,* vol. 12, no. 3, pp. 413-422.

Epstein, C.J., Sahud, M.A., Piel, C.F., Goodman, J.R., Bernfield, M.R., Kushner, J.H. & Ablin, A.R. 1972, "Hereditary macrothrombocytopathia, nephritis and deafness", *The American Journal of Medicine,* vol. 52, no. 3, pp. 299-310.

Fang, J., Wei, H., Sun, Y., Zhang, X., Liu, W., Chang, Q., Wang, R. & Gong, Y. 2013, "Regulation of podocalyxin expression in the kidney of streptozotocin-induced diabetic rats with Chinese herbs (Yishen capsule)", *BMC complementary and alternative medicine,* vol. 13, no. 76-6882-13-76.

Fanning, A.S., Ma, T.Y. & Anderson, J.M. 2002, "Isolation and functional characterization of the actin binding region in the tight junction protein ZO-1", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 16, no. 13, pp. 1835-1837.

Fievet, B.T., Gautreau, A., Roy, C., Del Maestro, L., Mangeat, P., Louvard, D. & Arpin, M. 2004, "Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin", *The Journal of Cell Biology,* vol. 164, no. 5, pp. 653-659.

Fioretto, P., Steffes, M.W., Sutherland, D.E. & Mauer, M. 1995, "Sequential renal biopsies in insulin-dependent diabetic patients: structural factors associated with clinical progression", *Kidney International,* vol. 48, no. 6, pp. 1929-1935.

Forbes, J.M., Bonnet, F., Russo, L.M., Burns, W.C., Cao, Z., Candido, R., Kawachi, H., Allen, T.J., Cooper, M.E., Jerums, G. & Osicka, T.M. 2002, "Modulation of nephrin in the diabetic kidney: association with systemic hypertension and increasing albuminuria", *Journal of Hypertension,* vol. 20, no. 5, pp. 985-992.

Forsblom, C.M., Eriksson, J.G., Ekstrand, A.V., Teppo, A.M., Taskinen, M.R. & Groop, L.C. 1995, "Insulin resistance and abnormal albumin excretion in non-diabetic first-degree relatives of patients with NIDDM", *Diabetologia,* vol. 38, no. 3, pp. 363-369.

Foster, L.J. & Klip, A. 2000, "Mechanism and regulation of GLUT-4 vesicle fusion in muscle and fat cells", *American Journal of Physiology. Cell Physiology,* vol. 279, no. 4, pp. 877-890.

Fukasawa, H., Bornheimer, S., Kudlicka, K. & Farquhar, M.G. 2009, "Slit diaphragms contain tight junction proteins", *Journal of the American Society of Nephrology : JASN,* vol. 20, no. 7, pp. 1491-1503.

Fulcher, F.K., Smith, B.T., Russ, M. & Patel, Y.M. 2008, "Dual role for myosin II in GLUT4-mediated glucose uptake in 3T3-L1 adipocytes", *Experimental Cell Research,* vol. 314, no. 17, pp. 3264-3274.

Garg, P., Verma, R., Cook, L., Soofi, A., Venkatareddy, M., George, B., Mizuno, K., Gurniak, C., Witke, W. & Holzman, L.B. 2010, "Actin-depolymerizing factor cofilin-1 is necessary in maintaining mature podocyte architecture", *The Journal of Biological Chemistry,* vol. 285, no. 29, pp. 22676-22688.

Gerich, J.E. 1993, "Control of glycaemia", *Bailliere's Clinical Endocrinology and Metabolism,* vol. 7, no. 3, pp. 551-586.

Gerke, P., Huber, T.B., Sellin, L., Benzing, T. & Walz, G. 2003, "Homodimerization and heterodimerization of the glomerular podocyte proteins nephrin and NEPH1", *Journal of the American Society of Nephrology : JASN,* vol. 14, no. 4, pp. 918-926.

Gerke, P., Sellin, L., Kretz, O., Petraschka, D., Zentgraf, H., Benzing, T. & Walz, G. 2005, "NEPH2 is located at the glomerular slit diaphragm, interacts with nephrin and is cleaved from podocytes by metalloproteinases", *Journal of the American Society of Nephrology : JASN,* vol. 16, no. 6, pp. 1693-1702.

Gigante, M., Pontrelli, P., Montemurno, E., Roca, L., Aucella, F., Penza, R., Caridi, G., Ranieri, E., Ghiggeri, G.M. & Gesualdo, L. 2009, "CD2AP mutations are associated with sporadic nephrotic syndrome and focal segmental glomerulosclerosis (FSGS)",

Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, vol. 24, no. 6, pp. 1858-1864.

Golay, A., Swislocki, A.L., Chen, Y.D., Jaspan, J.B. & Reaven, G.M. 1986, "Effect of obesity on ambient plasma glucose, free fatty acid, insulin, growth hormone, and glucagon concentrations", *The Journal of Clinical Endocrinology and Metabolism,* vol. 63, no. 2, pp. 481-484.

Gordon, A.M., Homsher, E. & Regnier, M. 2000, "Regulation of contraction in striated muscle", *Physiological Reviews,* vol. 80, no. 2, pp. 853-924.

Greinacher, A., Nieuwenhuis, H.K. & White, J.G. 1990, "The Sebastian platelet syndrome. A new form of hereditary thrombocytopenia with giant thrombocytes and inclusion bodies in granulocytes", *Beitrage zur Infusionstherapie = Contributions to infusion therapy,* vol. 26, pp. 383-385.

Groop, L., Ekstrand, A., Forsblom, C., Widen, E., Groop, P.H., Teppo, A.M. & Eriksson, J. 1993, "Insulin resistance, hypertension and microalbuminuria in patients with type 2 (non-insulin-dependent) diabetes mellitus", *Diabetologia,* vol. 36, no. 7, pp. 642-647.

Groop, P.H., Forsblom, C. & Thomas, M.C. 2005, "Mechanisms of disease: Pathway-selective insulin resistance and microvascular complications of diabetes", *Nature Clinical Practice Endocrinology & Metabolism*, vol. 1, no. 2, pp. 100-110.

Groop, P.H., Thomas, M.C., Moran, J.L., Waden, J., Thorn, L.M., Makinen, V.P., Rosengard-Barlund, M., Saraheimo, M., Hietala, K., Heikkila, O., Forsblom, C. & FinnDiane Study Group 2009, "The presence and severity of chronic kidney disease predicts all-cause mortality in type 1 diabetes", *Diabetes,* vol. 58, no. 7, pp. 1651-1658.

Guder, W.G. & Ross, B.D. 1984, "Enzyme distribution along the nephron", *Kidney International,* vol. 26, no. 2, pp. 101-111.

Guzman, J., Jauregui, A.N., Merscher-Gomez, S., Maiguel, D., Muresan, C., Mitrofanova, A., Diez-Sampedro, A., Szust, J., Yoo, T.H., Villarreal, R., Pedigo, C., Molano, R.D., Johnson, K., Kahn, B., Hartleben, B., Huber, T.B., Saha, J., Burke, G.W.,3rd, Abel, E.D., Brosius, F.C. & Fornoni, A. 2014, "Podocyte-specific GLUT4-deficient mice have

fewer and larger podocytes and are protected from diabetic nephropathy", *Diabetes,* vol. 63, no. 2, pp. 701-714.

Hall, P.A., Jung, K., Hillan, K.J. & Russell, S.E. 2005, "Expression profiling the human septin gene family", *The Journal of Pathology,* vol. 206, no. 3, pp. 269-278.

Hall, P.A. & Russell, S.E. 2004, "The pathobiology of the septin gene family", *The Journal of Pathology,* vol. 204, no. 4, pp. 489-505.

Hara, M., Yanagihara, T., Kihara, I., Higashi, K., Fujimoto, K. & Kajita, T. 2005, "Apical cell membranes are shed into urine from injured podocytes: a novel phenomenon of podocyte injury", *Journal of the American Society of Nephrology : JASN,* vol. 16, no. 2, pp. 408-416.

Haraldsson, B., Nystrom, J. & Deen, W.M. 2008, "Properties of the glomerular barrier and mechanisms of proteinuria", *Physiological Reviews,* vol. 88, no. 2, pp. 451-487.

Harjutsalo, V. & Groop, P.H. 2014, "Epidemiology and risk factors for diabetic kidney disease", *Advances in Chronic Kidney Disease*s, vol. 21, no. 3, pp. 260-266.

Hartwell, L.H. 1971, "Genetic control of the cell division cycle in yeast. II. Genes controlling DNA replication and its initiation", *Journal of Molecular Biology,* vol. 59, no. 1, pp. 183-194.

Hasslacher, C., Stech, W., Wahl, P. & Ritz, E. 1985, "Blood pressure and metabolic control as risk factors for nephropathy in type 1 (insulin-dependent) diabetes", *Diabetologia,* vol. 28, no. 1, pp. 6-11.

Hays, T., Ma'ayan, A., Clark, N.R., Tan, C.M., Teixeira, A., Teixeira, A., Choi, J.W., Burdis, N., Jung, S.Y., Bajaj, A.O., O'Malley, B.W., He, J.C., Hyink, D.P. & Klotman, P.E. 2014, "Proteomics Analysis of the Non-Muscle Myosin Heavy Chain IIa-Enriched Actin-Myosin Complex Reveals Multiple Functions within the Podocyte", *PloS One,* vol. 9, no. 6, pp. e100660.

He, F.F., Zhang, C., Chen, S., Deng, B.Q., Wang, H., Shao, N., Tian, X.J., Fang, Z., Sun, X.F., Liu, J.S., Zhu, Z.H. & Meng, X.F. 2011, "Role of CD2-associated protein in

albumin overload-induced apoptosis in podocytes", *Cell Biology International,* vol. 35, no. 8, pp. 827-834.

Heasman, S.J. & Ridley, A.J. 2008, "Mammalian Rho GTPases: new insights into their functions from in vivo studies", *Nature reviews. Molecular Cell Biology,* vol. 9, no. 9, pp. 690-701.

Heilig, C., Zaloga, C., Lee, M., Zhao, X., Riser, B., Brosius, F. & Cortes, P. 1995, "Immunogold localization of high-affinity glucose transporter isoforms in normal rat kidney", *Laboratory Investigation; a Journal of Technical Methods and Pathology,* vol. 73, no. 5, pp. 674-684.

Heilig, C.W., Liu, Y., England, R.L., Freytag, S.O., Gilbert, J.D., Heilig, K.O., Zhu, M., Concepcion, L.A. & Brosius, F.C.,3rd 1997, "D-glucose stimulates mesangial cell GLUT1 expression and basal and IGF-I-sensitive glucose uptake in rat mesangial cells: implications for diabetic nephropathy", *Diabetes,* vol. 46, no. 6, pp. 1030-1039.

Heyworth, P.G., Robinson, J.M., Ding, J., Ellis, B.A. & Badwey, J.A. 1997, "Cofilin undergoes rapid dephosphorylation in stimulated neutrophils and translocates to ruffled membranes enriched in products of the NADPH oxidase complex. Evidence for a novel cycle of phosphorylation and dephosphorylation", *Histochemistry and Cell Biology,* vol. 108, no. 3, pp. 221-233.

Hirabayashi, S., Mori, H., Kansaku, A., Kurihara, H., Sakai, T., Shimizu, F., Kawachi, H. & Hata, Y. 2005, "MAGI-1 is a component of the glomerular slit diaphragm that is tightly associated with nephrin", *Laboratory Investigation; a Journal of Technical Methods and Pathology,* vol. 85, no. 12, pp. 1528-1543.

Hofmann, F., Bernhard, D., Lukowski, R. & Weinmeister, P. 2009, "cGMP regulated protein kinases (cGK)", *Handbook of Experimental Pharmacology,* vol. 191, pp. 137-62.

Hsu, S.C., Hazuka, C.D., Roth, R., Foletti, D.L., Heuser, J. & Scheller, R.H. 1998, "Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments", *Neuron,* vol. 20, no. 6, pp. 1111-1122.

Hu, Q., Nelson, W.J. & Spiliotis, E.T. 2008, "Forchlorfenuron alters mammalian septin assembly, organization, and dynamics", *The Journal of Biological Chemistry*, vol. 283, no. 43, pp. 29563-29571.

Huber, T.B., Hartleben, B., Kim, J., Schmidts, M., Schermer, B., Keil, A., Egger, L., Lecha, R.L., Borner, C., Pavenstadt, H., Shaw, A.S., Walz, G. & Benzing, T. 2003, "Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling", *Molecular and Cellular Biology,* vol. 23, no. 14, pp. 4917-4928.

Hugo, C., Nangaku, M., Shankland, S.J., Pichler, R., Gordon, K., Amieva, M.R., Couser, W.G., Furthmayr, H. & Johnson, R.J. 1998, "The plasma membrane-actin linking protein, ezrin, is a glomerular epithelial cell marker in glomerulogenesis, in the adult kidney and in glomerular injury", *Kidney International,* vol. 54, no. 6, pp. 1934-1944.

Hutchings, N.J., Clarkson, N., Chalkley, R., Barclay, A.N. & Brown, M.H. 2003, "Linking the T cell surface protein CD2 to the actin-capping protein CAPZ via CMS and CIN85", *The Journal of Biological Chemistry,* vol. 278, no. 25, pp. 22396-22403.

Hyvonen, M.E., Saurus, P., Wasik, A., Heikkila, E., Havana, M., Trokovic, R., Saleem, M., Holthofer, H. & Lehtonen, S. 2010, "Lipid phosphatase SHIP2 downregulates insulin signalling in podocytes", *Molecular and Cellular Endocrinology,* vol. 328, no. 1-2, pp. 70-79.

Ijaz, A., Tejada, T., Catanuto, P., Xia, X., Elliot, S.J., Lenz, O., Jauregui, A., Saenz, M.O., Molano, R.D., Pileggi, A., Ricordi, C. & Fornoni, A. 2009, "Inhibition of C-jun N-terminal kinase improves insulin sensitivity but worsens albuminuria in experimental diabetes", *Kidney International,* vol. 75, no. 4, pp. 381-388.

Inoguchi, T., Li, P., Umeda, F., Yu, H.Y., Kakimoto, M., Imamura, M., Aoki, T., Etoh, T., Hashimoto, T., Naruse, M., Sano, H., Utsumi, H. & Nawata, H. 2000, "High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells", *Diabetes,* vol. 49, no. 11, pp. 1939-1945.

Inoue, T., Yaoita, E., Kurihara, H., Shimizu, F., Sakai, T., Kobayashi, T., Ohshiro, K., Kawachi, H., Okada, H., Suzuki, H., Kihara, I. & Yamamoto, T. 2001, "FAT is a

component of glomerular slit diaphragms", *Kidney International,* vol. 59, no. 3, pp. 1003-1012.

International Diabetes Federation. 2013, "IDF Diabetes Atlas, 6th edition", Melbourne, Australia: International Diabetes Federation, http://www.idf.org/diabetesatlas.

Iseki, K., Ikemiya, Y., Kinjo, K., Inoue, T., Iseki, C. & Takishita, S. 2004, "Body mass index and the risk of development of end-stage renal disease in a screened cohort", *Kidney International*, vol. 65, no. 5, pp. 1870-1876.

Itani, S.I., Ruderman, N.B., Schmieder, F. & Boden, G. 2002, "Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha", *Diabetes,* vol. 51, no. 7, pp. 2005-2011.

Jauregui, A., Mintz, D.H., Mundel, P. & Fornoni, A. 2009, "Role of altered insulin signaling pathways in the pathogenesis of podocyte malfunction and microalbuminuria", *Current Opinion in Nephrology and Hypertension,* vol. 18, no. 6, pp. 539-545.

Jeansson, M. & Haraldsson, B. 2006, "Morphological and functional evidence for an important role of the endothelial cell glycocalyx in the glomerular barrier", *American Journal of Physiology. Renal Physiology,* vol. 290, no. 1, pp. 111-116.

Jim, B., Ghanta, M., Qipo, A., Fan, Y., Chuang, P.Y., Cohen, H.W., Abadi, M., Thomas, D.B. & He, J.C. 2012, "Dysregulated nephrin in diabetic nephropathy of type 2 diabetes: a cross sectional study", *PloS One,* vol. 7, no. 5, pp. e36041.

Joberty, G., Perlungher, R.R., Sheffield, P.J., Kinoshita, M., Noda, M., Haystead, T. & Macara, I.G. 2001, "Borg proteins control septin organization and are negatively regulated by Cdc42", *Nature Cell Biology,* vol. 3, no. 10, pp. 861-866.

Johnstone, D.B., Ikizler, O., Zhang, J. & Holzman, L.B. 2013, "Background strain and the differential susceptibility of podocyte-specific deletion of Myh9 on murine models of experimental glomerulosclerosis and HIV nephropathy", *PloS One,* vol. 8, no. 7, pp. e67839.

Johnstone, D.B., Zhang, J., George, B., Leon, C., Gachet, C., Wong, H., Parekh, R. & Holzman, L.B. 2011, "Podocyte-specific deletion of Myh9 encoding nonmuscle myosin heavy chain 2A predisposes mice to glomerulopathy", *Molecular and Cellular Biology,* vol. 31, no. 10, pp. 2162-2170.

Jones, N., Blasutig, I.M., Eremina, V., Ruston, J.M., Bladt, F., Li, H., Huang, H., Larose, L., Li, S.S., Takano, T., Quaggin, S.E. & Pawson, T. 2006, "Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes", *Nature,* vol. 440, no. 7085, pp. 818-823.

Joo, E., Surka, M.C. & Trimble, W.S. 2007, "Mammalian SEPT2 is required for scaffolding nonmuscle myosin II and its kinases", *Developmental Cell,* vol. 13, no. 5, pp. 677-690.

Kadowaki, T., Yamauchi, T., Kubota, N., Hara, K., Ueki, K. & Tobe, K. 2006, "Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome", *The Journal of Clinical Investigation,* vol. 116, no. 7, pp. 1784-1792.

Kaplan, J.M., Kim, S.H., North, K.N., Rennke, H., Correia, L.A., Tong, H.Q., Mathis, B.J., Rodriguez-Perez, J.C., Allen, P.G., Beggs, A.H. & Pollak, M.R. 2000, "Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis", *Nature Genetics,* vol. 24, no. 3, pp. 251-256.

Kerjaschki, D. 2001, "Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis", *The Journal of Clinical Investigation,* vol. 108, no. 11, pp. 1583-1587.

Kerjaschki, D., Sharkey, D.J. & Farquhar, M.G. 1984, "Identification and characterization of podocalyxin--the major sialoprotein of the renal glomerular epithelial cell", *The Journal of Cell Biology,* vol. 98, no. 4, pp. 1591-1596.

Kestilä, M., Lenkkeri, U., Mannikko, M., Lamerdin, J., McCready, P., Putaala, H., Ruotsalainen, V., Morita, T., Nissinen, M., Herva, R., Kashtan, C.E., Peltonen, L., Holmberg, C., Olsen, A. & Tryggvason, K. 1998, "Positionally cloned gene for a novel glomerular protein--nephrin--is mutated in congenital nephrotic syndrome", *Molecular Cell,* vol. 1, no. 4, pp. 575-582.

Kim, J.M., Wu, H., Green, G., Winkler, C.A., Kopp, J.B., Miner, J.H., Unanue, E.R. & Shaw, A.S. 2003, "CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility", *Science,* vol. 300, no. 5623, pp. 1298-1300.

Kimmelstiel, P. & Wilson, C. 1936, "Intercapillary Lesions in the Glomeruli of the Kidney", *American Journal of Pathology,* vol. 12, pp. 83-98.

King, A.J. 2012, "The use of animal models in diabetes research", *British Journal of Pharmacology,* vol. 166, no. 3, pp. 877-894.

Kinoshita, M. 2003, "Assembly of mammalian septins", *Journal of Biochemistry,* vol. 134, no. 4, pp. 491-496.

Kinoshita, M., Field, C.M., Coughlin, M.L., Straight, A.F. & Mitchison, T.J. 2000, "Self- and actin-templated assembly of Mammalian septins", *Developmental Cell*, vol. 3, no. 6, pp. 791-802.

Kojima, K., Davidovits, A., Poczewski, H., Langer, B., Uchida, S., Nagy-Bojarski, K., Hovorka, A., Sedivy, R. & Kerjaschki, D. 2004, "Podocyte flattening and disorder of glomerular basement membrane are associated with splitting of dystroglycan-matrix interaction", *Journal of the American Society of Nephrology: JASN,* vol. 15, no. 8, pp. 2079-2089.

Koop, K., Eikmans, M., Baelde, H.J., Kawachi, H., De Heer, E., Paul, L.C. & Bruijn, J.A. 2003, "Expression of podocyte-associated molecules in acquired human kidney diseases", *Journal of the American Society of Nephrology : JASN,* vol. 14, no. 8, pp. 2063-2071.

Kopp, J.B., Smith, M.W., Nelson, G.W., Johnson, R.C., Freedman, B.I., Bowden, D.W., Oleksyk, T., McKenzie, L.M., Kajiyama, H., Ahuja, T.S., Berns, J.S., Briggs, W., Cho, M.E., Dart, R.A., Kimmel, P.L., Korbet, S.M., Michel, D.M., Mokrzycki, M.H., Schelling, J.R., Simon, E., Trachtman, H., Vlahov, D. & Winkler, C.A. 2008, "MYH9 is a major-effect risk gene for focal segmental glomerulosclerosis", *Nature Genetics,* vol. 40, no. 10, pp. 1175-1184.

Koshikawa, M., Mukoyama, M., Mori, K., Suganami, T., Sawai, K., Yoshioka, T., Nagae, T., Yokoi, H., Kawachi, H., Shimizu, F., Sugawara, A. & Nakao, K. 2005, "Role of p38 mitogen-activated protein kinase activation in podocyte injury and proteinuria in experimental nephrotic syndrome", *Journal of the American Society of Nephrology: JASN,* vol. 16, no. 9, pp. 2690-2701.

Kramer, H.J., Nguyen, Q.D., Curhan, G. & Hsu, C.Y. 2003, "Renal insufficiency in the absence of albuminuria and retinopathy among adults with type 2 diabetes mellitus", *JAMA : the journal of the American Medical Association,* vol. 289, no. 24, pp. 3273-3277.

Kreidberg, J.A., Donovan, M.J., Goldstein, S.L., Rennke, H., Shepherd, K., Jones, R.C. & Jaenisch, R. 1996, "Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis", *Development,* vol. 122, no. 11, pp. 3537-3547.

Kremer, B.E., Adang, L.A. & Macara, I.G. 2007, "Septins regulate actin organization and cell-cycle arrest through nuclear accumulation of NCK mediated by SOCS7", *Cell,* vol. 130, no. 5, pp. 837-850.

Kurella, M., Lo, J.C. & Chertow, G.M. 2005, "Metabolic syndrome and the risk for chronic kidney disease among nondiabetic adults", *Journal of the American Society of Nephrology : JASN,* vol. 16, no. 7, pp. 2134-2140.

Kurihara, H., Anderson, J.M., Kerjaschki, D. & Farquhar, M.G. 1992, "The altered glomerular filtration slits seen in puromycin aminonucleoside nephrosis and protamine sulfate-treated rats contain the tight junction protein ZO-1", *The American Journal of Pathology,* vol. 141, no. 4, pp. 805-816.

Lander, H.M., Tauras, J.M., Ogiste, J.S., Hori, O., Moss, R.A. & Schmidt, A.M. 1997, "Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress", *The Journal of Biological Chemistry,* vol. 272, no. 28, pp. 17810-17814.

Langham, R.G., Kelly, D.J., Cox, A.J., Thomson, N.M., Holthofer, H., Zaoui, P., Pinel, N., Cordonnier, D.J. & Gilbert, R.E. 2002, "Proteinuria and the expression of the podocyte

slit diaphragm protein, nephrin, in diabetic nephropathy: effects of angiotensin converting enzyme inhibition", *Diabetologia,* vol. 45, no. 11, pp. 1572-1576.

Lee, J. & Pilch, P.F. 1994, "The insulin receptor: structure, function, and signaling", *The American Journal of Physiology,* vol. 266, no. 2, pp. 319-334.

Lehtonen, S., Lehtonen, E., Kudlicka, K., Holthofer, H. & Farquhar, M.G. 2004, "Nephrin forms a complex with adherens junction proteins and CASK in podocytes and in Madin-Darby canine kidney cells expressing nephrin", *The American Journal of Pathology,* vol. 165, no. 3, pp. 923-936.

Lehtonen, S., Ora, A., Olkkonen, V.M., Geng, L., Zerial, M., Somlo, S. & Lehtonen, E. 2000, "In vivo interaction of the adapter protein CD2-associated protein with the type 2 polycystic kidney disease protein, polycystin-2", *Journal of Biological Chemistry*, vol. 275, no. 42, pp. 32888–32893.

Lehtonen, S., Ryan, J.J., Kudlicka, K., Iino, N., Zhou, H. & Farquhar, M.G. 2005, "Cell junction-associated proteins IQGAP1, MAGI-2, CASK, spectrins, and alpha-actinin are components of the nephrin multiprotein complex", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 102, no. 28, pp. 9814-9819.

Lehtonen, S., Tienari, J., Londesborough, A., Pirvola, U., Ora, A., Reima, I. & Lehtonen E. 2008, "CD2-associated protein is widely expressed and differentially regulated during embryonic development", *Differentiation*, vol. 76, no. 5, pp. 506-517.

Lehtonen, S., Zhao, F. & Lehtonen, E. 2002, "CD2-associated protein directly interacts with the actin cytoskeleton", *American Journal of Physiology. Renal Physiology,* vol. 283, no. 4, pp. 734-743.

Leinonen, E., Hurt-Camejo, E., Wiklund, O., Hulten, L.M., Hiukka, A. & Taskinen, M.R. 2003, "Insulin resistance and adiposity correlate with acute-phase reaction and soluble cell adhesion molecules in type 2 diabetes", *Atherosclerosis,* vol. 166, no. 2, pp. 387-394.

Lennon, R., Pons, D., Sabin, M.A., Wei, C., Shield, J.P., Coward, R.J., Tavare, J.M., Mathieson, P.W., Saleem, M.A. & Welsh, G.I. 2009, "Saturated fatty acids induce

insulin resistance in human podocytes: implications for diabetic nephropathy", *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association,* vol. 24, no. 11, pp. 3288-3296.

Leung, T., Chen, X.Q., Manser, E. & Lim, L. 1996, "The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton", *Molecular and Cellular Biology,* vol. 16, no. 10, pp. 5313-5327.

Lewko, B., Bryl, E., Witkowski, J.M., Latawiec, E., Golos, M., Endlich, N., Hahnel, B., Koksch, C., Angielski, S., Kriz, W. & Stepinski, J. 2005, "Characterization of glucose uptake by cultured rat podocytes", *Kidney & Blood Pressure Research,* vol. 28, no. 1, pp. 1-7.

Lewko, B. & Stepinski, J. 2009, "Hyperglycemia and mechanical stress: targeting the renal podocyte", *Journal of Cellular Physiology,* vol. 221, no. 2, pp. 288-295.

Li, C., Ruotsalainen, V., Tryggvason, K., Shaw, A.S. & Miner, J.H. 2000, "CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere", *American Journal of Physiology. Renal Physiology,* vol. 279, no. 4, pp. 785-792.

Li, H., Lemay, S., Aoudjit, L., Kawachi, H. & Takano, T. 2004, "SRC-family kinase Fyn phosphorylates the cytoplasmic domain of nephrin and modulates its interaction with podocin", *Journal of the American Society of Nephrology: JASN,* vol. 15, no. 12, pp. 3006-3015.

Like, A.A. & Rossini, A.A. 1976, "Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus", *Science,* vol. 193, no. 4251, pp. 415-417.

Liu, X.L., Done, S.C., Yan, K., Kilpelainen, P., Pikkarainen, T. & Tryggvason, K. 2004, "Defective trafficking of nephrin missense mutants rescued by a chemical chaperone", *Journal of the American Society of Nephrology : JASN,* vol. 15, no. 7, pp. 1731-1738.

Lopez, J.A., Burchfield, J.G., Blair, D.H., Mele, K., Ng, Y., Vallotton, P., James, D.E., & Hughes, W.E. 2009, "Identification of a distal GLUT4 trafficking event controlled by actin polymerization", *Molecular Biology of the Cell*, vol. 20, no. 17, pp. 3918-3929.

Lopez, J.P., Turner, J.R. & Philipson, L.H. 2010, "Glucose-induced ERM protein activation and translocation regulates insulin secretion", *American Journal of Physiology. Endocrinology and Metabolism,* vol. 299, no. 5, pp. 772-785.

Lynch, D.K., Winata, S.C., Lyons, R.J., Hughes, W.E., Lehrbach, G.M., Wasinger, V., Corthals, G., Cordwell, S. & Daly, R.J. 2003, "A Cortactin-CD2-associated protein (CD2AP) complex provides a novel link between epidermal growth factor receptor endocytosis and the actin cytoskeleton", *The Journal of Biological Chemistry,* vol. 278, no. 24, pp. 21805-21813.

MacIsaac, R.J., Tsalamandris, C., Panagiotopoulos, S., Smith, T.J., McNeil, K.J. & Jerums, G. 2004, "Nonalbuminuric renal insufficiency in type 2 diabetes", *Diabetes Care,* vol. 27, no. 1, pp. 195-200.

Marieb, E.N., 2001, "The urinary system. Human anatomy and physiology", *San Francisco Benjamin Cummings*, 5th ed.

Marsick, B.M., San Miguel-Ruiz, J.E. & Letourneau, P.C. 2012, "Activation of ezrin/radixin/moesin mediates attractive growth cone guidance through regulation of growth cone actin and adhesion receptors", *The Journal of Neuroscience: the Official Journal of the Society for Neuroscience,* vol. 32, no. 1, pp. 282-296.

Mathieson, P.W. 2012, "The podocyte cytoskeleton in health and in disease", *Clinincal Kidney Journal,* vol. 5, pp. 498-501.

Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S. & Tsukita, S. 1998, "Rho-kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association", *The Journal of Cell Biology,* vol. 140, no. 3, pp. 647-657.

Matsui, T., Yonemura, S., Tsukita, S. & Tsukita, S. 1999, "Activation of ERM proteins in vivo by Rho involves phosphatidyl-inositol 4-phosphate 5-kinase and not ROCK kinases", *Current Biology,* vol. 9, no. 21, pp. 1259-1262.

Mauer, S.M., Fish, A.J., Blau, E.B. & Michael, A.F. 1972, "The glomerular mesangium. I. Kinetic studies of macromolecular uptake in normal and nephrotic rats", *The Journal of Clinical Investigation,* vol. 51, no. 5, pp. 1092-1101.

Mauer, S.M., Steffes, M.W., Ellis, E.N., Sutherland, D.E., Brown, D.M. & Goetz, F.C. 1984, "Structural-functional relationships in diabetic nephropathy", *The Journal of Clinical Investigation,* vol. 74, no. 4, pp. 1143-1155.

McClain, D.A., Maegawa, H., Lee, J., Dull, T.J., Ulrich, A. & Olefsky, J.M. 1987, "A mutant insulin receptor with defective tyrosine kinase displays no biologic activity and does not undergo endocytosis", *The Journal of Biological Chemistry,* vol. 262, no. 30, pp. 14663-14671.

Meyer, C., Dostou, J., Nadkarni, V. & Gerich, J. 1998a, "Effects of physiological hyperinsulinemia on systemic, renal, and hepatic substrate metabolism", *The American Journal of Physiology,* vol. 275, no. 6, pp. 915-921.

Meyer, C., Dostou, J.M., Welle, S.L. & Gerich, J.E. 2002, "Role of human liver, kidney, and skeletal muscle in postprandial glucose homeostasis", *American Journal of Physiology. Endocrinology and Metabolism,* vol. 282, no. 2, pp. 419-427.

Meyer, C., Stumvoll, M., Nadkarni, V., Dostou, J., Mitrakou, A. & Gerich, J. 1998b, "Abnormal renal and hepatic glucose metabolism in type 2 diabetes mellitus", *The Journal of Clinical Investigation,* vol. 102, no. 3, pp. 619-624.

Meyer, C., Woerle, H.J., Dostou, J.M., Welle, S.L. & Gerich, J.E. 2004, "Abnormal renal, hepatic, and muscle glucose metabolism following glucose ingestion in type 2 diabetes", *American Journal of Physiology. Endocrinology and Metabolism,* vol. 287, no. 6, pp. 1049-1056.

Meyer, T.W., Bennett, P.H. & Nelson, R.G. 1999, "Podocyte number predicts long-term urinary albumin excretion in Pima Indians with Type II diabetes and microalbuminuria", *Diabetologia,* vol. 42, no. 11, pp. 1341-1344.

Michaud, J.L., Lemieux, L.I., Dube, M., Vanderhyden, B.C., Robertson, S.J. & Kennedy, C.R. 2003, "Focal and segmental glomerulosclerosis in mice with podocyte-specific

expression of mutant alpha-actinin-4", *Journal of the American Society of Nephrology : JASN,* vol. 14, no. 5, pp. 1200-1211.

Miele, C., Riboulet, A., Maitan, M.A., Oriente, F., Romano, C., Formisano, P., Giudicelli, J., Beguinot, F. & Van Obberghen, E. 2003, "Human glycated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced insulin receptor substrate (IRS) signaling through a protein kinase C alpha-mediated mechanism", *The Journal of Biological Chemistry,* vol. 278, no. 48, pp. 47376-47387.

Miettinen, A., Dekan, G. & Farquhar, M.G. 1990, "Monoclonal antibodies against membrane proteins of the rat glomerulus. Immunochemical specificity and immunofluorescence distribution of the antigens", *The American Journal of Pathology,* vol. 137, no. 4, pp. 929-944.

Mima, A., Ohshiro, Y., Kitada, M., Matsumoto, M., Geraldes, P., Li, C., Li, Q., White, G.S., Cahill, C., Rask-Madsen, C. & King, G.L. 2011, "Glomerular-specific protein kinase C-beta-induced insulin receptor substrate-1 dysfunction and insulin resistance in rat models of diabetes and obesity", *Kidney International,* vol. 79, no. 8, pp. 883-896.

Miner, J.H. 1999, "Renal basement membrane components", *Kidney International,* vol. 56, no. 6, pp. 2016-2024.

Miyauchi, K., Takiyama, Y., Honjyo, J., Tateno, M. & Haneda, M. 2009, "Upregulated IL-18 expression in type 2 diabetic subjects with nephropathy: TGF-beta1 enhanced IL-18 expression in human renal proximal tubular epithelial cells", *Diabetes Research and Clinical Practice,* vol. 83, no. 2, pp. 190-199.

Miyazaki, Y., Cersosimo, E., Triplitt, C. & DeFronzo, R.A. 2007, "Rosiglitazone decreases albuminuria in type 2 diabetic patients", *Kidney International,* vol. 72, no. 11, pp. 1367-1373.

Mogensen, C.E. 1971, "Maximum tubular reabsorption capacity for glucose and renal hemodynamcis during rapid hypertonic glucose infusion in normal and diabetic subjects", *Scandinavian Journal of Clinical and Laboratory Investigation,* vol. 28, no. 1, pp. 101-109.

Mogensen, C.E. & Christensen, C.K. 1984, "Predicting Diabetic Nephropathy in Insulin-Dependent Patients", *The New England Journal of Medicine,* vol. 311, pp. 89-93.

Molitch, M.E., Steffes, M., Sun, W., Rutledge, B., Cleary, P., de Boer, I.H., Zinman, B., Lachin, J. & Epidemiology of Diabetes Interventions and Complications Study Group 2010, "Development and progression of renal insufficiency with and without albuminuria in adults with type 1 diabetes in the diabetes control and complications trial and the epidemiology of diabetes interventions and complications study", *Diabetes Care,* vol. 33, no. 7, pp. 1536-1543.

Moller, N., Rizza, R.A., Ford, G.C. & Nair, K.S. 2001, "Assessment of postabsorptive renal glucose metabolism in humans with multiple glucose tracers", *Diabetes,* vol. 50, no. 4, pp. 747-751.

Moriyama, K., Iida, K. & Yahara, I. 1996, "Phosphorylation of Ser-3 of cofilin regulates its essential function on actin", *Genes to Cells: Devoted to Molecular & Cellular Mechanisms,* vol. 1, no. 1, pp. 73-86.

Moutzouris, D.A., Kitsiou, P.V., Talamagas, A.A., Drossopoulou, G.I., Kassimatis, T.I. & Katsilambros, N.K. 2007, "Chronic exposure of human glomerular epithelial cells to high glucose concentration results in modulation of high-affinity glucose transporters expression", *Renal Failure,* vol. 29, no. 3, pp. 353-358.

Mullins, R.D., Heuser, J.A. & Pollard, T.D. 1998, "The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 95, no. 11, pp. 6181-6186.

Mundel, P. & Shankland, S.J. 2002, "Podocyte biology and response to injury", *Journal of the American Society* of *Nephrology*, vol. 13, no. 12, pp. 3005-3015.

Müller, T., Rumpel, E., Hradetzky, S., Bollig, F., Wegner, H., Blumenthal, A., Greinacher, A., Endlich, K. & Endlich, N. 2011, "Non-muscle myosin IIA is required for the development of the zebrafish glomerulus", *Kidney International,* vol. 80, no. 10, pp. 1055-1063.

Mykkanen, L., Zaccaro, D.J., Wagenknecht, L.E., Robbins, D.C., Gabriel, M. & Haffner, S.M. 1998, "Microalbuminuria is associated with insulin resistance in nondiabetic subjects: the insulin resistance atherosclerosis study", *Diabetes,* vol. 47, no. 5, pp. 793-800.

Myrup, B., de Maat, M., Rossing, P., Gram, J., Kluft, C. & Jespersen, J. 1996, "Elevated fibrinogen and the relation to acute phase response in diabetic nephropathy", *Thrombosis Research,* vol. 81, no. 4, pp. 485-490.

Nagata, K., Asano, T., Nozawa, Y. & Inagaki, M. 2004, "Biochemical and cell biological analyses of a mammalian septin complex, Sept7/9b/11", *The Journal of Biological Chemistry,* vol. 279, no. 53, pp. 55895-55904.

Nakamura, A., Shikata, K., Hiramatsu, M., Nakatou, T., Kitamura, T., Wada, J., Itoshima, T., & Makino, H. 2005, "Serum interleukin-18 levels are associated with nephropathy and atherosclerosis in Japanese patients with type 2 diabetes", *Diabetes Care*, vol. 28, no. 12, pp. 2890-2895.

Nakamura, T., Ushiyama, C., Suzuki, S., Hara, M., Shimada, N., Ebihara, I. & Koide, H. 2000, "Urinary excretion of podocytes in patients with diabetic nephropathy", *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association,* vol. 15, no. 9, pp. 1379-1383.

Nathan, D.M., Cleary, P.A., Backlund, J.Y., Genuth, S.M., Lachin, J.M., Orchard, T.J., Raskin, P., Zinman, B. & Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study Research Group 2005, "Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes", *The New England Journal of Medicine,* vol. 353, no. 25, pp. 2643-2653.

Navarro, J.F., Milena, F.J., Mora, C., Leon, C. & Garcia, J. 2006, "Renal pro-inflammatory cytokine gene expression in diabetic nephropathy: effect of angiotensin-converting enzyme inhibition and pentoxifylline administration", *American Journal of Nephrology,* vol. 26, no. 6, pp. 562-570.

Neco, P., Giner, D., Viniegra, S., Borges, R., Villarroel, A. & Gutierrez, L.M. 2004, "New roles of myosin II during vesicle transport and fusion in chromaffin cells", *The Journal of Biological Chemistry,* vol. 279, no. 26, pp. 27450-27457.

Ng, T., Parsons, M., Hughes, W.E., Monypenny, J., Zicha, D., Gautreau, A., Arpin, M., Gschmeissner, S., Verveer, P.J., Bastiaens, P.I. & Parker, P.J. 2001, "Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility", *The EMBO Journal,* vol. 20, no. 11, pp. 2723-2741.

Nishida, E., Maekawa, S. & Sakai, H. 1984, "Cofilin, a protein in porcine brain that binds to actin filaments and inhibits their interactions with myosin and tropomyosin", *Biochemistry,* vol. 23, no. 22, pp. 5307-5313.

Ohman, T., Lietzen, N., Valimaki, E., Melchjorsen, J., Matikainen, S. & Nyman, T.A. 2010, "Cytosolic RNA recognition pathway activates 14-3-3 protein mediated signaling and caspase-dependent disruption of cytokeratin network in human keratinocytes", *Journal of Proteome Research,* vol. 9, no. 3, pp. 1549-1564.

Omata, W., Shibata, H., Li, L., Takata, K. & Kojima, I. 2000, "Actin filaments play a critical role in insulin-induced exocytotic recruitment but not in endocytosis of GLUT4 in isolated rat adipocytes", *The Biochemical Journal,* vol. 346, no. 2, pp. 321-328.

Orlando, R.A., Takeda, T., Zak, B., Schmieder, S., Benoit, V.M., McQuistan, T., Furthmayr, H. & Farquhar, M.G. 2001, "The glomerular epithelial cell anti-adhesin podocalyxin associates with the actin cytoskeleton through interactions with ezrin", *Journal of the American Society of Nephrology: JASN,* vol. 12, no. 8, pp. 1589-1598.

Ostalska-Nowicka, D., Zachwieja, J., Nowicki, M., Kaczmarek, E., Siwinska, A. & Witt, M. 2006, "Ezrin--a useful factor in the prognosis of nephrotic syndrome in children: an immunohistochemical approach", *Journal of Clinical Pathology,* vol. 59, no. 9, pp. 916-920.

Osterby, R. 1973, "Kidney structural abnormalities in early diabetes", *Advances in Metabolic Disorders,* vol. 2, no. Suppl 2, pp. 323-340.

Pagtalunan, M.E., Miller, P.L., Jumping-Eagle, S., Nelson, R.G., Myers, B.D., Rennke, H.G., Coplon, N.S., Sun, L. & Meyer, T.W. 1997, "Podocyte loss and progressive glomerular injury in type II diabetes", *The Journal of Clinical Investigation,* vol. 99, no. 2, pp. 342-348.

Palaniappan, L., Carnethon, M. & Fortmann, S.P. 2003, "Association between microalbuminuria and the metabolic syndrome: NHANES III", *American Journal of Hypertension,* vol. 16, no. 11, pp. 952-958.

Palmén, T., Lehtonen, S., Ora, A., Kerjaschki, D., Antignac, C., Lehtonen, E. & Holthöfer, H. 2002, "Interaction of endogenous nephrin and CD2-associated protein in mouse epithelial M-1 cell line", *Journal of the American Society* of *Nephrology*, vol. 13, no. 7, pp. 1766-1772.

Parvanova, A.I., Trevisan, R., Iliev, I.P., Dimitrov, B.D., Vedovato, M., Tiengo, A., Remuzzi, G. & Ruggenenti, P. 2006, "Insulin resistance and microalbuminuria: a cross-sectional, case-control study of 158 patients with type 2 diabetes and different degrees of urinary albumin excretion", *Diabetes,* vol. 55, no. 5, pp. 1456-1462.

Parving, H.H., Andersen, A.R., Smidt, U.M. & Svendsen, P.A. 1983, "Early aggressive antihypertensive treatment reduces rate of decline in kidney function in diabetic nephropathy", *Lancet,* vol. 1, no. 8335, pp. 1175-1179.

Parving, H.H., Oxenboll, B., Svendsen, P.A., Christiansen, J.S. & Andersen, A.R. 1982, "Early detection of patients at risk of developing diabetic nephropathy. A longitudinal study of urinary albumin excretion", *Acta Endocrinologica,* vol. 100, no. 4, pp. 550-555.

Patari, A., Forsblom, C., Havana, M., Taipale, H., Groop, P.H. & Holthofer, H. 2003, "Nephrinuria in diabetic nephropathy of type 1 diabetes", *Diabetes,* vol. 52, no. 12, pp. 2969-2974.

Pavenstädt, H., Kriz, W. & Kretzler, M. 2003, "Cell biology of the glomerular podocyte", *Physiological Reviews,* vol. 83, no. 1, pp. 253-307.

Perkins, B.A., Ficociello, L.H., Silva, K.H., Finkelstein, D.M., Warram, J.H. & Krolewski, A.S. 2003, "Regression of microalbuminuria in type 1 diabetes", *The New England Journal of Medicine,* vol. 348, no. 23, pp. 2285-2293.

Perrin, N.E., Torbjornsdotter, T.B., Jaremko, G.A. & Berg, U.B. 2006, "The course of diabetic glomerulopathy in patients with type I diabetes: a 6-year follow-up with serial biopsies", *Kidney International,* vol. 69, no. 4, pp. 699-705.

Peterson, L.C., Rao, K.V., Crosson, J.T. & White, J.G. 1985, "Fechtner syndrome--a variant of Alport's syndrome with leukocyte inclusions and macrothrombocytopenia", *Blood,* vol. 65, no. 2, pp. 397-406.

Pickup, J.C., Chusney, G.D. & Mattock, M.B. 2000, "The innate immune response and type 2 diabetes: evidence that leptin is associated with a stress-related (acute-phase) reaction", *Clinical Endocrinology,* vol. 52, no. 1, pp. 107-112.

Piwkowska, A., Rogacka, D., Angielski, S. & Jankowski, M. 2014, "Insulin stimulates glucose transport via protein kinase G type I alpha-dependent pathway in podocytes", *Biochemical and Biophysical Research Communications,* vol. 446, no. 1, pp. 328-334.

Piwkowska, A., Rogacka, D., Jankowski, M., Kocbuch, K. & Angielski, S. 2012, "Hydrogen peroxide induces dimerization of protein kinase G type Ialpha subunits and increases albumin permeability in cultured rat podocytes", *Journal of Cellular Physiology,* vol. 227, no. 3, pp. 1004-1016.

Piwkowska, A., Rogacka, D., Kasztan, M., Angielski, S. & Jankowski, M. 2013, "Insulin increases glomerular filtration barrier permeability through dimerization of protein kinase G type Ialpha subunits", *Biochimica et biophysica acta,* vol. 1832, no. 6, pp. 791-804.

Plomgaard, P., Bouzakri, K., Krogh-Madsen, R., Mittendorfer, B., Zierath, J.R. & Pedersen, B.K. 2005, "Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation", *Diabetes,* vol. 54, no. 10, pp. 2939-2945.

Plomgaard, P., Fischer, C.P., Ibfelt, T., Pedersen, B.K. & van Hall, G. 2008, "Tumor necrosis factor-alpha modulates human in vivo lipolysis", *The Journal of Clinical Endocrinology and Metabolism,* vol. 93, no. 2, pp. 543-549.

Putaala, H., Soininen, R., Kilpelainen, P., Wartiovaara, J. & Tryggvason, K. 2001, "The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death", *Human Molecular Genetics,* vol. 10, no. 1, pp. 1-8.

Qi, J., Xiao, Y.F., Zhang, D.J., Yang, G.R. & Huang, H.C. 2007, "High glucose downregulates the expression of podocalyxin protein in glomerular podocytes of mice", *Beijing da xue xue bao.Yi xue ban = Journal of Peking University. Health sciences,* vol. 39, no. 2, pp. 167-170.

Rantanen, M., Palmen, T., Patari, A., Ahola, H., Lehtonen, S., Astrom, E., Floss, T., Vauti, F., Wurst, W., Ruiz, P., Kerjaschki, D. & Holthofer, H. 2002, "Nephrin TRAP mice lack slit diaphragms and show fibrotic glomeruli and cystic tubular lesions", *Journal of the American Society of Nephrology : JASN,* vol. 13, no. 6, pp. 1586-1594.

Rees, D.A. & Alcolado, J.C. 2005, "Animal models of diabetes mellitus", *Diabetic Medicine : a Journal of the British Diabetic Association,* vol. 22, no. 4, pp. 359-370.

Regele, H.M., Fillipovic, E., Langer, B., Poczewki, H., Kraxberger, I., Bittner, R.E. & Kerjaschki, D. 2000, "Glomerular expression of dystroglycans is reduced in minimal change nephrosis but not in focal segmental glomerulosclerosis", *Journal of the American Society of Nephrology : JASN,* vol. 11, no. 3, pp. 403-412.

Regoli, M. & Bendayan, M. 1997, "Alterations in the expression of the alpha 3 beta 1 integrin in certain membrane domains of the glomerular epithelial cells (podocytes) in diabetes mellitus", *Diabetologia,* vol. 40, no. 1, pp. 15-22.

Reiser, J., Kriz, W., Kretzler, M. & Mundel, P. 2000, "The glomerular slit diaphragm is a modified adherens junction", *Journal of the American Society of Nephrology: JASN,* vol. 11, no. 1, pp. 1-8.

Reutens, A.T. & Atkins, R.C. 2011, "Epidemiology of diabetic nephropathy", *Contributions to Nephrology,* vol. 170, pp. 1-7.

Roden, M., Price, T.B., Perseghin, G., Petersen, K.F., Rothman, D.L., Cline, G.W. & Shulman, G.I. 1996, "Mechanism of free fatty acid-induced insulin resistance in humans", *The Journal of Clinical Investigation,* vol. 97, no. 12, pp. 2859-2865.

Rothman, J.E. 1994, "Intracellular membrane fusion", *Advances in Second Messenger and Phosphoprotein Research,* vol. 29, pp. 81-96.

Ruotsalainen, V., Ljungberg, P., Wartiovaara, J., Lenkkeri, U., Kestilä, M., Jalanko, H., Holmberg, C. & Tryggvason, K. 1999, "Nephrin is specifically located at the slit diaphragm of glomerular podocytes", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 96, no. 14, pp. 7962-7967.

Ryu, S., Chang, Y., Woo, H.Y., Lee, K.B., Kim, S.G., Kim, D.I., Kim, W.S., Suh, B.S., Jeong, C. & Yoon, K. 2009, "Time-dependent association between metabolic syndrome and risk of CKD in Korean men without hypertension or diabetes", *American Journal of Kidney Diseases : The Official Journal of the National Kidney Foundation,* vol. 53, no. 1, pp. 59-69.

Saleem, M.A., O'Hare, M.J., Reiser, J., Coward, R.J., Inward, C.D., Farren, T., Xing, C.Y., Ni, L., Mathieson, P.W. & Mundel, P.A. 2002, "A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression", *Journal of the American Society* of *Nephrology*, vol. 13, no. 3, pp. 630-638.

Saraheimo, M., Teppo, A.M., Forsblom, C., Fagerudd, J. & Groop, P.H. 2003, "Diabetic nephropathy is associated with low-grade inflammation in Type 1 diabetic patients", *Diabetologia,* vol. 46, no. 10, pp. 1402-1407.

Schalkwijk, C.G., Poland, D.C., van Dijk, W., Kok, A., Emeis, J.J., Drager, A.M., Doni, A., van Hinsbergh, V.W. & Stehouwer, C.D. 1999, "Plasma concentration of C-reactive protein is increased in type I diabetic patients without clinical macroangiopathy and correlates with markers of endothelial dysfunction: evidence for chronic inflammation", *Diabetologia,* vol. 42, no. 3, pp. 351-357.

Schiffer, M., Bitzer, M., Roberts, I.S., Kopp, J.B., ten Dijke, P., Mundel, P. & Bottinger, E.P. 2001, "Apoptosis in podocytes induced by TGF-beta and Smad7", *The Journal of Clinical Investigation,* vol. 108, no. 6, pp. 807-816.

Schiffer, M., Mundel, P., Shaw, A.S. & Bottinger, E.P. 2004, "A novel role for the adaptor molecule CD2-associated protein in transforming growth factor-beta-induced apoptosis", *The Journal of Biological Chemistry,* vol. 279, no. 35, pp. 37004-37012.

Schiffer, M., Susztak, K., Ranalletta, M., Raff, A.C., Bottinger, E.P. & Charron, M.J. 2005, "Localization of the GLUT8 glucose transporter in murine kidney and regulation in vivo in nondiabetic and diabetic conditions", *American Journal of Physiology. Renal Physiology,* vol. 289, no. 1, pp. 186-193.

Schnabel, E., Anderson, J.M. & Farquhar, M.G. 1990, "The tight junction protein ZO-1 is concentrated along slit diaphragms of the glomerular epithelium", *The Journal of Cell Biology,* vol. 111, no. 3, pp. 1255-1263.

Schnaper, H.W., Kopp, J.B., Poncelet, A.C., Hubchak, S.C., Stetler-Stevenson, W.G., Klotman, P.E. & Kleinman, H.K. 1996, "Increased expression of extracellular matrix proteins and decreased expression of matrix proteases after serial passage of glomerular mesangial cells", *Journal of Cell Science,* vol. 109, no. 10, pp. 2521-2528.

Scholey, J.M., Taylor, K.A. & Kendrick-Jones, J. 1980, "Regulation of non-muscle myosin assembly by calmodulin-dependent light chain kinase", *Nature,* vol. 287, no. 5779, pp. 233-235.

Schoolwerth, A.C., Smith, B.C. & Culpepper, R.M. 1988, "Renal gluconeogenesis", *Mineral and Electrolyte Metabolism,* vol. 14, no. 6, pp. 347-361.

Schwarz, K., Simons, M., Reiser, J., Saleem, M.A., Faul, C., Kriz, W., Shaw, A.S., Holzman, L.B. & Mundel, P. 2001, "Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin", *The Journal of Clinical Investigation,* vol. 108, no. 11, pp. 1621-1629.

Sellers, J.R. 2000, "Myosins: a diverse superfamily", *Biochimica et biophysica acta,* vol. 1496, no. 1, pp. 3-22.

Sellers, J.R., Pato, M.D. & Adelstein, R.S. 1981, "Reversible phosphorylation of smooth muscle myosin, heavy meromyosin, and platelet myosin", *The Journal of Biological Chemistry,* vol. 256, no. 24, pp. 13137-13142.

Senn, J.J., Klover, P.J., Nowak, I.A. & Mooney, R.A. 2002, "Interleukin-6 induces cellular insulin resistance in hepatocytes", *Diabetes,* vol. 51, no. 12, pp. 3391-3399.

Sever, S., Altintas, M.M., Nankoe, S.R., Moller, C.C., Ko, D., Wei, C., Henderson, J., del Re, E.C., Hsing, L., Erickson, A., Cohen, C.D., Kretzler, M., Kerjaschki, D., Rudensky, A., Nikolic, B. & Reiser, J. 2007, "Proteolytic processing of dynamin by cytoplasmic cathepsin L is a mechanism for proteinuric kidney disease", *The Journal of Clinical Investigation,* vol. 117, no. 8, pp. 2095-2104.

Sharma, K., Ramachandrarao, S., Qiu, G., Usui, H.K., Zhu, Y., Dunn, S.R., Ouedraogo, R., Hough, K., McCue, P., Chan, L., Falkner, B. & Goldstein, B.J. 2008, "Adiponectin regulates albuminuria and podocyte function in mice", *The Journal of Clinical Investigation,* vol. 118, no. 5, pp. 1645-1656.

Shih, N.Y., Li, J., Cotran, R., Mundel, P., Miner, J.H. & Shaw, A.S. 2001, "CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain", *American Journal of Pathology*, vol. 159, no. 6, pp. 2303-2308.

Shih, N.Y., Li, J., Karpitskii, V., Nguyen, A., Dustin, M.L., Kanagawa, O., Miner, J.H. & Shaw, A.S. 1999, "Congenital nephrotic syndrome in mice lacking CD2-associated protein", *Science,* vol. 286, no. 5438, pp. 312-315.

Shin, K., Fogg, V.C. & Margolis, B. 2006, "Tight junctions and cell polarity", *Annual Review of Cell and Developmental Biology,* vol. 22, pp. 207-235.

Shirato, I., Sakai, T., Kimura, K., Tomino, Y. & Kriz, W. 1996, "Cytoskeletal changes in podocytes associated with foot process effacement in Masugi nephritis", *The American Journal of Pathology,* vol. 148, no. 4, pp. 1283-1296.

Siu, B., Saha, J., Smoyer, W.E., Sullivan, K.A. & Brosius, F.C.,3rd 2006, "Reduction in podocyte density as a pathologic feature in early diabetic nephropathy in rodents: prevention by lipoic acid treatment", *BMC Nephrology,* vol. 7, pp. 6.

Skupien, J., Warram, J.H., Smiles, A.M., Niewczas, M.A., Gohda, T., Pezzolesi, M.G., Cantarovich, D., Stanton, R. & Krolewski, A.S. 2012, "The early decline in renal function in patients with type 1 diabetes and proteinuria predicts the risk of end-stage renal disease", *Kidney International,* vol. 82, no. 5, pp. 589-597.

Smoyer, W.E., Mundel, P., Gupta, A. & Welsh, M.J. 1997, "Podocyte alpha-actinin induction precedes foot process effacement in experimental nephrotic syndrome", *The American Journal of Physiology,* vol. 273, no. 1, pp. 150-157.

Smoyer, W.E. & Ransom, R.F. 2002, "Hsp27 regulates podocyte cytoskeletal changes in an in vitro model of podocyte process retraction", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 16, no. 3, pp. 315-326.

Soldatos, G. & Cooper, M.E. 2008, "Diabetic nephropathy: important pathophysiologic mechanisms", *Diabetes Research and Clinical Practice,* vol. 82, Suppl 1, pp. 75-79.

Sollner, T.H. 2003, "Regulated exocytosis and SNARE function (Review)", *Molecular Membrane Biology,* vol. 20, no. 3, pp. 209-220.

St John, P.L. & Abrahamson, D.R. 2001, "Glomerular endothelial cells and podocytes jointly synthesize laminin-1 and -11 chains", *Kidney International,* vol. 60, no. 3, pp. 1037-1046.

Stall, R., Ramos, J., Kent Fulcher, F. & Patel, Y.M. 2014, "Regulation of myosin IIA and filamentous actin during insulin-stimulated glucose uptake in 3T3-L1 adipocytes", *Experimental Cell Research,* vol. 322, no. 1, pp. 81-88.

Stambe, C., Atkins, R.C., Hill, P.A. & Nikolic-Paterson, D.J. 2003, "Activation and cellular localization of the p38 and JNK MAPK pathways in rat crescentic glomerulonephritis", *Kidney International,* vol. 64, no. 6, pp. 2121-2132.

Steffes, M.W., Schmidt, D., McCrery, R., Basgen, J.M. & International Diabetic Nephropathy Study Group 2001, "Glomerular cell number in normal subjects and in type 1 diabetic patients", *Kidney International,* vol. 59, no. 6, pp. 2104-2113.

Steimle, P.A., Fulcher, F.K. & Patel, Y.M. 2005, "A novel role for myosin II in insulin-stimulated glucose uptake in 3T3-L1 adipocytes", *Biochemical and Biophysical Research Communications,* vol. 331, no. 4, pp. 1560-1565.

Stout, L.C., Kumar, S. & Whorton, E.B. 1994, "Insudative lesions--their pathogenesis and association with glomerular obsolescence in diabetes: a dynamic hypothesis based on single views of advancing human diabetic nephropathy", *Human Pathology,* vol. 25, no. 11, pp. 1213-1227.

Stöckli, J., Fazakerley, D.J. & James, D.E. 2011, "GLUT4 exocytosis", *Journal of Cell Science,* vol. 124, no. 24, pp. 4147-4159.

Stumvoll, M., Chintalapudi, U., Perriello, G., Welle, S., Gutierrez, O. & Gerich, J. 1995, "Uptake and release of glucose by the human kidney. Postabsorptive rates and responses to epinephrine", *The Journal of Clinical Investigation,* vol. 96, no. 5, pp. 2528-2533.

Sugano, M., Yamato, H., Hayashi, T., Ochiai, H., Kakuchi, J., Goto, S., Nishijima, F., Iino, N., Kazama, J.J., Takeuchi, T., Mokuda, O., Ishikawa, T. & Okazaki, R. 2006, "High-fat diet in low-dose-streptozotocin-treated heminephrectomized rats induces all features of human type 2 diabetic nephropathy: a new rat model of diabetic nephropathy", *Nutrition, metabolism, and cardiovascular diseases : NMCD,* vol. 16, no. 7, pp. 477-484.

Suh, J.H. & Miner, J.H. 2013, "The glomerular basement membrane as a barrier to albumin", *Nature Reviews. Nephrology,* vol. 9, no. 8, pp. 470-477.

Susztak, K., Raff, A.C., Schiffer, M. & Bottinger, E.P. 2006, "Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy", *Diabetes,* vol. 55, no. 1, pp. 225-233.

Svitkina, T.M. & Borisy, G.G. 1999, "Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia", *The Journal of Cell Biology,* vol. 145, no. 5, pp. 1009-1026.

Taft, J.L., Nolan, C.J., Yeung, S.P., Hewitson, T.D. & Martin, F.I. 1994, "Clinical and histological correlations of decline in renal function in diabetic patients with proteinuria", *Diabetes,* vol. 43, no. 8, pp. 1046-1051.

Takeda, T., Go, W.Y., Orlando, R.A. & Farquhar, M.G. 2000, "Expression of podocalyxin inhibits cell-cell adhesion and modifies junctional properties in Madin-Darby canine kidney cells", *Molecular Biology of the Cell,* vol. 11, no. 9, pp. 3219-3232.

Takeda, T., McQuistan, T., Orlando, R.A. & Farquhar, M.G. 2001, "Loss of glomerular foot processes is associated with uncoupling of podocalyxin from the actin cytoskeleton", *The Journal of Clinical Investigation,* vol. 108, no. 2, pp. 289-301.

Tay, Y.C., Wang, Y., Kairaitis, L., Rangan, G.K., Zhang, C. & Harris, D.C. 2005, "Can murine diabetic nephropathy be separated from superimposed acute renal failure?", *Kidney International,* vol. 68, no. 1, pp. 391-398.

Tejada, T., Catanuto, P., Ijaz, A., Santos, J.V., Xia, X., Sanchez, P., Sanabria, N., Lenz, O., Elliot, S.J. & Fornoni, A. 2008, "Failure to phosphorylate AKT in podocytes from mice with early diabetic nephropathy promotes cell death", *Kidney International,* vol. 73, no. 12, pp. 1385-1393.

Tesch, G.H. & Allen, T.J. 2007, "Rodent models of streptozotocin-induced diabetic nephropathy", *Nephrology*, vol. 12, no. 3, pp. 261-266.

Theriot, J.A. 1997, "Accelerating on a treadmill: ADF/cofilin promotes rapid actin filament turnover in the dynamic cytoskeleton", *The Journal of Cell Biology,* vol. 136, no. 6, pp. 1165-1168.

Thomas, G., Sehgal, A.R., Kashyap, S.R., Srinivas, T.R., Kirwan, J.P. & Navaneethan, S.D. 2011, "Metabolic syndrome and kidney disease: a systematic review and meta-analysis", *Clinical Journal of the American Society of Nephrology: CJASN,* vol. 6, no. 10, pp. 2364-2373.

Thorn, L.M., Forsblom, C., Fagerudd, J., Thomas, M.C., Pettersson-Fernholm, K., Saraheimo, M., Waden, J., Ronnback, M., Rosengard-Barlund, M., Bjorkesten, C.G., Taskinen, M.R., Groop, P.H. & FinnDiane Study Group 2005, "Metabolic syndrome in type 1 diabetes: association with diabetic nephropathy and glycemic control (the FinnDiane study)", *Diabetes Care,* vol. 28, no. 8, pp. 2019-2024.

Tian, W., Zhang, Z. & Cohen, D.M. 2000, "MAPK signaling and the kidney", *American Journal of Physiology. Renal physiology,* vol. 279, no. 4, pp. 593-604.

Tolonen, N., Forsblom, C., Thorn, L., Waden, J., Rosengard-Barlund, M., Saraheimo, M., Heikkila, O., Pettersson-Fernholm, K., Taskinen, M.R., Groop, P.H. & FinnDiane Study Group 2008, "Relationship between lipid profiles and kidney function in patients with type 1 diabetes", *Diabetologia,* vol. 51, no. 1, pp. 12-20.

Tomas, E., Tsao, T.S., Saha, A.K., Murrey, H.E., Zhang Cc, C., Itani, S.I., Lodish, H.F. & Ruderman, N.B. 2002, "Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 99, no. 25, pp. 16309-16313.

Tong, P., Khayat, Z.A., Huang, C., Patel, N., Ueyama, A. & Klip, A. 2001, "Insulin-induced cortical actin remodelling promotes GLUT4 insertion at muscle cell membrane ruffles", *The Journal of Clinical Investigation,* vol. 108, no. 3, pp. 371-381.

Topham, P.S., Kawachi, H., Haydar, S.A., Chugh, S., Addona, T.A., Charron, K.B., Holzman, L.B., Shia, M., Shimizu, F. & Salant, D.J. 1999, "Nephritogenic mAb 5-1-6 is directed at the extracellular domain of rat nephrin", *The Journal of Clinical Investigation,* vol. 104, no. 11, pp. 1559-1566.

Tsukita, S. & Yonemura, S. 1999, "Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins", *The Journal of Biological Chemistry,* vol. 274, no. 49, pp. 34507-34510.

Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.C. & Tsubokawa, M. 1985, "Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes", *Nature,* vol. 313, no. 6005, pp. 756-761.

Vaheri, A., Carpen, O., Heiska, L., Helander, T.S., Jaaskelainen, J., Majander-Nordenswan, P., Sainio, M., Timonen, T. & Turunen, O. 1997, "The ezrin protein family: membrane-cytoskeleton interactions and disease associations", *Current Opinion in Cell Biology,* vol. 9, no. 5, pp. 659-666.

Vega, I.E. & Hsu, S.C. 2003, "The septin protein Nedd5 associates with both the exocyst complex and microtubules and disruption of its GTPase activity promotes aberrant neurite sprouting in PC12 cells", *Neuroreport,* vol. 14, no. 1, pp. 31-37.

Veis, J.H. 1993, "An overview of mesangial cell biology", *Contributions to Nephrology,* vol. 104, pp. 115-126.

Verma, R., Kovari, I., Soofi, A., Nihalani, D., Patrie, K. & Holzman, L.B. 2006, "Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization", *The Journal of Clinical Investigation,* vol. 116, no. 5, pp. 1346-1359.

Viberti, G.C., Bilous, R.W., Mackintosh, D. & Keen, H. 1983, "Monitoring glomerular function in diabetic nephropathy. A prospective study", *American Journal of Medicine*, vol. 74, pp. 256-264.

Viberti, G.C., Jarrett, R.J. & Keen, H. 1982, "Microalbuminuria as prediction of nephropathy in diabetics", *Lancet,* vol. 2, no. 8298, pp. 611.

Vicente-Manzanares, M., Ma, X., Adelstein, R.S. & Horwitz, A.R. 2009, "Non-muscle myosin II takes centre stage in cell adhesion and migration", *Nature reviews. Molecular Cell Biology,* vol. 10, no. 11, pp. 778-790.

Vinciguerra, M. & Foti, M. 2006, "PTEN and SHIP2 phosphoinositide phosphatases as negative regulators of insulin signalling", *Archives of Physiology and Biochemistry,* vol. 112, no. 2, pp. 89-104.

Wang, Y., Heilig, K., Saunders, T., Minto, A., Deb, D.K., Chang, A., Brosius, F., Monteiro, C. & Heilig, C.W. 2010, "Transgenic overexpression of GLUT1 in mouse glomeruli produces renal disease resembling diabetic glomerulosclerosis", *American Journal of Physiology. Renal Physiology,* vol. 299, no. 1, pp. 99-111.

Ward, S.M., Weins, A., Pollak, M.R. & Weitz, D.A. 2008, "Dynamic viscoelasticity of actin cross-linked with wild-type and disease-causing mutant alpha-actinin-4", *Biophysical Journal,* vol. 95, no. 10, pp. 4915-4923.

Wartiovaara, J., Ofverstedt, L.G., Khoshnoodi, J., Zhang, J., Makela, E., Sandin, S., Ruotsalainen, V., Cheng, R.H., Jalanko, H., Skoglund, U. & Tryggvason, K. 2004, "Nephrin strands contribute to a porous slit diaphragm scaffold as revealed by electron tomography", *The Journal of Clinical Investigation,* vol. 114, no. 10, pp. 1475-1483.

Watson, R.T., Kanzaki, M. & Pessin, J.E. 2004, "Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes", *Endocrine Reviews,* vol. 25, no. 2, pp. 177-204.

Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H. & Rothman, J.E. 1998, "SNAREpins: minimal machinery for membrane fusion", *Cell,* vol. 92, no. 6, pp. 759-772.

Weins, A., Schlondorff, J.S., Nakamura, F., Denker, B.M., Hartwig, J.H., Stossel, T.P. & Pollak, M.R. 2007, "Disease-associated mutant alpha-actinin-4 reveals a mechanism for regulating its F-actin-binding affinity", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 104, no. 41, pp. 16080-16085.

Welsh, G.I., Hale, L.J., Eremina, V., Jeansson, M., Maezawa, Y., Lennon, R., Pons, D.A., Owen, R.J., Satchell, S.C., Miles, M.J., Caunt, C.J., McArdle, C.A., Pavenstadt, H., Tavare, J.M., Herzenberg, A.M., Kahn, C.R., Mathieson, P.W., Quaggin, S.E., Saleem, M.A. & Coward, R.J. 2010, "Insulin signaling to the glomerular podocyte is critical for normal kidney function", *Cell Metabolism,* vol. 12, no. 4, pp. 329-340.

White, K.E., Bilous, R.W. & Diabiopsies Study Group 2004, "Structural alterations to the podocyte are related to proteinuria in type 2 diabetic patients", *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association,* vol. 19, no. 6, pp. 1437-1440.

White, K.E., Bilous, R.W., Marshall, S.M., El Nahas, M., Remuzzi, G., Piras, G., De Cosmo, S. & Viberti, G. 2002, "Podocyte number in normotensive type 1 diabetic patients with albuminuria", *Diabetes,* vol. 51, no. 10, pp. 3083-3089.

Wirthensohn, G. & Guder, W.G. 1986, "Renal substrate metabolism", *Physiological Reviews,* vol. 66, no. 2, pp. 469-497.

Wiseman, M.J., Saunders, A.J., Keen, H. & Viberti, G. 1985, "Effect of blood glucose control on increased glomerular filtration rate and kidney size in insulin-dependent diabetes", *The New England Journal of Medicine,* vol. 312, no. 10, pp. 617-621.

Woerle, H.J., Popa, E., Dostou, J., Welle, S., Gerich, J. & Meyer, C. 2002, "Exogenous insulin replacement in type 2 diabetes reverses excessive hepatic glucose release, but not excessive renal glucose release and impaired free fatty acid clearance", *Metabolism: Clinical and Experimental,* vol. 51, no. 11, pp. 1494-1500.

Wolf, G., Chen, S. & Ziyadeh, F.N. 2005, "From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy", *Diabetes,* vol. 54, no. 6, pp. 1626-1634.

Yaddanapudi, S., Altintas, M.M., Kistler, A.D., Fernandez, I., Moller, C.C., Wei, C., Peev, V., Flesche, J.B., Forst, A.L., Li, J., Patrakka, J., Xiao, Z., Grahammer, F., Schiffer, M., Lohmuller, T., Reinheckel, T., Gu, C., Huber, T.B., Ju, W., Bitzer, M., Rastaldi, M.P., Ruiz, P., Tryggvason, K., Shaw, A.S., Faul, C., Sever, S. & Reiser, J. 2011, "CD2AP in mouse and human podocytes controls a proteolytic program that regulates cytoskeletal structure and cellular survival", *The Journal of Clinical Investigation,* vol. 121, no. 10, pp. 3965-3980.

Yamada, E., Saito, T., Okada, S., Takahashi, H., Ohshima, K., Hashimoto, K., Satoh, T., Mori, M., Okada, J. & Yamada, M. 2014, "Synip phosphorylation is required for insulin-stimulated Glut4 translocation and glucose uptake in podocyte", *Endocrine Journal,* vol. 61, no. 5, pp. 523-527.

Yang, M., Zhang, Y., Pan, J., Sun, J., Liu, J., Libby, P., Sukhova, G.K., Doria, A., Katunuma, N., Peroni, O.D., Guerre-Millo, M., Kahn, B.B., Clement, K. & Shi, G.P. 2007, "Cathepsin L activity controls adipogenesis and glucose tolerance", *Nature Cell Biology,* vol. 9, no. 8, pp. 970-977.

Yerneni, K.K., Bai, W., Khan, B.V., Medford, R.M. & Natarajan, R. 1999, "Hyperglycemia-induced activation of nuclear transcription factor kappaB in vascular smooth muscle cells", *Diabetes*, vol. 48, no. 4, pp. 855-864.

Yip, J., Mattock, M.B., Morocutti, A., Sethi, M., Trevisan, R. & Viberti, G. 1993,"Insulin resistance in insulin-dependent diabetic patients with microalbuminuria", *Lancet,* vol. 342, no. 8876, pp. 883-887.

Yonezawa, N., Nishida, E. & Sakai, H. 1985, "pH control of actin polymerization by cofilin", *The Journal of Biological Chemistry,* vol. 260, no. 27, pp. 14410-14412.

Zhang, H., Saha, J., Byun, J., Schin, M., Lorenz, M., Kennedy, R.T., Kretzler, M., Feldman, E.L., Pennathur, S. & Brosius, F.C., 3rd 2008, "Rosiglitazone reduces renal and plasma markers of oxidative injury and reverses urinary metabolite abnormalities in the amelioration of diabetic nephropathy", *American Journal of Physiology. Renal Physiology,* vol. 295, no. 4, pp. F1071-81.

Zhang, H., Schin, M., Saha, J., Burke, K., Holzman, L.B., Filipiak, W., Saunders, T., Xiang, M., Heilig, C.W. & Brosius, F.C., 3rd 2010, "Podocyte-specific overexpression of GLUT1 surprisingly reduces mesangial matrix expansion in diabetic nephropathy in mice", *American Journal of Physiology. Renal Physiology,* vol. 299, no. 1, pp. F91-8.

Zhang, Y., Conti, M.A., Malide, D., Dong, F., Wang, A., Shmist, Y.A., Liu, C., Zerfas, P., Daniels, M.P., Chan, C.C., Kozin, E., Kachar, B., Kelley, M.J., Kopp, J.B. & Adelstein, R.S. 2012, "Mouse models of MYH9-related disease: mutations in nonmuscle myosin II-A", *Blood,* vol. 119, no. 1, pp. 238-250.

Zhu, J., Sun, N., Aoudjit, L., Li, H., Kawachi, H., Lemay, S. & Takano, T. 2008, "Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes", *Kidney International,* vol. 73, no. 5, pp. 556-566.

Zigmond, S.H. 1996, "Signal transduction and actin filament organization", *Current Opinion in Cell Biology,* vol. 8, no. 1, pp. 66-73.

Zimmet, P., Alberti, K.G. & Shaw, J. 2001, "Global and societal implications of the diabetes epidemic", *Nature,* vol. 414, no. 6865, pp. 782-787.