PODOCYTE PATHOLOGY AND NEPHROPATHY

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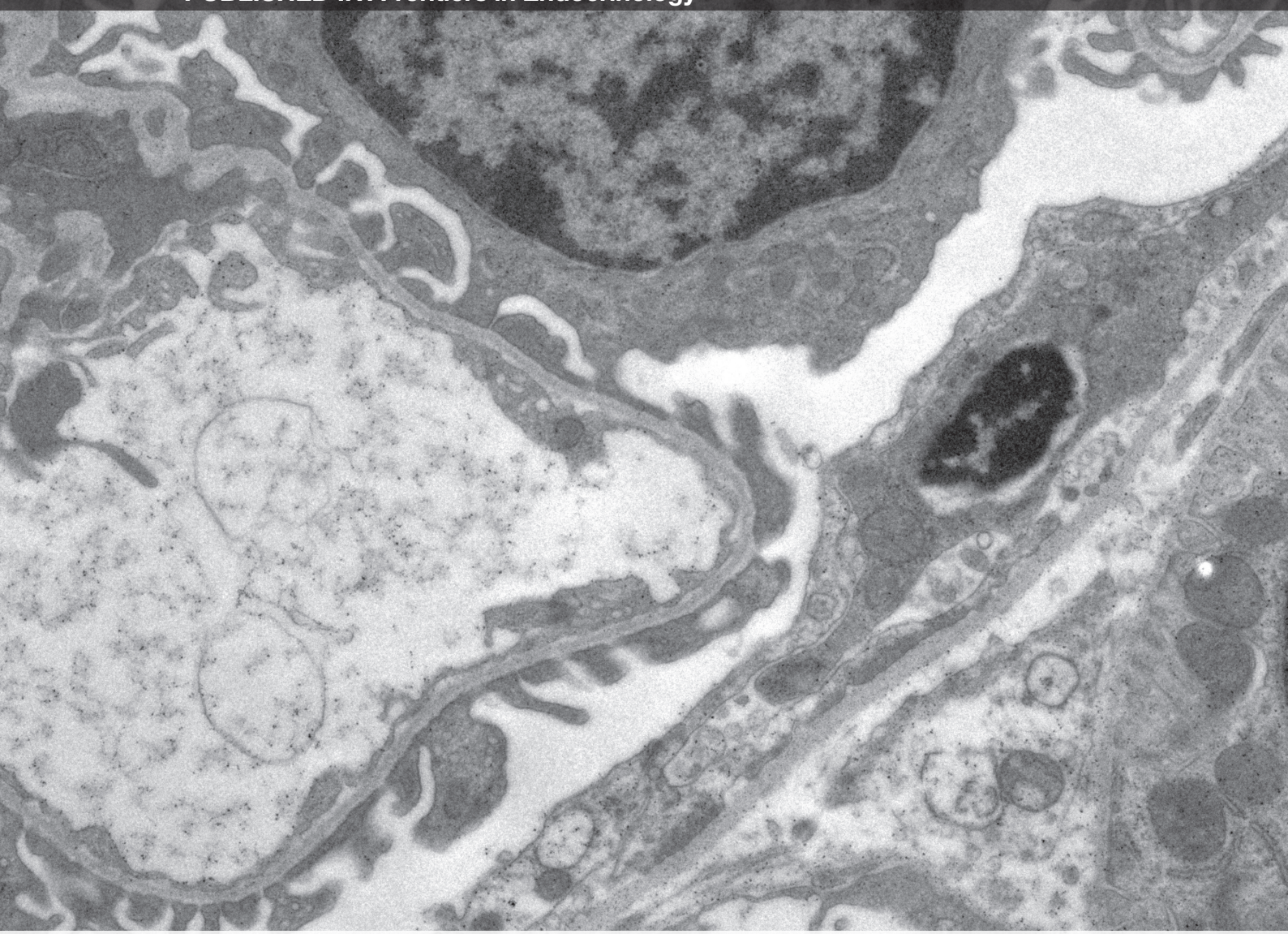

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PODOCYTE PATHOLOGY AND NEPHROPATHY

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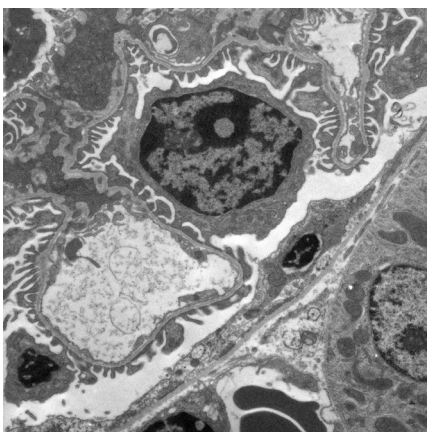

Electron micrograph showing a section through the edge of a mouse glomerulus with a peripheral Bowman's capsule. Centre stage is a podocyte with 8 cytoplasmic processes holding the cell body and its darkly stained nucleus onto the basement membrane of two glomerular capillaries. The basement membrane shows additional profiles of podocyte foot processes forming the glomerular filtration barrier of the capillary wall. The clear space surrounding the podocyte and up to the capsular edge is urinary space which collects filtrate from the capillaries. Image courtesy of Dr Chris Neal and Dr Louise Farmer, Bristol Renal, University of Bristol.

The understanding of the pathogenesis of diabetic nephropathy (DN) has advanced considerably in the last few years. Much has been learned about the natural history, the relative lack of significance of microalbuminuria in reflecting underlying pathological change, questionable effects of ACEs and ARBs on the progression of nephropathy, the emergence of new biomarkers such as Cystatin and the role of cytokines, inflammatory molecules and adhesion molecules.

Podocytes, the cells with limited ability to replenish and to repair, play a pivotal role in glomerular filtration. In recent years these cells have become the focus for research on pathogenesis of DN as well as other nephropathies. A recent review from the NIH has identified new insights into the pathophysiology, the genetics and the role of the podocytes and some of the important new metabolic pathways such as mTOR or autophagy which may be targeting the podocyte. Knowledge is emerging about the role of podocyte as a part of immune system and about the role of growth factors and cytokines in regulation of podocyte functions.

Presented in this e-book articles highlight recent advances in our understanding of the pathogenesis of kidney pathology and the role of podocytes in this process.

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EDITORIAL
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Editorial: Podocyte pathology and nephropathy

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Keywords: podocytes, diabetes, kidney disease, proteinuria, foot processes, exosomes

Podocytes, glomerular visceral cells of epithelial origin, are the direct target of both immune and non-immune forms of injury in many diverse glomerular diseases. They do not typically proliferate in the mature kidney, which means that damaged cells are not replaced by new ones. It is now generally accepted that dysfunction or loss of these cells underlies progression of almost all glomerulopathies. Progressing glomerular diseases, of which diabetic nephropathy (DN) is the most common, are the most frequent cause of end-stage renal disease. Therefore, identification of the pathways that lead to podocyte injury is essential for developing more effective, targeted therapies. The articles presented in this research topic give a comprehensive overview of recent discoveries into the mechanisms of podocyte impairment and discuss the possibilities of podocyte-targeted therapies.

Each podocyte contacts its neighboring cells via a slit diaphragm (SD) connecting their interdigitating foot processes (FPs). Via transmembrane adhesion receptors in the basolateral domain of the FP, the podocytes interact with the glomerular basement membrane (GBM). Both these forms of adhesions are crucial for maintaining podocyte structure, which in turn determines the integrity and permeability of the glomerular filtration barrier. As highlighted by Lennon et al. (1), the cell–cell and cell–matrix junctions drive the coordinated response of podocytes to environmental cues in order to regulate glomerular filtration. Recent findings indicate that there are two distinct FP types, anchoring FPs (AFPs) and ordinary FPs (OFPs), both of which are involved in the regulation of fluid outflow from the subpodocyte space (2). The components of SD and adhesion complexes in FPs transduce signals from outside of the podocyte to the actin cytoskeleton inside the cell. Up to now, almost 100 actin associated proteins have been discovered in mammalian podocytes, with distribution specific for apical, SD, and basal domains of the FP membrane (2). Dysregulation of signaling is likely to lead to actin reorganization and podocyte foot process effacement, which is typically observed in proteinuric diseases. In contrast to traditional interpretations of this loss of shape as a pathological derangement, Kriz et al. (3) indicate that adhesions are reinforced in effaced FPs and therefore it seems to be a protective mechanism against detachment. Alterations in podocyte phenotype and structure are particularly prominent when proteinuria reaches nephrotic range. It is accepted that loss of the specialized podocyte morphology is associated with transition from epithelial to a more mesenchymal phenotype irrespective of the underlying causes that include both genetic defects and mediators from the microenvironment. However, May et al. (4) note that podocytes display partial features of both mesenchymal and epithelial cells. Therefore, dependent on the clinical conditions, dedifferentiation in disease could result in regression to either of these states. Upon treatment, these changes are reversible only if the insult is not very severe, as for example in minimal change disease (MCD). In focal segmental glomerulosclerosis (FSGS), phenotypic dedifferentiation of podocytes is not only irreversible but progressive.

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Using a rat model, Kriz et al. (3) have performed a detailed structural study demonstrating how podocytes reinforce attachment to the GBM and how they detach. It appears that if the protective mechanisms fail, viable podocytes, mostly in clusters, detach from the GBM. Some of them may reach the renal pelvis as living cells, while other may develop contacts to the parietal epithelium, forming crescents that connect glomerular capillaries with the Bowman's capsule. Podocyte depletion represents one of the earliest cellular lesions affecting the diabetic kidney, and decreased number of podocytes in glomeruli is the strongest predictor of progression of both type 1 and 2 DN. Activation of protein kinase C (PKC) seems to play a critical role in pathogenesis of DN. Teng et al. (5) point out that conventional as well as atypical forms of PKC, which play a pivotal role in the regulation of podocyte physiology, may be a destructive factor when hyperactivated in disease conditions. PKC activation results in downregulation of podocyte and SD structural proteins such as P-cadherin or β-catenin, which may contribute to the disruption of podocyte integrity. In DN, the PKC isoforms may also mediate the high glucose-induced overproduction of VEGF and increased TGFβ signaling in podocytes, with subsequent impairment of the glomerular filtration barrier. Recent findings reveal that abnormal intracellular accumulation of sphingolipids modulates podocyte functions in glomerular disorders of both genetic and non-genetic origin. Based on their experimental results, Merscher and Fornoni (6) report that in FSGS, suPAR-dependent αVβ3 integrin activation decreased expression of sphingomyelin-like phosphodiesterase 3b (SMPDL3b) resulting in increased accumulation of sphingomyelin, which is associated with remodeling of the podocyte actin cytoskeleton, loss of stress fibers, and a shift from a migratory to an apoptotic phenotype. In contrast, in the diabetic kidney SMPDL3b expression was elevated, nonetheless rendering podocytes more susceptible to apoptosis. These observations indicate that podocyte responses to sphingolipids are complex and require additional research.

Hereditary, but also sporadic, nephrotic syndrome (NS) is frequently associated with mutations in podocyte genes encoding functional and structural proteins. However, currently known mutations explain <40% of NS cases (7). Moreover, immunosuppression appears to be effective in about 8–10% of genetic disorders. Recent advances in our knowledge about the podocyte transcriptosome and proteasome have led to identification and characterization of novel disease-causing variants and disease-modifying genes. At present, almost 50 podocyte genes directly associated with human NS have been identified. It has also become apparent that monogenic defects correlating with some morphological changes in podocytes do not explain completely the pathogenesis of congenital podocytopathies.

An understanding of the mechanisms of podocyte impairment will allow for the design of targeted therapeutic approaches that may prevent deterioration of glomerular function, e.g., in allograft recipients. The frequency of recurrent as well as newly developed post-transplant kidney diseases, such as DN, is relatively high and now it is clear that podocytes are the initial site of injury. Detailed pathogenesis of post-transplant diabetes is not known; however, numerous risk factors have been identified (8). Although direct treatment of genetic disorders still remains a question for the future, certain biochemical pathways in podocytes already seem to be a promising target for current therapies. For example, it has been shown recently that SMPDL3b in podocyte lipid rafts is a direct target for rituximab, which prevents downregulation of this protein and podocyte injury (7). Furthermore, calcineurin inhibitors (CNIs) that, together with glucocorticoids and mTOR inhibitors, have been traditionally used as anti-inflammatory agents have been shown to also act directly on podocytes (9). Diverse actions of CNIs on podocytes include stabilizing the actin cytoskeleton and inhibiting NFAT-dependent podocyte apoptosis. Other immunosuppressive drugs, such as abatacept or belatacept, also directly target the podocytes, suggesting this is an exciting area for future research (8).

Early, sensitive, and specific diagnosis of ongoing podocyte injury is still lacking. Microalbuminuria, which results from podocyte dysfunction, has been accepted as the earliest marker of DN. However, patients presenting with microalbuminuria already show advanced damage of the glomerular filtration barrier (10), while nephrinuria may precede this manifestation (11). It has been shown recently that urinary podocyte damage biomarkers correlate with cystatin C and eGFR even in normoalbuminuric patients with type 2 diabetes (11). Yet, due to technical problems, such as low quantities of podocyte proteins or their proteolytic digestion, using free urinary podocyte proteins as diagnostic markers appears to be questionable. Musante et al. (12) describe a promising new method in which exosomes carrying specific podocyte biomarkers are isolated from urine. In one pilot study, the levels of urinary exosomal mRNA for cystatin C showed a marked upregulation after the induction of podocyte damage in the puromycin aminonucleoside nephrosis (PAN) rat model (13). However, this was accompanied by the *de novo* expression of cystatin C not only in podocytes but also in tubular epithelial cells. On the other hand, in an experimental model of DN, which is associated with podocyte injury, cystatin C immunoreactivity in tubules was not changed (14). So far, there are only few studies referring to the urinary exosomal mRNA, and data concerning levels and cellular origin of cystatin C mRNA in urine are still missing. Nevertheless, correlation between cystatin C and podocyte-specific genes in urinary exosomes could probably allow for early specific diagnosis of podocyte damage prior to the symptomatic podocytopathy.

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References

1. Lennon R, Randles MJ, Humphries MJ. The importance of podocyte adhesion for a healthy glomerulus. *Front Endocrinol* (2014) **5**:160. doi:10.3389/fendo.2014.00160
2. Neal CR. Podocytes . What's under yours? (podocytes and foot processes and how they change in nephropathy). *Front Endocrinol* (2015) **6**:9. doi:10.3389/fendo.2015.00009
3. Kriz W, Hahnel B, Hosser H, Rosener S, Waldherr R. Structural analysis of how podocytes detach from the glomerular basement membrane under

hypertrophic stress. *Front Endocrinol* (2014) **5**:207. doi:10.3389/fendo.2014.00207
4. May CJ, Saleem M, Welsh GI. Podocyte dedifferentiation: a specialized process for a specialized cell. *Front Endocrinol* (2014) **5**:148. doi:10.3389/fendo.2014.00148
5. Teng B, Duong M, Tossidou I, Yu X, Schiffer M. Role of protein kinase C in podocytes and development of glomerular damage in diabetic nephropathy. *Front Endocrinol* (2014) **5**:179. doi:10.3389/fendo.2014.00179
6. Merscher S, Fornoni A. Podocyte pathology and nephropathy – sphingolipids in glomerular diseases. *Front Endocrinol* (2014) **5**:127. doi:10.3389/fendo.2014.00127
7. Bierzynska A, Soderquest K, Koziell A. Genes and podocytes – new insights into mechanisms of podocytopathy. *Front Endocrinol* (2014) **5**:226. doi:10.3389/fendo.2014.00226
8. Peev V, Reiser J, Alachkar N. Diabetes mellitus in the transplanted kidney. *Front Endocrinol* (2014) **5**:141. doi:10.3389/fendo.2014.00141
9. Spurney RF. Non-immunologic actions of calcineurin inhibitors in proteinuric kidney diseases. *Front Endocrinol* (2014) **5**:181. doi:10.3389/fendo.2014.00181
10. Glassock RJ. Is the presence of microalbuminuria a relevant marker of kidney disease? *Curr Hypertens Rep* (2010) **12**(5):364–8. doi:10.1007/s11906-010-0133-3
11. Petrica L, Vlad A, Gluhovschi G, Gadalean F, Dumitrascu V, Gluhovschi C, et al. Proximal tubule dysfunction is associated with podocyte damage biomarkers nephrin and vascular endothelial growth factor in type 2 diabetes mellitus patients: a cross-sectional study. *PLoS One* (2014) **9**(11):e112538. doi:10.1371/journal.pone.0112538
12. Musante L, Tataruch DE, Holthofer H. Use and isolation of urinary exosomes as biomarkers for diabetic nephropathy. *Front Endocrinol* (2014) **5**:149. doi:10.3389/fendo.2014.00149
13. Spanu S, van Roeyen CR, Denecke B, Floege J, Muhlfeld AS. Urinary exosomes: a novel means to non-invasively assess changes in renal gene and protein expression. *PLoS One* (2014) **9**(10):e109631. doi:10.1371/journal.pone.0109631
14. Togashi Y, Miyamoto Y. Urinary cystatin C as a biomarker for diabetic nephropathy and its immunohistochemical localization in kidney in Zucker diabetic fatty (ZDF) rats. *Exp Toxicol Pathol* (2013) **65**(5):615–22. doi:10.1016/j.etp.2012.06.005

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REVIEW ARTICLE
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Podocytes . . . What's under yours? (Podocytes and foot processes and how they change in nephropathy)

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Most of the described structures of podocytes in health and disease have been inferred from light and electron microscopic studies of rodent models. The variation in filtration barrier features is measured on micrographs, the aim being statistical significance. This is the technical campaign waged against kidney disease but this approach can be misleading. The signaling cascades and connectivity of the podocyte and foot processes (FPs) are inferred from *in vitro* studies that at best blurr the reality of the *in vivo* state. This review will outline actin signaling connectivity and the key differences in the structural and functional domains squeezed into the FPs and the relationship of these domains to other parts of the podocyte. It covers the changes in podocytes during nephropathy concentrating on FP and finally proposes an alternative interpretation of FP ultrastructure derived from articles published over the last 60 years.

Keywords: podocyte foot processes, nephropathy, glomerular filtration barrier, subpodocyte space, podocyte cytoskeleton, actin cytoskeleton

INTRODUCTION

The podocyte has an intrinsic part to play in forming and maintaining the glomerular filtration barrier (GFB), but the relevance of the various structural components of the GFB in disease is complex (**Figure 1**). For instance, the glomerular basement membrane (GBM) not only serves as a barrier to protein *in vivo* but also requires the slit diaphragm (SD) to prevent albumin passage from the capillary lumen into urinary space (1) (**Figure 2A**). In addition to SDs, the glycocalyx overlying the endothelial cells restricts macromolecular passage and ensures that plasma albumin is largely excluded from the GFB (2). However, it is mutations of SD proteins that are strongly linked to the occurrence of proteinuria (3–5), on this basis SDs are assumed to be the weakest point. The GBM after development is maintained by the podocyte with dysfunction leading to GBM disruption. However, changes in the underlying GBM can lead to podocyte dysfunction, which comes first in disease is unclear.

This review will outline the current view on podocyte structure focusing on the podocyte foot processes (FPs) with an overview of associated FP actin signaling and connectivity. Additionally, regulation and instigation of structural changes associated with disease states will be outlined. Finally, alternative interpretations of FP structure and FP changes will be advanced based on the presence of a subpodocyte space (SPS) (**Figure 1**).

"NORMAL" PODOCYTE STRUCTURE

Podocyte ultrastructure has been investigated for over half a century (6) with the GFB model being developed continuously (7). The urinary space of the glomerulus has been described as free to the movement of fluid and small solutes with the GFB being the single fluid resistance between the blood and urinary spaces (8).

A large number of reviews and articles published in the scientific literature cover the current description of normal podocyte structure. Briefly, the cell body has many major processes (MP) attached to it, primary, then secondary, and some tertiary MPs branching from the cell body. FPs emerge at right angles to the MPs and make contact with the underlying GBM (9) (**Figures 1** and **2A**).

Foot processes from neighboring podocytes interdigitate with each other forming a SD with its heavy investment of specific proteins and structures (10). The importance of podocyte SD structure is highlighted by exact homologs in insect nephrocyte SDs employing similar conserved proteins for hemolymph filtration (11). The highly conserved structure and function between insects and vertebrates illustrates that nephrocytes and podocytes are from a very old cell lineage having evolved on filtration barriers *at least* since the vertebrate/insect last common ancestor over half a billion years ago (11).

PODOCYTE CYTOSKELETON

Aside from the longitudinally oriented actin bundles in the central apical part of FPs, there are three membrane associated domains (**Figure 2A**). A sub-plasmalemmal network of cortical branched actin filaments connects the SD domains (SDD), the basal domain (BD), and the apical domain (AD) to the actin bundle (12, 13). At the end of FP adjoining major processes (MPs), actin bundles connect to MP intermediate filaments and microtubules running back to the central cell body (14–17) (**Figures 2A,B**). The complete FP cytoskeleton is reported as having the components necessary to oppose the forces of a high pressure distensible glomerular capillary wall (18–20). The FP actin network should not be confused with MP and cell body actin stress fibers observed in both *in vitro* and *in vivo* podocytes (21).

Podocyte actin filaments organized into stress fibers are in contrast to the FP actin network attached to the cell membrane thru focal adhesions (18). They regulate cell motility and any sustained

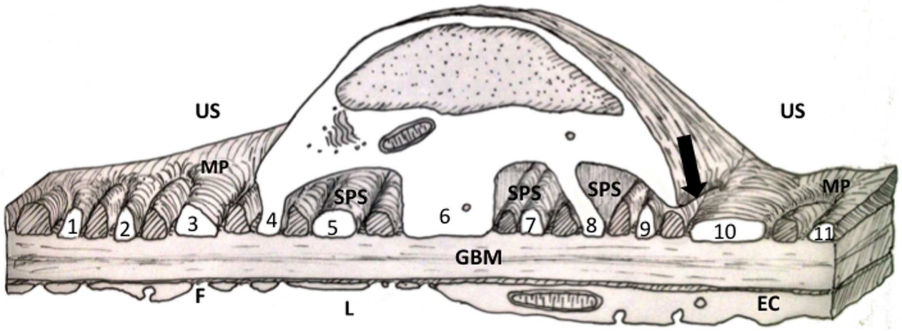

FIGURE 1 | Diagram of a thick section through the cell body of a podocyte showing foot processes (FPs). FPs arising from the two major processes (MP) in the diagram are labeled with numbers. Other FPs (cross hatched) interdigitating with the numbered FPs would have emerged from the MP of a podocyte neighbor (for further discussion of this figure see the text). EC, endothelial cell; F, fenestration; GBM, glomerular basement membrane; MP, major process; L, capillary lumen; US, urinary space; SPS, subpodocyte space.

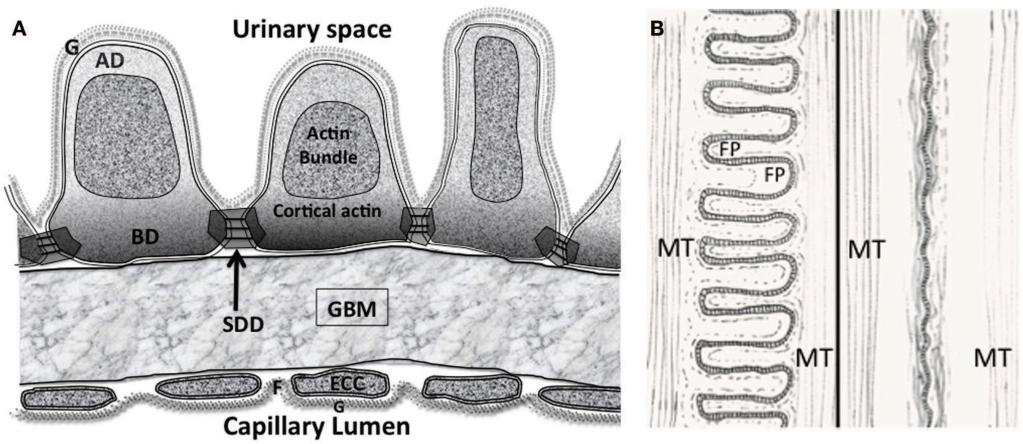

FIGURE 2 | (A) The glomerular filtration barrier (GFB) with the disposition of membrane associated domains in foot processes (FPs). Diagram shows three FPs with apical domain (AD), basal domain (BD), and slit diaphragm domains (SDD) outlined. The apical actin bundles are shown, other actin networks cortical actin (sub-plasmalemmal actin) surrounding the actin bundle are attached to the various membrane domains. The cortical actin meshwork in BD and SDD is thicker than AD. ECC, endothelial cell cytoplasm; F, fenestration; G, glycocalyx. **(B)** Interdigitating foot process (FP) pattern in health (left) and after effacement in disease (right). The dashed line in FP marks the boundary of the actin bundles. MT – microtubules in the major processes. Actin bundles and cortical actin either reduce to a diffuse band of actin after effacement or disperse with effacement.

cell body contractions, the structure is similar to that of sarcomeres in myocytes with myosin filaments, α-actinin, and regulators like tropomyosin (22). Tropomyosin is only found in the cell body of podocytes the equivalent regulator in FP actin networks being synaptopodin (23).

Small GTPase Rho associated protein kinase ROCK as well as intracellular calcium levels are the main upstream regulators of myosin activity. Rho and calcium pathways regulate actin stress fiber dynamics of the cell body (24) as well as the cortical actin and actin bundles of FP but with different regulators (see above).

Microtubules and intermediate filaments are not part of FP structure. Differentiated podocytes *in vivo* have a mesenchymal intermediate filament pattern with the cell body and MPs expressing vimentin and desmin (17, 25, 26) and intermediate filament associated proteins plectin and p250 protein (27, 28).

ENDOCYTOSIS AND EXOCYTOSIS IN FOOT PROCESSES

While endothelial cells and podocytes produce the GBM in development, the fully differentiated podocyte assembles and secretes matrix components into the GBM via the BD. This maintains the GBM meshwork of collagen IV, laminin, fibronectin, entactin, agrin, and perlecan (29). Cargos from production sites closer to the cell body (Golgi apparatus, rough ER) necessarily require transport to FPs via MP microtubules (see below). Endocytotic transmembrane proteins exist along the BD membrane (e.g., megalin) and motor proteins that bind membranes to actin such as myo1e are recruited in clathrin endocytosis (30). Ultimately, these cargoes will require transport to the cell body.

MAJOR PROCESS MICROTUBULAR TRANSPORT

The tubulin subunits form a stiff 24 nm-thick tubular structure along the length of MPs and connect the cell body with FP actin

networks. The minus-ends of microtubules can be located in the cell body microtubule organizing center with the plus-end (fast growing) at the cell periphery, resulting in a neuronal "plus-end-distal" orientation (31). However, podocyte microtubules are orientated both ways, allowing transport and elongation in either direction along MPs (16, 32). Central to distal cargo transport was shown with movements of virus (33) and also vesicular cargos were moved from a Golgi apparatus under the control of rab8 (34), another small GTPase.

Evidence of distal to central movements has been seen with Wilms tumor 1 interacting protein (WT1P) an actin associated protein (AAP) in FP, which translocates from the SDD to the nucleus via dynein-microtubule transporters (35) [Stress signaling initiates loss of WTIP from SDD and suggests a mechanism that transmits changes in podocyte morphology to the nucleus (35)]. The genes coding for Nephrin (SDD) and podocalyxin (AD) are dramatically downregulated in mice with decreased levels of WT1 gene expression (36).

The CHO1/MKLP1 (kinesin superfamily motor protein) is responsible for elongation of minus-end-distal microtubules in podocyte processes (16). Microtubular associated protein 4 (MAP4) could be another microtubule forming molecule in podocytes (37) with phosphorylation slowing microtubule assembly along the process.

FOOT PROCESS ACTIN CONNECTIVITY

An ever increasing number of actin regulators adapters and associated proteins are being discovered around the cortical actin of the FP membrane domains and around the apical actin bundle (see **Box 1**). The distribution of FP proteins around the different domains is crucial with different biochemistries setting different signaling. Some of the major interactions with actin involved in FP structural change are described below and in **Figure 3** [for a fuller description of the protein complexes see references (13, 38, 39)]. The type of actin interaction by the final signal molecule in the pathway is also described in **Figure 3**.

SLIT DIAPHRAGM DOMAIN ACTIN COUPLING IN FPs

In the SD region cortical (sub-plasmalemmal), actin is connected to the molecules of the SD complex in the membrane (SDD **Figure 2A**). ZO-1 (47), catenins (48), CD2AP (49), and podocin (50) and the like serve as adapter molecules between the slit membrane molecules nephrin, Neph, and P-cadherin and actin microfilaments. α-actinin 4 acts as a particularly busy signaling node (**Figure 3A**), it is notably abundant in podocyte FPs and colocalizes with all actin associated with the FPs (51). Arp2/3 also represents a prominent signaling node. Other classical actin binding proteins function in different modes – such as the non-muscle myosin Myo1e, which signals between synaptopodin and actin and Myo1c interacts directly with nephrin and Neph1 ensuring insertion in the SDD (38, 39).

APICAL DOMAIN ACTIN COUPLING IN FPs

In the AD (**Figure 3C**), Syndecans I and IV, podocalyxin GLEPP1, and podoendin are major contributors to the surface negative charge on the apical surface (52–54), which is said to repel proteins and act as a spacer molecule preventing FPs from getting too close (55). Compared to the SDD, less is known about actin signaling in the AD. Podocalyxin is joined to the sub-plasmalemmal actin system via NHERF2 and ezrin (56) podocalyxin complex disruption results in changes in FP structure (57). Podocalyxin null mice fail to form FPs (58); even though these changes are in an apical position away from the SDD and BD.

BASAL DOMAIN ACTIN COUPLING IN FPs

A thicker meshwork of actin is found in the BD facing the GBM and also the lateral SDD actin network. In the BD of FPs (**Figure 3B**) dystroglycan connects actin to agrin in the GBM independently of α-actinin 4 but a large part of the signaling again centers around α-actinin 4. Megalin, a transmembrane endocytotic receptor glycoprotein links to actin via α-actinin 4 and synaptopodin (59, 60) and the integrin complex (5) acts through α actinin 4.

Signaling from the GBM to the integrins can regulate actin dynamics in outside-in signaling (OIS; **Figure 3B**) but inside-out signaling (IOS; **Figure 3B**) also occurs where actin signaling to the integrin complex alters integrin adhesiveness. Inside-out IOS signaling can also signal via arp2/3 and vinculin (**Figure 3B**) (61).

PROMINENT FP SIGNALING NODES

Common signaling components of the three domains are highlighted in black throughout **Figure 3**. These are seen as prominent nodes and important in the different signaling pathways of the

Box 1 | Podocyte actin associated proteins.

The cytoskeletal research impetus has been on the actin cytoskeleton since the only identified mutants in patients have all been based around this part of the cytoskeleton and no mutations have been identified so far in microtubule or intermediate filament associated genes (40). Any disruption of microtubule function with colchicine or vinblastine did not appear to alter FP dynamics in the short term (41) (although the trafficking of cargoes between the cell body and the GBM should result in GBM disruption in the longer term). Any proteinuric kidney disease that can not be traced to podocytic actin filament disruption is traceable to slit diaphragm change (42).

Podocyte actin research momentum has revealed a huge number of actin associated proteins. A list compiled by Faul showed that 94 actin associated proteins (AAPs) have been discovered in cultured mammalian podocytes up to 2013. This can be narrowed down to a list of 26 that have mutations associated with human disease or genetic modification resulting in experimental proteinuria (43). How these AAPs fully interact with the individual podocyte actin domains and networks especially in the FPs remains to be determined.

The list is ever growing: two further podocyte cytoskeletal proteins have been found upregulated in the FPs of puromycin aminonucleoside nephropathy (PAN) rats and also human disease (44). Survivin (Birc5 gene) colocalizes with synaptopodin and is an actin binder, survivin knockdown with siRNA rearranges the actin cytoskeleton (45). Another potential is Arc/Arg3.1 (Activity regulated cytoskeleton associated protein), which acts as a crucial mediator for actin polymerization in other cells. Its possible Arc is associated with podocyte endocytotic control, weirdly, the gene was upregulated but not the protein in PAN rats (44, 46).

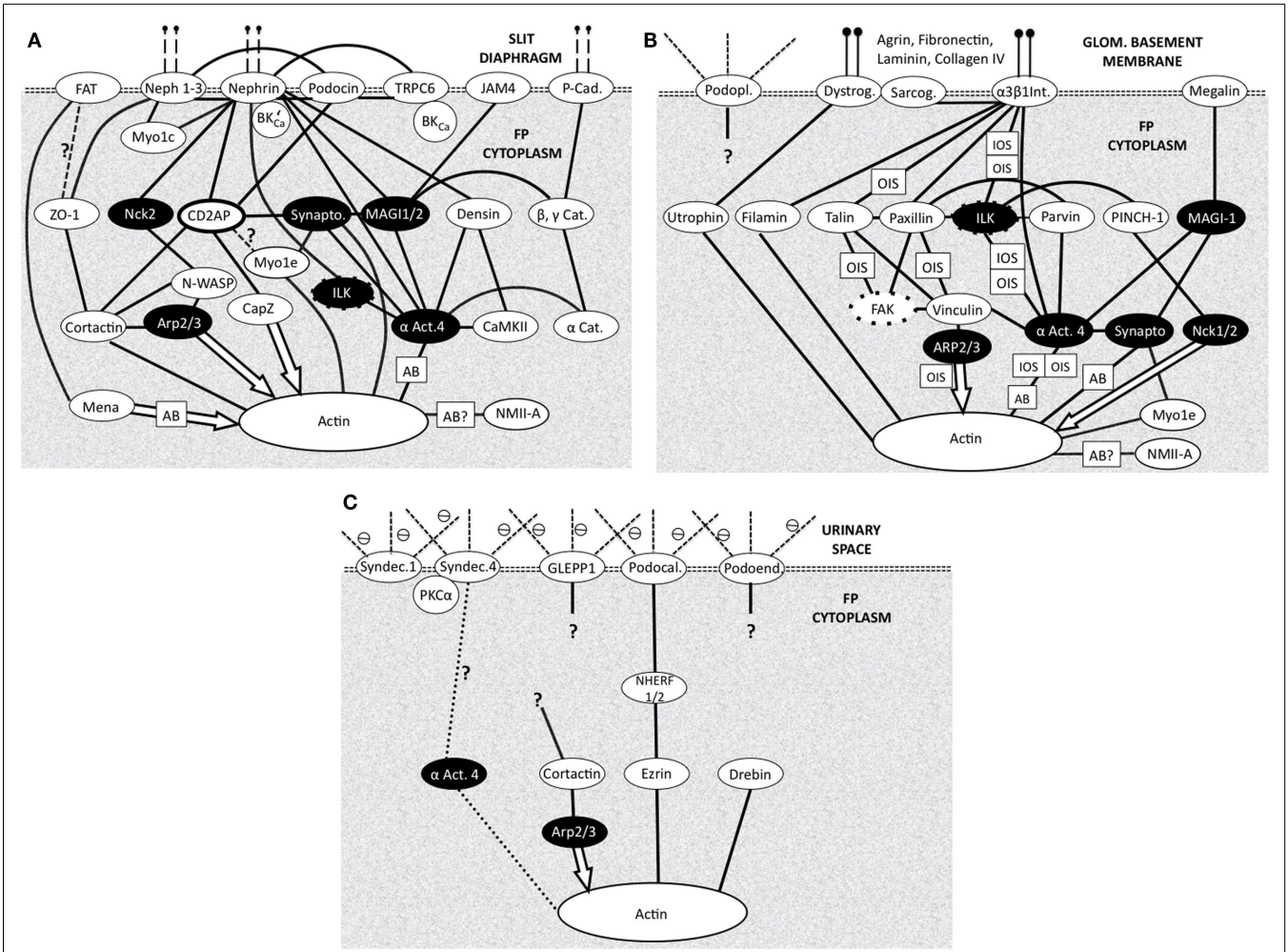

FIGURE 3 | Foot process membrane and associated domains with membrane proteins at the top then adapter and effector proteins leading to actin. Each domain is connected to the underlying cortical actin network and to the other domains. (N.B. Not all protein interactions are shown) all are bidirectional biochemical interactions except effector molecule interactions (arrows). Question marks show uncertainty. White text on black shows common adapter/effector molecules across the three domains. Most actin interactions will be with local cortical actin with some potential longer range actin bundle interactions (AB) Figure adapted from Refs. (13, 38, 39) . **(A)** Slit diaphragm domain (SDD). Vertical dashed lines at the top indicate connectivity across the SD to another FP. Cat, catenin; αAct4-α, actinin4; Cad, cadherin; Synapto, synaptopodin; BK'_{Ca} – $BK_{Ca,}$ splice variant, which binds Nephrin; Myo1c, myosin 1c; Myo1e, myosin 1e; NMIIA, non-muscle myosin II A; *Actin action*: *Mena, organizes long actin into bundles inhibits capping. Cortactin, promotes branching. Arp 2/3, branching and actin nucleation. CapZ, actin end capping.* α *Act.4, actin bundler and anchorer. Synaptopodin, actin bundler.* **(B)** Basal domain (BD). Vertical lines at the top indicate attachment to GBM components. Dystrog, dystroglycan; Sarcog, sarcoglycan; α3 β1 Int, α3 β1 integrin; αAct4, α actinin 4; Podopl, podoplanin (gp38); OIS, outside-in signaling (integrins regulate actin dynamics); IOS, inside-out signaling (actin regulates integrin adhesiveness); NMIIA, non-muscle myosin type IIA. *Actin action*: *Filamin, binds 2 actins at large angles (to make networks). Utropin, binds along actin length. NMIIA, motor protein binding 2 actins. Myo1e, motor protein binding membrane to actin. Nck1/2, actin polymerization.* **(C)** Apical Domain (AD). Dashed lines at the top indicate proteoglycan part of the membrane proteins in urinary space. Syndec, syndecan; αAct4, -α actinin 4; ⊖, negative charge on proteoglycans (glycocalyx) *Actin action*: *Ezrin, actin binder. Drebin, actin binder.*

three domains. α-actinin 4, Arp2/3, integrin-linked kinase (ILK), and synaptopodin are highlighted below.

Irrespective of membrane domain α-actinin 4 represents a prominent signaling node in the three domains but with noticeably different connectivity to the cortical actin and actin bundles of the FP. The network associations in **Figure 3** show α-actinin 4 to be closely associated with the cortical actin network and also active in the actin bundles of FP. Immuno-staining of α-actinin 4 shows location in only the actin bundles in rats (12), whereas in human podocytes its confined to SDD or BD and not in an apical actin bundle position (62). The close association with other proteins in complexes could cloak the α-actinin 4 antigenic site as it interacts directly with integrins in BD (**Figure 3B**) and forms a complex with nephrin and ILK in SDD (63) (**Figure 3A**). This highlights caution in interpretation of immuno-staining results.

Arp2/3 is in a separate signaling pathway to α-actinin 4 in SDD but both interact with vinculin in BD (**Figure 3B**). Since Arp2/3 interaction with actin is as an effector then only outside-in

signaling (OIS) is possible. Its associations in the AD are still being defined (**Figure 3C**).

Integrin-linked kinase (**Figures 3A,B**) seems to function as an adaptor that biochemically and functionally connects the BD and SDD complexes (13, 61). In the SDD, ILK physically interacts with nephrin to form a ternary complex, and α-actinin-4 in ILK/nephrin complex formation (63) in BD one of its functions is in relaying signals from integrins to actin (61) (**Figure 3B**).

Synaptopodin can be described as a scaffolding protein connecting the signaling complexes of the SDD and the BD by virtue of binding to α-actinin and the actin cytoskeleton (13). Small GTPases like RhoA act as regulators of actin networks and synaptopodin is involved in the promotion of RhoA-mediated actin fibril formation at the SDD or BD next to the GBM. However, in AD podocalyxin can activate RhoA (64).

PODOCYTE CHANGES IN DISEASE

Disruptions in podocyte biochemistry will alter podocyte structure in disease. The structural changes will be outlined in this next section and examples given of the underlying signaling.

REDUCTION IN PODOCYTE NUMBER

A reduction in podocyte number per glomerulus is a feature of FSGS (65, 66). Wharram showed a direct relationship between reduction in the podocyte population and extent of glomerulosclerosis in rats (67), podocyte depletion marks an inability of the glomerulus to replace podocytes. Detachment of podocytes due to disruption of cell adhesion proteins in the BD (68) allows recovery of them as viable cells from urine (69). Apoptosis regulated through cell cycle regulatory proteins (cyclin) and/or caspase routes can be an early feature in some disease as in type 2 diabetic kidney disease (70).

Podocyte loss is prevalent in type 2 diabetes (with microalbuminuria) the lower density of podocytes per glomerulus was the strongest predictor of renal disease progression (71). Again, podocyte loss in type 2 diabetic nephropathy scaled with the progression of the disease (72). However, in type 1 diabetic kidney disease the frequency of all glomerular cells varied with age in one study but was *not* reduced in the diabetic compared to control. The glomerulus appeared to regulate its architecture to maintain a constant podocyte density (73, 74). Although the second study did show a correlation between podocyte loss and proteinuria it was uncertain whether it was cause or effect of the nephropathy (74).

PODOCYTE DIVISION

How do neighboring podocytes fill in the gaps where podocytes are lost? Podocytes show no evidence of division *in vivo* (75), so how mature podocytes regenerate is unclear. Replacements from bone marrow have been demonstrated in mice (76, 77) other possibilities include the parietal cells of the Bowman's capsule, which can migrate from the urinary pole to the vascular stalk and there differentiate into new podocytes (78–80). Using serial multiphoton imaging of podocin-confetti mice in a renal fibrosis model the appearance of a new podocyte was noted within 24 h (81). Thus, some proportion of the podocyte population or its precursors has a highly dynamic, motile, and migratory phenotype, which must involve actin stress fibers.

PODOCYTE HYPERTROPHY

Cell expansion is one possibility for filling the podocyte gaps in disease, aging rat podocytes have been shown to increase in size up to twofold. This starts as a non-stressed hypertrophy and progresses to a severe hypertrophy with a reduction in (functional) SD proteins, fattening of FPs and increased proteinuria. Loss of SDs mark a reduction in filtration capacity; however, the resultant GFB still has a podocyte cover. The final stage involves more podocyte loss and glomerulosclerosis (82). Podocyte hypertrophy occurs with glomerular capillary hypertension ultimately leading to progressive glomerulosclerosis (83).

The controllers and regulators of hypertrophy are not completely resolved. A neuronal protein ubiquitin C-terminal hydrolase L1 (UCH-L1) appears to induce podocyte hypertrophy in Membranous Glomerular Nephritis (MGN) by increasing the total protein content by promoting cytoplasmic accumulation of proteins such as Cdk inhibitors ($p27^{Kip1}$) (84). Modification of both UCH-L1 activity and levels could reduce podocyte hypertrophy therapeutically in MGN.

The cyclin-dependent kinase inhibitor $p27^{Kip1}$ is a major regulator of the podocyte hypertrophic response to hyperglycemia *in vitro* (85) and *in vivo* in a mouse type 2 diabetes model (86) and levels of $p27^{Kip1}$ radically increase in experimental nephritis (87). However, different Cdk inhibitors appear important in podocytes *in vitro*, (88) compared to *in vivo*.

Another regulator of hypertrophy is GLUT4 (an insulin downstream effector), deficiency in podocytes results in fewer hypertrophic cells. GLUT4 also protects mice from the development of diabetic nephropathy (no proteinuria). There is a possibility that podocyte hypertrophy concomitant with podocyte loss may be associated with a protective mechanism avoiding proteinuria.

Genetic deletion of mTOR complex 1 (mTORC1) in mouse podocytes induced proteinuria and progressive glomerulosclerosis. However, increased mTOR activity accompanied human diabetic nephropathy, characterized by early glomerular hypertrophy and hyperfiltration. These results demonstrate the requirement for tightly balanced mTOR activity in podocyte homeostasis and suggest that mTOR inhibition can protect podocytes and prevent progressive diabetic nephropathy (89).

FP EFFACEMENT

The next response of FPs to disease or abnormal conditions is to retract toward the MP, any remaining FPs spread out and the normal interdigitating nature of the FPs gets simplified and smoothed into an undulation (**Figure 2B**). In a review on FP effacement, Kriz et al. pointed out that the process may be an adaptive and protective response in order to escape cell detachment or to cover bare areas where cells have been removed (90). This is in contrast to the old idea of damage and injury.

Effacement can be rapidly induced after only a few minutes of protamine sulfate, which reduces podocyte surface charge (41) with no observable change in SDs which apparently remain intact. α-actinin4 was found in effaced FP basal actin networks consistent with FP effacement being an adaptive change in cell shape, reinforcing the supportive role of podocytes (91).

ACTIN NETWORK FAILURE

Foot process effacement occurs with the disruption or dispersion of its actin networks. Rat glomerulopathy effacement occurs with buildup of a meshwork of intercrossing actin fibers on the FP BD adjacent to the GBM (91, 92). In contrast, the effacement of complement-mediated injury (93) or PAN (94) dissociated the basal actin cytoskeleton from matrix-attached integrins producing a transient dispersion of actin microfilament structure. How these structural changes relate or integrate with all the FP actin domains is unknown (12).

It seems there is a fine biochemical balancing act over activation or dispersion of actin basal networks, both resulting in FP effacement. PAN FP effacement was preceded by raised α-actinin 4 expression (91, 95) and transgenic overexpression of α-actinin 4 in mice yields an FSGS phenotype (96). However, mutations of ACTN4, reducing the levels of normal α-actinin 4, causes a late-onset FSGS (97) and the mutant crosslinkage with actin causes loss of nephrin from the SD. In signaling α-actinin-4 is heavily linked to both SDD (Nephrin, JAM4, p Cadherin; **Figure 3A**) and also the BD (via MAGI1 – megalin, α3 β1 integrin; **Figure 3B**) and possibly AD (**Figure 3C**).

The SDD, AD, and BDs of the FPs are physically linked to the FP cortical actin and central actin bundles, actin is the structural denominator in podocyte function and dysfunction (5, 98). Its clear that any interference with the three FP domains (BD, AD, SDD) and associated cortical actin then affects the FP actin bundles. These rearrange into either a dense network of short branching actin filaments (similar to cortical actin meshwork) or a dispersed network, both arrangements resulting in FP effacement and proteinuria.

SD LOSS PROMOTING DISEASE

Reduction in cellular levels, mutation or dislocation of SD proteins leads to SD loss or disruption and FP effacement. SD protein mutations or closely associated FP protein mutations signpost FSGS. Nephrin, Podocin, CD2AP, PLCε1, and MYO1E (non-muscle myosin) are autosomal recessive; α-actinin-4, TRPC6, and INF2 (Inverted Formin 2) are autosomal dominant (99). The dislocation or loss of only one SD component is necessary and effacement can occur, in Passive Heymann Nephritis for instance, it is Nephrin alone that appears to dissociate from SDs (100).

SMALL GTPase REGULATORS OF PODOCYTE ACTIN

The small GTPase group of proteins appears throughout in regulatory roles involving actin and podocyte shape change. Signals from the SD to actin and the microtubular system are critical in maintaining normal position, shape and functioning for the FPs. Critical regulators are RhoA, Rac1, and Cdc42. A GTPase activating protein binds CD2AP, which is present in the Nephrin complex (101), also nephrin can activate Rac 1 through a phosphoinositide pathway involving another nephrin binder Fyn (102) (not represented in **Figure 3A**).

Synaptopodin RhoA interaction seems to be critical for Actin fibre regulation (103). SD domain TRPC6 mediated calcium influx activates RhoA and inhibits podocyte motility. Either an increase or decrease in motility due to RhoA changes lead to FP effacement, too much or too little disrupts the barrier (104).

RhoA and Rac1 also appear in a similar balanced mechanistic model of podocyte disruption involving a circulating factor and podocyte protein expression. FSGS and DKD (diabetic kidney disease) have elevated levels of circulating soluble urokinase plasminogen activator receptor (suPAR) but the podocyte expression of acid sphingomyelinase-like phosphodiesterase 3b (SMPDL3b) is elevated only in DKD (105).

In FSGS, high SuPAR levels lead to Rac 1 activation and a migratory phenotype. In DKD, high suPAR levels competitively bind with high SMPDL3b, allowing RhoA activation and increased apoptosis (**Table 1**). Healthy podocytes with an absence of proteinuria mark a balance between the two.

PROBLEMS WITH THE CURRENT STRUCTURAL MODEL

The pathways of actin signaling highlighted here show that the different domains are complex highly modified evolved structures that have radically different signaling pathways in closely adjacent domains. From above it seems that disruption of often only a single element of the actin linked pathways in FP in any of the membrane associated domains leads to effacement. A more recent interpretation of podocyte structure raises the question of how basic FP signaling pathways are arranged in FP structure.

The view above simply assumes one type of FP and attached SD; however, a discovery 60 years ago, which was revisited in the 1980s and in 2000s challenges this view. The presence of a subpodocyte space (SPS) was highlighted early on in EM investigations of podocytes. Gautier et al. in 1951 (106) first highlighted the presence of "lacunaire peri capillaire" (pericapillary lacunae) under podocyte cell bodies. Elias studied the SPS in the 1980s (107) but again this was ignored in favor of a simple urinary space concept.

More recently, the SPS was entirely reconstructed from electron micrographs and showed that these little urinary spaces were restrictive to fluid outflow (108–110) (**Figure 1**). Evidence has since emerged from two labs, which found SPS *in vivo* to be a restrictive space, which trapped 10 kDa macromolecules (111, 112) but was not restrictive to smaller 450 Da molecules (111). Mathematical modeling of the SPS predicted that the fluid resistance of SPS plus GFB was 2.5 times the "ordinary" uncovered GFB and exquisitely sensitive to changes in the width of the exit regions

Table 1 | The consequences of circulating levels (+, +++) of suPAR and podocyte expression levels (+, +++) of SMPDL3b on the activation (⇑⇑) or inactivation (⇓⇓) of integrins ($\alpha V\beta_3$).

FSGS	Healthy	DKD
SMPDL3b+	SMPDL3b+	SMPDL3b+++
suPAR+++	suPAR+	suPAR+++
$\alpha V\beta_3$ ⇑⇑	$\alpha V\beta_3$ ⇔	$\alpha V\beta_3$ ⇓⇓
RhoA ⇓ Rac1 ⇑	RhoA ⇔ Rac1 ⇔	RhoA ⇑ Rac1⇓
Migration	–	Apoptosis
Proteinuria	–	Proteinuria

High levels of SMPDL3b and suPAR competitively bind and inactivate $\alpha V\beta_3$. Subsequently a different small GTPase. is activated leading to either a migratory or apoptotic phenotype. [Tabulated from Yoo et al. (105)].

leading from the SPS (arrow in **Figure 1**) (109). This is a problem for structure-based models predicting fluid permeability without accounting for an SPS contribution to the barrier (113)**.**

In essence, an SPS is formed by the cell body and some supporting MPs of the podocyte covering up and roofing over more than 50% of the urinary side of the GFB (108–110). The covered GFB comprises underlapping MPs and attached FPs sitting on GBM with the usual fenestrated endothelium on the luminal side (**Figure 1**).

FP REDEFINITION

At first glance the GFB underlying the SPS seems to be identical to the GFB in non-SPS areas with SDs and domains formed between FPs of neighboring podocytes. However, not all FPs in SPS arise from MPs because some FPs emerge directly from the cell body (**Figure 1**. foot processes 4, 6 and 8). These processes still run parallel with other FPs and TEM image reconstruction shows them to be extended columns supporting the cell body (109).

These major FPs were originally referred to as anchoring processes (108, 109), here we call them anchoring foot processes (AFP) for clarity (**Figure 4**). The original name was applied when these processes were found to widen under transiently increased perfusion pressure (approximately 30 s), as if they were an adjustable anchor for the podocyte cell body on the GBM (108, 109). The smaller ordinary FPs (OFPs), which have all the cytoskeletal machinery for shape change did *not* widen, so AFPs appeared to be more sensitive to pressure than OFPs.

Looking at AFPs in more detail (**Figure 4**), there is an SD domain, a BD but an AD stretching up to the underside of the podocyte cell body (an SPS "ceiling" domain). If the OFPs and AFPs are considered separately, some initial questions arise:

CONTRACTILE/CYTOSKELETAL STRUCTURES

1. Do AFPs have OFP type actin bundles, cortical actin, and membrane associated domains?
2. Do AFPs have cell centric actin stress fibers?
3. Since AFPs are a form of MP do they have microtubules?
4. Do AFPs have a combination of 1, 2, and 3.

SIGNALING NETWORKS

1. What signaling networks and pathways are specific to AFPs compared to OFPs? Broadly, do the signaling pathways of "FPs" in **Figure 3** occur in AFP?
2. With an increase in perfusion pressure how could the filtrate flow (through the SD) or mechanical stretch (of the podocyte membrane) be transduced allowing the AFP widening response? (see below)

A POSSIBLE AFP STRETCH/FLOW TRANSDUCTION MECHANISM

Some of the mechanical stretch due to increased perfusion pressure will get exerted at the SD with the components being put under increased wall stress (hoop or paraxial stress due to the shape of the underlying capillary). Similarly, any increased filtrate flow due to increased perfusion pressure will have to pass through the SDs but exerting an increased radial stress on extracellular SD components pulling and stretching the SDD membranes. Thus, either increased wall stress or filtrate flow will both induce stretch deformations at the SD membranes.

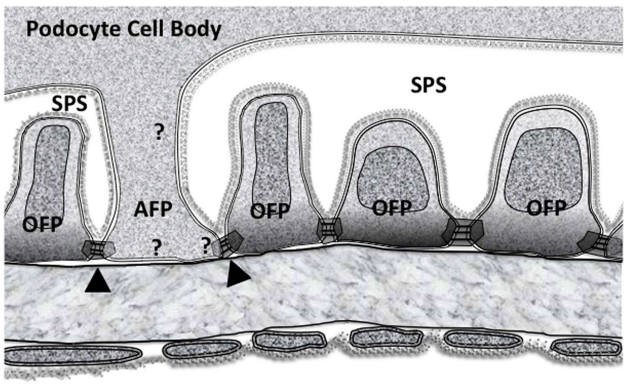

FIGURE 4 | The subpodocyte space (SPS) GFB showing the disposition of membrane domains in foot processes as in Figure 2. Ordinary foot processes (OFP) have signaling networks that are well defined. The less frequent anchoring foot processes (AFP) could have some of the features of (OFP) but must have some differences based upon their altered response to increased perfusion pressure. Arrowheads mark the possible asymmetric slit diaphragm between OFP and AFP. Question marks show the possibility of differences in all three membrane associated domains.

While *in vitro* studies show that the identity of the podocyte mechanosensor remains unclear (114) one possible transduction pathway could involve stretch sensitive BK_{Ca} channels (115), which are bound to the SD protein complexes (both Nephrin and TRPC6. **Figure 3A**). Large conductance calcium activated potassium channels or BK_{Ca} channels could provide a mechanism with TRPC6 to fine-tune Ca^{2+} influx during normal glomerular function. The Slo1 subunits of BK_{Ca} bind to TRPC6 channels (116) (**Figure 3A**), which are expressed in the SD domain, cell body, and throughout the MP (117). The interactions between BK_{Ca} channels and actin filaments are complex and are likely to have multiple effects on the overall activity of BK_{Ca} channels (118). However, acute depolymerization of podocyte actin with cytochalasin-D did not affect BK_{Ca} channels (119), suggesting that any stretch activated response in AFP is not mediated by actin. While actin is thought of as being the mediator of protrusion and contraction, microtubules can also fulfill this function (118, 120).

How could differential responses to the same stretch stimulus arise in adjacent AFP and OFP, which share the same SD? This variability could be due to BK_{Ca} alone since the *KCNMA1* gene that codes for the 4 Slo1 subunits of BK_{Ca} has 35 exons, which can be alternatively spliced at 7 different sites. Thus, BK_{Ca} can have a multiplicity of variants sensitive to various pressure ranges but crucially, lots of different BK_{Ca} splice variants (and heteromers) can exist in a *single* cell (121–123). Its not unreasonable to hypothesize high stretch sensitivity splice variants/heteromers in AFP and low stretch sensitivity splice variants in OFP of the same cell and also on the other side of an SDD in an adjacent OFP. This could give the necessary asymmetric response in neighboring FPs. Highly stretch sensitive BK_{Ca} splice variants/heteromers should accordingly be located along AFP borders (SPS) and absent from OFP ones if this hypothesis works.

Crucially, in the rapid movement of AFP in response to pressure changes integrins can also alter their adhesive characteristics

in response to cellular events via "inside-out" signaling (61) (IOS in **Figure 3B**), allowing them to take up the extra space on the GBM.

The alternative to a local stretch sensor is whole podocyte stretch sensing, with either BK_{Ca} or another mechanosensor. Here, the differential AFP/OFP mechanisms would rely on different AFP and OFP effector signaling pathways.

EFFACEMENT, ANCHOR WIDENING AND SPS LOSS

Widening of AFPs and the reduction of SPS under an elevated stimulus or a modified response could be a mechanism resulting in the production of effacement. This type of effacement appears to occur in many published electron micrographs [e.g., Figure 11 in (124)] and begs the question how much podocyte FP effacement is AFP widening in addition to OFP changes. Since SPS is a partially enclosed region, which accumulates/concentrates 10 kDa Dextran molecules but not molecules below 450 Da (111, 112) loss of this space points to a dysfunction in macromolecular transport by podocytes. Thus, effacement and proteinuria could fit, at least partly, with AFP widening and SPS loss in disease.

AFP MICROTUBULE TRANSPORT

Since AFPs are MPs (arising direct from cell bodies) its possible they have microtubules, podocyte MTs are oriented to convey cargoes both ways to and from the cell body along the MPs (16, 32). AFP associated microtubules might be organized for delivery to the periphery (i.e., GBM structural proteins from perinuclear Golgi apparatus) or delivery to the cell body (i.e., cargoes endocytosed from the GBM matrix) or both. The MPs that stretch away from the podocyte peri-nuclear region carrying many OFPs may transport in a different fashion to AFPs in SPS. The subtle distinctions between cytoplasmic transport domains in OFPs and AFPs might not be easy to determine.

CONCLUSION

Clearly, any structural differences, defining characteristics and functional interactions surrounding AFPs and OFPs and any effects on SPS will need to be defined under normal *in vivo* podocyte conditions before sensible re-assessments of effacement or podocyte structural change can be made in disease.

REFERENCES

1. Jarad G, Cunningham J, Shaw AS, Miner JH. Proteinuria precedes podocyte abnormalities inLamb2-/- mice, implicating the glomerular basement membrane as an albumin barrier. *J Clin Invest* (2006) **116**(8):2272–9. doi:10.1172/JCI28414
2. Satchell S. The role of the glomerular endothelium in albumin handling. *Nat Rev Nephrol* (2013) **9**(12):717–25. doi:10.1038/nrneph.2013.197
3. Wartiovaara J, Ofverstedt LG, Khoshnoodi J, Zhang J, Mäkelä E, Sandin S, et al. Nephrin strands contribute to a porous slit diaphragm scaffold as revealed by electron tomography. *J Clin Invest* (2004) **114**(10):1475–83. doi:10.1172/JCI22562C1
4. Tryggvason K, Wartiovaara J. Molecular basis of glomerular permselectivity. *Curr Opin Nephrol Hypertens* (2001) **10**(4):543–9. doi:10.1097/00041552-200107000-00009
5. Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *J Clin Invest* (2001) **108**(11):1583–7. doi:10.1172/JCI200114629
6. Kanwar YS, Venkatachalam MA. Ultrastructure of glomerulus and juxtaglomerular apparatus. In: Windhager EE, editor. *Handbook of Physiology*. New York, NY: Oxford University Press (1992). p. 3–40.
7. Hall B. Studies of normal glomerular structure by electron microscopy. *5th American Conference on Nephrotic Syndrome*. New York (1954).
8. Deen WM, Lazzara MJ, Myers BD. Structural determinants of glomerular permeability. *Am J Physiol Renal Physiol* (2001) **281**(4):F579–96.
9. Welsh GI, Saleem MA. The podocyte cytoskeleton – key to a functioning glomerulus in health and disease. *Nat Rev Nephrol* (2012) **8**(1):14–21. doi:10.1038/nrneph.2011.151
10. Grahammer F, Schell C, Huber TB. The podocyte slit diaphragm – from a thin grey line to a complex signalling hub. *Nat Rev Nephrol* (2013) **9**(10):587–98. doi:10.1038/nrneph.2013.169
11. Weavers H, Prieto-Sánchez S, Grawe F, Garcia-López A, Artero R, Wilsch-Bräuninger M, et al. The insect nephrocyte is a podocyte-like cell with a filtration slit diaphragm. *Nature* (2009) **457**(7227):322–6. doi:10.1038/nature07526
12. Ichimura K, Kurihara H, Sakai T. Actin filament organization of foot processes in rat podocytes. *J Histochem Cytochem* (2003) **51**(12):1589–600. doi:10.1177/002215540305101203
13. Faul C, Asanuma K, Yanagida-Asanuma E, Kim K, Mundel P. Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. *Trends Cell Biol* (2007) **17**(9):428–37. doi:10.1016/j.tcb.2007.06.006
14. Cortes P, Méndez M, Riser BL, Guérin CJ, Rodríguez-Barbero A, Hassett C, et al. F-actin fiber distribution in glomerular cells: structural and functional implications. *Kidney Int* (2000) **58**(6):2452–61. doi:10.1046/j.1523-1755.2000.00428.x
15. Farquhar MG, Vernier RL, Good RA. An electron microscope study of the glomerulus in nephrosis, glomerulonephritis, and lupus erythematosus. *J Exp Med* (1957) **106**(5):649–60. doi:10.1084/jem.106.5.649
16. Kobayashi N, Mundel P. A role of microtubules during the formation of cell processes in neuronal and non-neuronal cells. *Cell Tissue Res* (1998) **291**(2):163–74. doi:10.1007/s004410050988
17. Vasmant D, Maurice M, Feldmann G. Cytoskeleton ultrastructure of podocytes and glomerular endothelial cells in man and in the rat. *Anat Rec* (1984) **210**(1):17–24. doi:10.1002/ar.1092100104
18. Drenckhahn D, Franke RP. Ultrastructural organization of contractile and cytoskeletal proteins in glomerular podocytes of chicken, rat, and man. *Lab Invest* (1988) **59**(5):673–82.
19. Kriz W, Elger M, Mundel P, Lemley KV. Structure-stabilizing forces in the glomerular tuft. *J Am Soc Nephrol* (1995) **5**(10):1731–9.
20. Kriz W, Mundel P, Elger M. The contractile apparatus of podocytes is arranged to counteract GBM expansion. *Contrib Nephrol* (1994) **107**:1–9.
21. Endlich N, Kress KR, Reiser J, Uttenweiler D, Kriz W, Mundel P, et al. Podocytes respond to mechanical stress in vitro. *J Am Soc Nephrol* (2001) **12**(3):413–22.
22. Katoh K, Kano Y, Noda Y. Rho-associated kinase-dependent contraction of stress fibres and the organization of focal adhesions. *J R Soc Interface* (2011) **8**(56):305–11. doi:10.1098/rsif.2010.0419
23. Wong JS, Iorns E, Rheault MN, Ward TM, Rashmi P, Weber U, et al. Rescue of tropomyosin deficiency in *Drosophila* and human cancer cells by synaptopodin reveals a role of tropomyosin alpha in RhoA stabilization. *EMBO J* (2012) **31**(4):1028–40. doi:10.1038/emboj.2011.464
24. Kawano Y, Yoshimura T, Kaibuchi K. Smooth muscle contraction by small GTPase Rho. *Nagoya J Med Sci* (2002) **65**(1–2):1–8.
25. Stamenkovic I, Skalli O, Gabbiani G. Distribution of intermediate filament proteins in normal and diseased human glomeruli. *Am J Pathol* (1986) **125**(3):465–75.
26. Yaoita E, Franke WW, Yamamoto T, Kawasaki K, Kihara I. Identification of renal podocytes in multiple species: higher vertebrates are vimentin positive/lower vertebrates are desmin positive. *Histochem Cell Biol* (1999) **111**(2):107–15. doi:10.1007/s004180050340
27. Yaoita E, Wiche G, Yamamoto T, Kawasaki K, Kihara I. Perinuclear distribution of plectin characterizes visceral epithelial cells of rat glomeruli. *Am J Pathol* (1996) **149**(1):319–27.
28. Kurihara H, Sunagawa N, Kobayashi T, Kimura K, Takasu N, Shike T. Monoclonal antibody P-31 recognizes a novel intermediate filament-associated protein (p250) in rat podocytes. *Am J Physiol* (1998) **274**(5 Pt 2):F986–97.
29. Miner JH. Renal basement membrane components. *Kidney Int* (1999) **56**(6):2016–24. doi:10.1046/j.1523-1755.1999.00785.x
30. Cheng J, Grassart A, Drubin DG. Myosin 1E coordinates actin assembly and cargo trafficking during clathrin-mediated endocytosis. *Mol Biol Cell* (2012) **23**(15):2891–904. doi:10.1091/mbc.E11-04-0383

31. Baas PW, Black MM, Banker GA. Changes in microtubule polarity orientation during the development of hippocampal neurons in culture. *J Cell Biol* (1989) **109**(6 Pt 1):3085–94. doi:10.1083/jcb.109.6.3085
32. Kidd GJ, Andrews SB, Trapp BD. Organization of microtubules in myelinating Schwann cells. *J Neurocytol* (1994) **23**(12):801–10. doi:10.1007/BF01268092
33. Simons M, Saffrich R, Reiser J, Mundel P. Directed membrane transport is involved in process formation in cultured podocytes. *J Am Soc Nephrol* (1999) **10**(8):1633–9.
34. Huber LA, Pimplikar S, Parton RG, Virta H, Zerial M, Simons K. Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. *J Cell Biol* (1993) **123**(1):35–45. doi:10.1083/jcb.123.1.35
35. Kim JH, Konieczkowski M, Mukherjee A, Schechtman S, Khan S, Schelling JR, et al. Podocyte injury induces nuclear translocation of WTIP via microtubule-dependent transport. *J Biol Chem* (2010) **285**(13):9995–10004. doi:10.1074/jbc.M109.061671
36. Guo JK, Menke AL, Gubler MC, Clarke AR, Harrison D, Hammes A, et al. WT1 is a key regulator of podocyte function: reduced expression levels cause crescentic glomerulonephritis and mesangial sclerosis. *Hum Mol Genet* (2002) **11**(6):651–9. doi:10.1093/hmg/11.6.651
37. Kobayashi N, Reiser J, Schwarz K, Sakai T, Kriz W, Mundel P. Process formation of podocytes: morphogenetic activity of microtubules and regulation by protein serine/threonine phosphatase PP2A. *Histochem Cell Biol* (2001) **115**(3):255–66. doi:10.1007/s004180000242
38. Noris MR, Remuzzi G. Non-muscle myosins and the podocyte. *Clin Kidney J* (2012) **5**(2):94–101. doi:10.1093/ckj/sfs032
39. Arif E, Wagner MC, Johnstone DB, Wong HN, George B, Pruthi PA, et al. Motor protein Myo1c is a podocyte protein that facilitates the transport of slit diaphragm protein Neph1 to the podocyte membrane. *Mol Cell Biol* (2011) **31**(10):2134–50. doi:10.1128/MCB.05051-11
40. Gbadegesin RA, Winn MP, Smoyer WE. Genetic testing in nephrotic syndrome – challenges and opportunities. *Nat Rev Nephrol* (2013) **9**(3):179–84. doi:10.1038/nrneph.2012.286
41. Kerjaschki D. Polycation-induced dislocation of slit diaphragms and formation of cell junctions in rat kidney glomeruli: the effects of low temperature, divalent cations, colchicine, and cytochalasin B. *Lab Invest* (1978) **39**(5):430–40.
42. Kriz W, LeHir M. Pathways to nephron loss starting from glomerular diseases-insights from animal models. *Kidney Int* (2005) **67**(2):404–19. doi:10.1111/j.1523-1755.2005.67097.x
43. Faul C. *The Podocyte Cytoskeleton: Key to a Functioning Glomerulus in Health and Disease, in Podocytopathy (Contrib. Nephrol.)*. Basel: Karger (2014). p. 22–53.
44. Miao J, Fan Q, Cui Q, Zhang H, Chen L, Wang S, et al. Newly identified cytoskeletal components are associated with dynamic changes of podocyte foot processes. *Nephrol Dial Transplant* (2009) **24**(11):3297–305. doi:10.1093/ndt/gfp338
45. Li X, Zhang X, Li X, Ding F, Ding J. The role of survivin in podocyte injury induced by puromycin aminonucleoside. *Int J Mol Sci* (2014) **15**(4):6657–73. doi:10.3390/ijms15046657
46. Liu Y, Zhou QX, Hou YY, Lu B, Yu C, Chen J, et al. Actin polymerization-dependent increase in synaptic Arc/Arg3.1 expression in the amygdala is crucial for the expression of aversive memory associated with drug withdrawal. *J Neurosci* (2012) **32**(35):12005–17. doi:10.1523/JNEUROSCI.0871-12.2012
47. Kurihara H, Anderson JM, Kerjaschki D, Farquhar MG. The altered glomerular filtration slits seen in puromycin aminonucleoside nephrosis and protamine sulfate-treated rats contain the tight junction protein ZO-1. *Am J Pathol* (1992) **141**(4):805–16.
48. Reiser J, Kriz W, Kretzler M, Mundel P. The glomerular slit diaphragm is a modified adherens junction. *J Am Soc Nephrol* (2000) **11**(1):1–8.
49. Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, et al. Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* (1999) **286**(5438):312–5. doi:10.1126/science.286.5438.312
50. Huber TB, Simons M, Hartleben B, Sernetz L, Schmidts M, Gundlach E, et al. Molecular basis of the functional podocin-nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains. *Hum Mol Genet* (2003) **12**(24):3397–405. doi:10.1093/hmg/ddg360
51. Lachapelle M, Bendayan M. Contractile proteins in podocytes: immunocytochemical localization of actin and alpha-actinin in normal and nephrotic rat kidneys. *Virchows Arch B Cell Pathol Incl Mol Pathol* (1991) **60**(2):105–11. doi:10.1007/BF02899534
52. Kerjaschki D, Sharkey DJ, Farquhar MG. Identification and characterization of podocalyxin – the major sialoprotein of the renal glomerular epithelial cell. *J Cell Biol* (1984) **98**(4):1591–6. doi:10.1083/jcb.98.4.1591
53. Huang TW, Langlois JC. Podoendin. A new cell surface protein of the podocyte and endothelium. *J Exp Med* (1985) **162**(1):245–67. doi:10.1084/jem.162.1.245
54. Sawada H, Stukenbrok H, Kerjaschki D, Farquhar MG. Epithelial polyanion (podocalyxin) is found on the sides but not the soles of the foot processes of the glomerular epithelium. *Am J Pathol* (1986) **125**(2):309–18.
55. Takeda T, Go WY, Orlando RA, Farquhar MG. Expression of podocalyxin inhibits cell-cell adhesion and modifies junctional properties in Madin-Darby canine kidney cells. *Mol Biol Cell* (2000) **11**(9):3219–32. doi:10.1091/mbc.11.9.3219
56. Orlando RA, Takeda T, Zak B, Schmieder S, Benoit VM, McQuistan T, et al. The glomerular epithelial cell anti-adhesin podocalyxin associates with the actin cytoskeleton through interactions with ezrin. *J Am Soc Nephrol* (2001) **12**(8):1589–98.
57. Takeda T, McQuistan T, Orlando RA, Farquhar MG. Loss of glomerular foot processes is associated with uncoupling of podocalyxin from the actin cytoskeleton. *J Clin Invest* (2001) **108**(2):289–301. doi:10.1172/JCI200112539
58. Doyonnas R, Kershaw DB, Duhme C, Merkens H, Chelliah S, Graf T, et al. Anuria, omphalocele, and perinatal lethality in mice lacking the CD34-related protein podocalyxin. *J Exp Med* (2001) **194**(1):13–27. doi:10.1084/jem.194.1.13
59. Patrie KM, Drescher AJ, Goyal M, Wiggins RC, Margolis B. The membrane-associated guanylate kinase protein MAGI-1 binds megalin and is present in glomerular podocytes. *J Am Soc Nephrol* (2001) **12**(4):667–77.
60. Patrie KM, Drescher AJ, Welihinda A, Mundel P, Margolis B. Interaction of two actin-binding proteins, synaptopodin and alpha-actinin-4, with the tight junction protein MAGI-1. *J Biol Chem* (2002) **277**(33):30183–90. doi:10.1074/jbc.M203072200
61. Blattner SM, Kretzler M. Integrin-linked kinase in renal disease: connecting cell-matrix interaction to the cytoskeleton. *Curr Opin Nephrol Hypertens* (2005) **14**(4):404–10. doi:10.1097/01.mnh.0000172730.67746.5b
62. Goode NP, Shires M, Khan TN, Mooney AF. Expression of alpha-actinin-4 in acquired human nephrotic syndrome: a quantitative immunoelectron microscopy study. *Nephrol Dial Transplant* (2004) **19**(4):844–51. doi:10.1093/ndt/gfg620
63. Dai C, Stolz DB, Bastacky SI, St-Arnaud R, Wu C, Dedhar S, et al. Essential role of integrin-linked kinase in podocyte biology: bridging the integrin and slit diaphragm signaling. *J Am Soc Nephrol* (2006) **17**(8):2164–75. doi:10.1681/ASN.2006010033
64. Schmieder S, Nagai M, Orlando RA, Takeda T, Farquhar MG. Podocalyxin activates RhoA and induces actin reorganization through NHERF1 and Ezrin in MDCK cells. *J Am Soc Nephrol* (2004) **15**(9):2289–98. doi:10.1097/01.ASN.0000135968.49899.E8
65. Wiggins RC. The spectrum of podocytopathies: a unifying view of glomerular diseases. *Kidney Int* (2007) **71**(12):1205–14. doi:10.1038/sj.ki.5002222
66. Kriz W. Podocyte is the major culprit accounting for the progression of chronic renal disease. *Microsc Res Tech* (2002) **57**(4):189–95. doi:10.1002/jemt.10072
67. Wharram BL, Goyal M, Wiggins JE, Sanden SK, Hussain S, Filipiak WE, et al. Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. *J Am Soc Nephrol* (2005) **16**(10):2941–52. doi:10.1681/ASN.2005010055
68. Sachs N, Sonnenberg A. Cell-matrix adhesion of podocytes in physiology and disease. *Nat Rev Nephrol* (2013) **9**(4):200–10. doi:10.1038/nrneph.2012.291
69. Petermann AT, Pippin J, Krofft R, Blonski M, Griffin S, Durvasula R, et al. Viable podocytes detach in experimental diabetic nephropathy: potential mechanism underlying glomerulosclerosis. *Nephron Exp Nephrol* (2004) **98**(4):e114–23. doi:10.1159/000081555
70. Verzola D, Gandolfo MT, Ferrario F, Rastaldi MP, Villaggio B, Gianiorio F, et al. Apoptosis in the kidneys of patients with type II diabetic nephropathy. *Kidney Int* (2007) **72**(10):1262–72. doi:10.1038/sj.ki.5002531
71. Meyer TW, Bennett PH, Nelson RG. Podocyte number predicts long-term urinary albumin excretion in Pima Indians with type II diabetes

and microalbuminuria. *Diabetologia* (1999) **42**(11):1341–4. doi:10.1007/s001250051447
72. Pagtalunan ME, Miller PL, Jumping-Eagle S, Nelson RG, Myers BD, Rennke HG, et al. Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* (1997) **99**(2):342–8. doi:10.1172/JCI119163
73. Steffes MW, Schmidt D, McCrery R, Basgen JM; International Diabetic Nephropathy Study Group. Glomerular cell number in normal subjects and in type 1 diabetic patients. *Kidney Int* (2001) **59**(6):2104–13. doi:10.1046/j.1523-1755.2001.0590062104.x
74. White KE, Bilous RW, Marshall SM, El Nahas M, Remuzzi G, Piras G, et al. Podocyte number in normotensive type 1 diabetic patients with albuminuria. *Diabetes* (2002) **51**(10):3083–9. doi:10.2337/diabetes.51.10.3083
75. Griffin SV, Petermann AT, Durvasula RV, Shankland SJ. Podocyte proliferation and differentiation in glomerular disease: role of cell-cycle regulatory proteins. *Nephrol Dial Transplant* (2003) **18**(Suppl 6):vi8–13. doi:10.1093/ndt/gfg1069
76. Sugimoto H, Mundel TM, Sund M, Xie L, Cosgrove D, Kalluri R. Bone-marrow-derived stem cells repair basement membrane collagen defects and reverse genetic kidney disease. *Proc Natl Acad Sci U S A* (2006) **103**(19):7321–6. doi:10.1073/pnas.0601436103
77. Prodromidi EI, Poulsom R, Jeffery R, Roufosse CA, Pollard PJ, Pusey CD, et al. Bone marrow-derived cells contribute to podocyte regeneration and amelioration of renal disease in a mouse model of Alport syndrome. *Stem Cells* (2006) **24**(11):2448–55. doi:10.1634/stemcells.2006-0201
78. Ronconi E, Sagrinati C, Angelotti ML, Lazzeri E, Mazzinghi B, Ballerini L, et al. Regeneration of glomerular podocytes by human renal progenitors. *J Am Soc Nephrol* (2009) **20**(2):322–32. doi:10.1681/ASN.2008070709
79. Romagnani P. Parietal epithelial cells: their role in health and disease. *Contrib Nephrol* (2011) **169**:23–36. doi:10.1159/000313943
80. Schulte K, Berger K, Boor P, Jirak P, Gelman IH, Arkill KP, et al. Origin of parietal podocytes in atubular glomeruli mapped by lineage tracing. *J Am Soc Nephrol* (2014) **25**(1):129–41. doi:10.1681/ASN.2013040376
81. Hackl MJ, Burford JL, Villanueva K, Lam L, Suszták K, Schermer B, et al. Tracking the fate of glomerular epithelial cells in vivo using serial multiphoton imaging in new mouse models with fluorescent lineage tags. *Nat Med* (2013) **19**(12):1661–6. doi:10.1038/nm.3405
82. Wiggins JE, Goyal M, Sanden SK, Wharram BL, Shedden KA, Misek DE, et al. Podocyte hypertrophy, "adaptation," and "decompensation" associated with glomerular enlargement and glomerulosclerosis in the aging rat: prevention by calorie restriction. *J Am Soc Nephrol* (2005) **16**(10):2953–66. doi:10.1681/ASN.2005050488
83. Kriz W, Kretzler M, Nagata M, Provoost AP, Shirato I, Uiker S, et al. A frequent pathway to glomerulosclerosis: deterioration of tuft architecture-podocyte damage-segmental sclerosis. *Kidney Blood Press Res* (1996) **19**(5):245–53. doi:10.1159/000174083
84. Lohmann F, Sachs M, Meyer TN, Sievert H, Lindenmeyer MT, Wiech T, et al. UCH-L1 induces podocyte hypertrophy in membranous nephropathy by protein accumulation. *Biochim Biophys Acta* (2014) **1842**(7):945–58. doi:10.1016/j.bbadis.2014.02.011
85. Rüster C, Bondeva T, Franke S, Förster M, Wolf G. Advanced glycation end-products induce cell cycle arrest and hypertrophy in podocytes. *Nephrol Dial Transplant* (2008) **23**(7):2179–91. doi:10.1093/ndt/gfn085
86. Wolf G, Schroeder R, Thaiss F, Ziyadeh FN, Helmchen U, Stahl RA. Glomerular expression of p27Kip1 in diabetic db/db mouse: role of hyperglycemia. *Kidney Int* (1998) **53**(4):869–79. doi:10.1111/j.1523-1755.1998.00829.x
87. Shankland SJ, Floege J, Thomas SE, Nangaku M, Hugo C, Pippin J, et al. Cyclin kinase inhibitors are increased during experimental membranous nephropathy: potential role in limiting glomerular epithelial cell proliferation in vivo. *Kidney Int* (1997) **52**(2):404–13. doi:10.1038/ki.1997.347
88. Petermann AT, Pippin J, Durvasula R, Pichler R, Hiromura K, Monkawa T, et al. Mechanical stretch induces podocyte hypertrophy in vitro. *Kidney Int* (2005) **67**(1):157–66. doi:10.1111/j.1523-1755.2005.00066.x
89. Gödel M, Hartleben B, Herbach N, Liu S, Zschiedrich S, Lu S, et al. Role of mTOR in podocyte function and diabetic nephropathy in humans and mice. *J Clin Invest* (2011) **121**(6):2197–209. doi:10.1172/JCI44774
90. Kriz W, Shirato I, Nagata M, LeHir M, Lemley KV. The podocyte's response to stress: the enigma of foot process effacement. *Am J Physiol Renal Physiol* (2013) **304**(4):F333–47. doi:10.1152/ajprenal.00478.2012
91. Shirato I, Sakai T, Kimura K, Tomino Y, Kriz W. Cytoskeletal changes in podocytes associated with foot process effacement in Masugi nephritis. *Am J Pathol* (1996) **148**(4):1283–96.
92. Kretzler M, Koeppen-Hagemann I, Kriz W. Podocyte damage is a critical step in the development of glomerulosclerosis in the uninephrectomised-desoxycorticosterone hypertensive rat. *Virchows Arch* (1994) **425**(2):181–93. doi:10.1007/BF00230355
93. Topham PS, Haydar SA, Kuphal R, Lightfoot JD, Salant DJ. Complement-mediated injury reversibly disrupts glomerular epithelial cell actin microfilaments and focal adhesions. *Kidney Int* (1999) **55**(5):1763–75. doi:10.1046/j.1523-1755.1999.00407.x
94. Whiteside CI, Cameron R, Munk S, Levy J. Podocytic cytoskeletal disaggregation and basement-membrane detachment in puromycin aminonucleoside nephrosis. *Am J Pathol* (1993) **142**(5):1641–53.
95. Smoyer WE, Mundel P, Gupta A, Welsh MJ. Podocyte alpha-actinin induction precedes foot process effacement in experimental nephrotic syndrome. *Am J Physiol* (1997) **273**(1):F150–7.
96. Michaud JL, Lemieux LI, Dubé M, Vanderhyden BC, Robertson SJ, Kennedy CR. Focal and segmental glomerulosclerosis in mice with podocyte-specific expression of mutant alpha-actinin-4. *J Am Soc Nephrol* (2003) **14**(5):1200–11. doi:10.1097/01.ASN.0000059864.88610.5E
97. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, et al. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* (2000) **24**(3):251–6. doi:10.1038/73456
98. Asanuma K, Mundel P. The role of podocytes in glomerular pathobiology. *Clin Exp Nephrol* (2003) **7**(4):255–9. doi:10.1007/s10157-003-0259-6
99. Rood IM, Deegens JK, Wetzels JF. Genetic causes of focal segmental glomerulosclerosis: implications for clinical practice. *Nephrol Dial Transplant* (2012) **27**(3):882–90. doi:10.1093/ndt/gfr771
100. Yuan H, Takeuchi E, Taylor GA, McLaughlin M, Brown D, Salant DJ. Nephrin dissociates from actin, and its expression is reduced in early experimental membranous nephropathy. *J Am Soc Nephrol* (2002) **13**(4):946–56.
101. Liu Y, Yerushalmi GM, Grigera PR, Parsons JT. Mislocalization or reduced expression of Arf GTPase-activating protein ASAP1 inhibits cell spreading and migration by influencing Arf1 GTPase cycling. *J Biol Chem* (2005) **280**(10):8884–92. doi:10.1074/jbc.M412200200
102. Zhu J, Sun N, Aoudjit L, Li H, Kawachi H, Lemay S, et al. Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes. *Kidney Int* (2008) **73**(5):556–66. doi:10.1038/sj.ki.5002691
103. Asanuma K, Yanagida-Asanuma E, Faul C, Tomino Y, Kim K, Mundel P. Synaptopodin orchestrates actin organization and cell motility via regulation of RhoA signalling. *Nat Cell Biol* (2006) **8**(5):485–91. doi:10.1038/ncb1400
104. Kistler AD, Altintas MM, Reiser J. Podocyte GTPases regulate kidney filter dynamics. *Kidney Int* (2012) **81**(11):1053–5. doi:10.1038/ki.2012.12
105. Yoo TH, Pedigo CE, Guzman J, Correa-Medina M, Wei C, Villarreal R, et al. Sphingomyelinase-like phosphodiesterase 3b expression levels determine podocyte injury phenotypes in glomerular disease. *J Am Soc Nephrol* (2014) **26**(1):133–47. doi:10.1681/ASN.2013111213
106. Gautier A, Bernhard W, Oberling C. [The existence of a pericapillary lacunar apparatus in the malpighian glomeruli revealed by electronic microscopy]. *C R Seances Soc Biol Fil* (1950) **144**(23–24):1605–7.
107. Elias H, Allara E, Elias PM, Murthy AS. The podocytes, re-examined. *Z Mikrosk Anat Forsch* (1965) **72**(2):344–65.
108. Neal CR, Crook H, Bell E, Harper SJ, Bates DO. Three-dimensional reconstruction of glomeruli by electron microscopy reveals a distinct restrictive urinary subpodocyte space. *J Am Soc Nephrol* (2005) **16**(5):1223–35. doi:10.1681/ASN.2004100822
109. Neal CR, Muston PR, Njegovan D, Verrill R, Harper SJ, Deen WM, et al. Glomerular filtration into the subpodocyte space is highly restricted under physiological perfusion conditions. *Am J Physiol Renal Physiol* (2007) **293**(6):F1787–98. doi:10.1152/ajprenal.00157.2007
110. Arkill KP, Qvortrup K, Starborg T, Mantell JM, Knupp C, Michel CC, et al. Resolution of the three dimensional structure of components of the glomerular filtration barrier. *BMC Nephrol* (2014) **15**:24. doi:10.1186/1471-2369-15-24
111. Salmon AH, Toma I, Sipos A, Muston PR, Harper SJ, Bates DO, et al. Evidence for restriction of fluid and solute movement across the glomerular capillary wall by the subpodocyte space. *Am J Physiol Renal Physiol* (2007) **293**(6):F1777–86. doi:10.1152/ajprenal.00187.2007

112. Kistler AD, Caicedo A, Abdulreda MH, Faul C, Kerjaschki D, Berggren PO, et al. In vivo imaging of kidney glomeruli transplanted into the anterior chamber of the mouse eye. *Sci Rep* (2014) **4**:3872. doi:10.1038/srep03872
113. Thomson SC, Blantz RC. Biophysics of glomerular filtration. *Compr Physiol* (2012) **2**(3):1671–99. doi:10.1002/cphy.c100089
114. Endlich N, Endlich K. The challenge and response of podocytes to glomerular hypertension. *Semin Nephrol* (2012) **32**(4):327–41. doi:10.1016/j.semnephrol.2012.06.004
115. Morton MJ, Hutchinson K, Mathieson PW, Witherden IR, Saleem MA, Hunter M. Human podocytes possess a stretch-sensitive, Ca2+-activated K+ channel: potential implications for the control of glomerular filtration. *J Am Soc Nephrol* (2004) **15**(12):2981–7. doi:10.1097/01.ASN.0000145046.24268.0D
116. Kim EY, Alvarez-Baron CP, Dryer SE. Canonical transient receptor potential channel (TRPC)3 and TRPC6 associate with large-conductance Ca2+-activated K+ (BKCa) channels: role in BKCa trafficking to the surface of cultured podocytes. *Mol Pharmacol* (2009) **75**(3):466–77. doi:10.1124/mol.108.051912
117. Reiser J, Polu KR, Möller CC, Kenlan P, Altintas MM, Wei C, et al. TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. *Nat Genet* (2005) **37**(7):739–44. doi:10.1038/ng1592
118. Dryer SE, Reiser J. TRPC6 channels and their binding partners in podocytes: role in glomerular filtration and pathophysiology. *Am J Physiol Renal Physiol* (2010) **299**(4):F689–701. doi:10.1152/ajprenal.00298.2010
119. Kim EY, Suh JM, Chiu YH, Dryer SE. Regulation of podocyte BK(Ca) channels by synaptopodin, Rho, and actin microfilaments. *Am J Physiol Renal Physiol* (2010) **299**(3):F594–604. doi:10.1152/ajprenal.00206.2010
120. Etienne-Manneville S. Actin and microtubules in cell motility: which one is in control? *Traffic* (2004) **5**(7):470–7. doi:10.1111/j.1600-0854.2004.00196.x
121. Kim EY, Choi KJ, Dryer SE. Nephrin binds to the COOH terminus of a large-conductance Ca2+-activated K+ channel isoform and regulates its expression on the cell surface. *Am J Physiol Renal Physiol* (2008) **295**(1):F235–46. doi:10.1152/ajprenal.00140.2008
122. Kim EY, Ridgway LD, Zou S, Chiu YH, Dryer SE. Alternatively spliced C-terminal domains regulate the surface expression of large conductance calcium-activated potassium channels. *Neuroscience* (2007) **146**(4):1652–61. doi:10.1016/j.neuroscience.2007.03.038
123. Beisel KW, Rocha-Sanchez SM, Ziegenbein SJ, Morris KA, Kai C, Kawai J, et al. Diversity of Ca2+-activated K+ channel transcripts in inner ear hair cells. *Gene* (2007) **386**(1–2):11–23. doi:10.1016/j.gene.2006.07.023
124. Pavenstadt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* (2003) **83**(1):253–307. doi:10.1152/physrev.00020.2002

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The importance of podocyte adhesion for a healthy glomerulus

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Podocytes are specialized epithelial cells that cover the outer surfaces of glomerular capillaries. Unique cell junctions, known as slit diaphragms, which feature nephrin and Neph family proteins in addition to components of adherens, tight, and gap junctions, connect adjacent podocyte foot processes. Single gene disorders affecting the slit diaphragm result in nephrotic syndrome in humans, characterized by massive loss of protein across the capillary wall. In addition to specialized cell junctions, interconnecting podocytes also adhere to the glomerular basement membrane (GBM) of the capillary wall. The GBM is a dense network of secreted, extracellular matrix (ECM) components and contains tissue-restricted isoforms of collagen IV and laminin in addition to other structural proteins and ECM regulators such as proteases and growth factors. The specialized niche of the GBM provides a scaffold for endothelial cells and podocytes to support their unique functions and human genetic mutations in GBM components lead to renal failure, thus highlighting the importance of cell–matrix interactions in the glomerulus. Cells adhere to ECM via adhesion receptors, including integrins, syndecans, and dystroglycan and in particular the integrin heterodimer α3β1 is required to maintain barrier integrity. Therefore, the sophisticated function of glomerular filtration relies on podocyte adhesion both at cell junctions and at the interface with the ECM. In health, the podocyte coordinates signals from cell junctions and cell–matrix interactions, in response to environmental cues in order to regulate filtration and as our understanding of mechanisms that control cell adhesion in the glomerulus develops, then insight into the effects of disease will improve. The ultimate goal will be to develop targeted therapies to prevent or repair defects in the filtration barrier and to restore glomerular function.

Keywords: podocyte, adhesion and signaling molecules, cell junction, extracellular matrix, nephrotic syndrome

INTRODUCTION

The glomerulus is a highly sophisticated organelle that performs selective filtration of circulating blood. With a diameter of between 110 and 280 μm in humans (1), the glomerulus is a spherical bundle of capillaries contained by a cellular Bowman's capsule. The capillaries are lined by fenestrated endothelial cells and covered by specialized epithelial cells known as podocytes (**Figure 1**). Between the cell layers, there is a thick glomerular basement membrane (GBM) providing a structural scaffold to support the capillary wall. Endothelial cells and their associated glycocalyx (2), the GBM, and podocytes together form the glomerular filtration barrier, which allows free permeability to water and small solutes but prevents the loss of macromolecules or cells from the blood into the primary filtrate. Each human kidney contains approximately 1 million glomeruli, and they perform this selective filtration to generate a remarkable 180 l of filtrate per day (3).

Glomerular disease is characterized by reduced barrier integrity with consequent loss into the urine of protein (proteinuria) and or blood cells (hematuria). Barrier dysfunction is characterized by flattening or effacement of podocyte foot processes, as visualized by electron microscopy (**Figure 2**). Causes of barrier disruption range from congenital disorders associated with genetic mutations to acquired disease linked to a range of inflammatory or metabolic disturbances, which may specifically target the glomerulus or be part of a wider systemic illness, such as diabetes mellitus. Persistent glomerular dysfunction with proteinuria leads to chronic and ultimately end-stage kidney disease with a rapidly accelerating impact on worldwide healthcare costs. Dialysis and transplantation therapies are not globally accessible and the risk of recurrent glomerular disease following transplantation can be as high as 30% (4). There are currently limited therapies to slow the progression of glomerular disease and, therefore, a significant need to build our understanding about both normal glomerular function and the dysfunction associated with pathology.

The integrity of the glomerular filtration barrier depends on both cell–cell adhesion and cell–matrix adhesion, which have been predominantly investigated in podocytes, although undoubtedly critical for the glomerular endothelium. Cell–cell and cell–matrix adhesion receptors connect adjacent cells or matrix ligands to the cellular cytoskeleton, and they are vital conduits for the

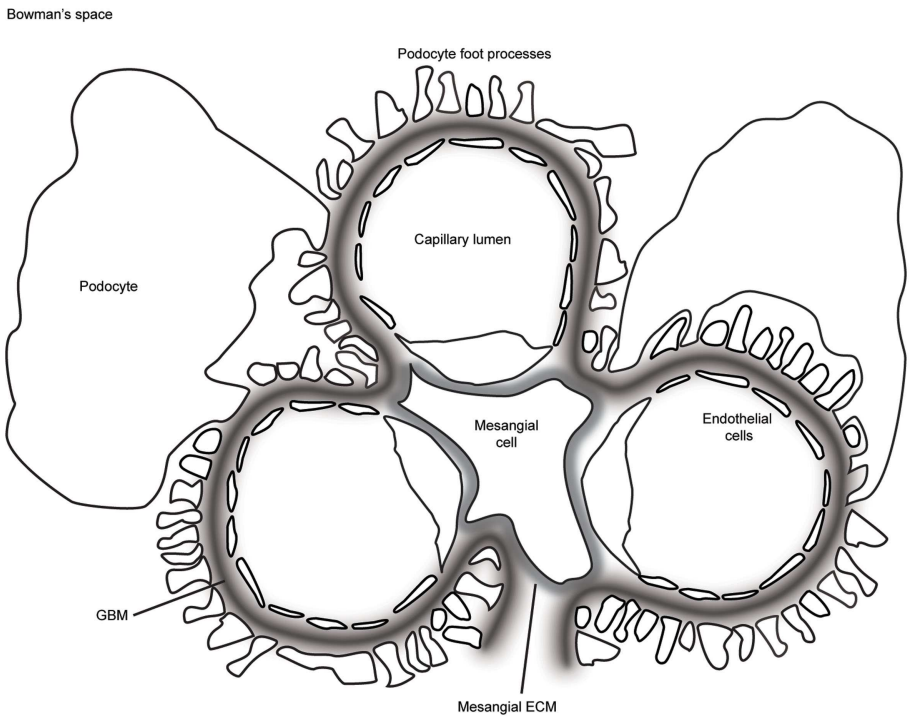

FIGURE 1 | A schematic representation of the glomerulus in cross-section. The glomerular capillaries are lined on the inside with fenestrated endothelial cells, which are attached to the glomerular basement membrane (GBM). Podocytes cover the outer aspect of GBM with large cell bodies and inter-digitating foot processes. Mesangial cells and their associated extracellular matrix (ECM) connect adjacent capillaries, and the capillary bundle is contained within Bowman's capsule.

transmission of signals in and out of cells. These receptors are highly conserved in evolution and are a fundamental requirement for tissue development. The glomerular filtration barrier is a highly complex structure, and we require deep understanding to appreciate how the barrier is formed during development, regulated in health, and disrupted in disease. This review focuses on the importance of podocyte adhesion for a healthy glomerulus. Cell–matrix adhesion is introduced initially with a review of the glomerular extracellular matrix (ECM) and our understanding about cell adhesion to ECM ligands. Cell–cell adhesion follows with a review of the unique podocyte slit diaphragm. Finally, we discuss the prospects for therapy to target defects in adhesion for patients with glomerular disease.

THE GLOMERULAR EXTRACELLULAR MATRIX

Extracellular matrix is essential for multicellular life providing a structural scaffold with appropriate mechanical properties to support adjacent cells (5). It comprises a complex network of glycosaminoglycans and fibrous proteins, which are synthesized and secreted by cells. Podocytes and glomerular endothelial cells adhere to ECM networks via cell surface receptors. This cell–ECM interface forms a signaling platform that controls all aspects of cell fate decisions, including shape, growth, differentiation, and survival (5, 6). In addition to this signaling platform, the ECM modulates cell–cell signaling by sequestering secreted growth factors and cytokines, forming reservoirs for controlled release (5).

Basement membranes are condensed sheets of ECM with a supramolecular assembly built around two major networks of laminin and collagen IV. In the glomerulus, ECM is organized as the GBM of the capillary walls and basement membrane of Bowman's capsule, in addition to the loose mesangial ECM between capillary loops (**Figure 1**). The mature GBM is thicker than most basement membranes (300–350 nm in humans), and it represents a fusion of two membranes, one derived from podocytes and the second from endothelial cells during glomerular development (7). The study of human glomerular disease led to the discovery of tissue-restricted isoforms of laminin and collagen IV in the mature GBM, and these are key components of this specialized extracellular niche (8, 9).

Laminins are self-assembling heterotrimeric glycoproteins (10, 11) and an absolute requirement for basement membrane formation (12, 13). All laminin heterotrimers contain α, β, and γ chains. The trimeric protein has a cruciform shape with one long arm and three short arms and the short arms contain the amino terminal (LN) domains of the laminin heterotrimer. These short arms also form the nodes within the laminin network, exclusively via interactions between the chain specific LN domains (14–16). The long arms contain the globular (LG) domains with cell surface receptor binding sites (17–21).

The developing GBM contains laminin α5β1γ1 (laminin-511), but the mature GBM comprises predominately laminin-521. A complete laminin-521 network is the key for a functional GBM

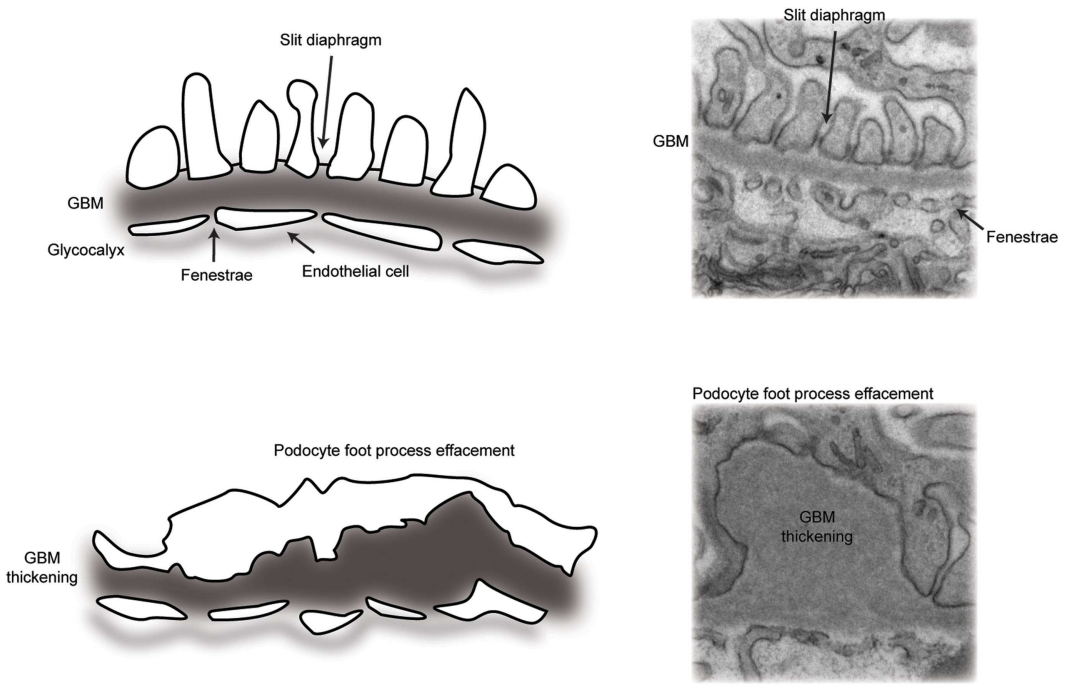

FIGURE 2 | A schematic representation of the glomerular filtration barrier. (Top panel) transmission electron micrograph and schematic of the normal architecture of the multi-layered glomerular filtration barrier. (Bottom panel) transmission electron micrograph and schematic of glomerular filtration barrier defects, including loss of slit diaphragms and podocyte foot process effacement, in addition to thickening of the glomerular basement membrane.

as mutations in *LAMB2*, the gene encoding the laminin β2 chain, cause Pierson syndrome in human beings. Affected individuals have a spectrum of pathology dependent on the type of mutation with truncating mutations causing congenital nephrotic syndrome, microcoria, muscular hypotonia, and neurodevelopmental deficit (22, 23). *Lamb2* mutations in mice are also associated with glomerular dysfunction. Mice with null mutations die after 3 weeks of age with severe proteinuria and neuromuscular defects (24). These animals have accumulation of ectopic laminin chains in the GBM, including α1, α2, α3, β1, β3, and γ2; however, these chains do not compensate for the loss of the β2 chain, possibly due to low expression or the absence of a complete laminin network (25). The theory that insufficient expression of laminin chains accounts for the observed lack of compensation is supported by the finding that podocyte overexpression of *Lamb1* in *Lamb2* null mice ameliorates proteinuria (26).

Unlike the laminin network, the collagen IV network is dispensable for basement membrane formation; however, it appears to be important for strength and stability (27). Collagen IV forms heterotrimers comprising three alpha chain combinations (α1α1α2, α3α4α5, or α5α5α6). Each alpha chain contains three distinct domains; an amino terminal 7S domain rich in cysteines and lysines, which is essential for inter-chain crosslinking through disulfide bonds and lysine/hydroxylysine crosslinks; a long collagenous repeat domain, around 1400 amino acids in length; and a carboxy terminal non-collagenous domain (NC1) (28). A novel chemical bond, not previously identified in biomolecules, the sulfilimine bond (-S=N-), was recently discovered in collagen IV. This bond crosslinks lysine/hydroxylysine-211 and methionine-93 of adjoining protomers in the NC1 domains of both collagen IV α1α1α2 and α3α4α5, which may provide additional resistance of the network to mechanical strain (29). Furthermore, peroxidasin, an enzyme found in basement membranes, catalyzes the formation of the sulfilime bond (30), and in ground breaking recent work, ionic bromide was shown to be a cofactor required for peroxidasin-catalyzed formation of the sulfilimine crosslinks in collagen IV networks (31), thus describing the first known essential function for bromine in animals.

From the capillary loop stage of glomerular development, the GBM comprises predominantly α3α4α5 networks of collagen IV, and as with laminin, the developmental collagen IV transition is critical for GBM maturation. Mutations leading to a reduction or absence of the α3α4α5 networks cause human Alport syndrome characterized by a renal phenotype of hematuria, proteinuria, and progressive renal failure (28, 32). The GBM in Alport syndrome has increased collagen IV α1α1α2, which is unable to compensate for the lack of the α3α4α5 network. As a consequence, the GBM develops splits and a typical basket-weave appearance, leading to speculation that mechanical strain cannot be tolerated perhaps due to fewer disulfide bonds in the α1α1α2 network relative to α3α4α5 and consequently a weaker GBM. This concept is further supported by the observation that reducing mechanical strain in the glomerulus with angiotensin-converting enzyme (ACE) inhibitors, which lower blood pressure as well as transcapillary filtration pressure, significantly delays disease progression in Alport syndrome (33–35).

The laminin and collagen IV networks are indirectly linked via nidogens (36) and the heparan sulfate proteoglycans, perlecan (37, 38), and agrin (39). Podocyte-specific deletion of agrin from the GBM resulted in a significant reduction in the negative charge associated with the barrier, however, alone or combined with knockout of perlecan, agrin deletion was not associated with proteinuria, therefore questioning the role of charge selection in glomerular filtration (40). Nidogen 1 and 2 are dumbbell-shaped proteins and bind to both laminin and collagen IV. Mice with knockout of either nidogen 1 or 2 are viable and have normal basement membranes. Deletion of both isoforms, however, causes perinatal lethality (41). This is consistent with a degree of redundancy in their ability to bind collagen IV and laminin. Surprisingly, the GBM has a normal appearance even in the double (*Nid-1*, *Nid-2*) knockout. This suggests that nidogen is dispensable for the formation of the GBM, but again it may be required for the GBM to resist mechanical strain. Taken together, it is likely that agrin, perlecan, and nidogens are important for overall basement membrane strength by contributing to the crosslinking of the collagen and laminin networks to each other and to the cell surface.

While these and other investigations detail the composition of the glomerular ECM, it has been more challenging to elucidate the relative position of ECM proteins in the GBM. However, a systematic analysis of the spatial arrangement of ECM components within basement membranes, with respect to each other and to their cell–adhesion receptors, was recently performed using super resolution microscopy (42). This investigation found two separate laminin networks, one produced by podocytes the other produced by endothelial cells. The collagen IV α3α4α5 network was distributed along the center of the GBM alongside nidogen, consistent with its putative crosslinking function. The human GBM is approximately twofold thicker than the mouse GBM, and interestingly, this study found increased thickness of the human collagen IV a3a4a5 network, and potentially, an additional layer of laminin-521 closer to the center of the GBM (42).

Thus, candidate-based investigations of the glomerular ECM have significantly advanced our understanding about key components. However, more recently unbiased, global approaches have shown that the glomerular ECM is a highly complex extracellular niche. In our own proteomic analysis of human glomerular ECM, we identified 144 structural and regulatory ECM proteins and found that more than 50% were expressed in the GBM (43). Together with the analysis of cell-derived ECM produced by glomerular cells in culture, we found a common core of highly connected and clustered ECM proteins, which may be important for ECM assembly (44). Overall, the glomerular ECM is a complex scaffold of interacting proteins, which are likely to be highly dynamic and are unique in order to support the complex function of the glomerular filtration barrier.

PODOCYTE ADHESION TO THE GBM

In order to adhere to the GBM, podocytes and endothelial cells utilize transmembrane adhesion receptors and the cell–matrix adhesion of podocytes was recently reviewed elsewhere (45). Adhesion receptors contain extracellular domains, which can bind to specific ECM proteins and intracellular domains that recruit effector proteins and link adhesion receptors to the cell cytoskeleton (**Figure 3**). A major family of proteins responsible for cell–ECM adhesion is the integrins.

Integrins are αβ-heterodimers that propagate signals from within the cell to the immediate extracellular environment in addition to outside–in signaling. All integrins link to the actin cytoskeleton, with the exception of α6β4, which links to intermediate filaments (6). Conformational changes in these receptors are central to the regulation of integrin receptor activity. Integrins adopt either a low affinity bent conformation, a primed or active high affinity extended conformation, or a ligand occupied state (46). Integrins form 24 different αβ combinations (6), which have differing affinities for ECM ligands and also have differential recruitment of proteins to their cytoplasmic domains (47). Laminin-binding integrins include α1β1, α2β1, α3β1, α6β1, α10β1, and α7β1, and the collagen binding integrins are α1β1, α2β1, α10β1, α11β1, and αXβ2 (48).

Upon integrin engagement of the ECM, there is integrin clustering and activation. Integrins lack intrinsic enzymatic activity; therefore, in order to propagate signals into the cell, active integrins must recruit a number of adaptor and effector proteins into sites known as focal adhesions. At least 232 protein components are recruited to adhesion complexes in a cell type and context dependent manner, demonstrating the potential for adhesion signaling to bring about different cellular outcomes (49). In addition, global analyses of adhesion complexes using mass spectrometry suggest an even larger number of proteins may be recruited to focal adhesions (50–52). Examples of the groups of proteins recruited to sites of active integrins are adaptors, actin remodeling proteins, signaling proteins, GTPase regulators including guanine-nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) in addition to numerous serine, threonine and tyrosine kinases, and phosphatases. This signaling nexus controls all aspects of cell fate and so does not just act as a mere anchoring point for cell attachment but also a signaling hub to alter cell behavior. Furthermore, integrin association and cross talk with other transmembrane receptors such as syndecans increases the potential for regulation of integrin-mediated adhesion (53, 54).

The α3β1 heterodimer is the most highly expressed integrin on the podocyte cell surface, and is thought to be the most important link between the podocyte and the GBM (55–57). Homozygous mutations in *ITGA3,* the gene encoding integrin α3, in humans leads to congenital nephrotic syndrome, interstitial lung disease and epidermolysis bullosa (58) with defects in the GBM. In addition, a mutation in *ITGA3* causing a gain of glycosylation and preventing α3β1 dimer formation causes fatal interstitial lung disease and congenital nephrotic syndrome (59). This phenotype is recapitulated in mice lacking the integrin α3 subunit, which die within the first day of life due to developmental defects in the kidneys and lungs, including loss of specialized podocyte morphology and thickened irregular GBMs (60). Moreover, podocyte-specific deletion of *Itga3* in the mouse also resulted in a disorganized GBM with thickening and protrusions and an inability of podocytes to form mature foot processes (61). Human mutations affecting integrin-β1 have not been described, to date, and this may be due to embryonic lethality since integrin-β1 forms at least 12 heterodimers. However, the role of this integrin has been studied with podocyte-specific deletion of *Itgb1* in the mouse. This resulted

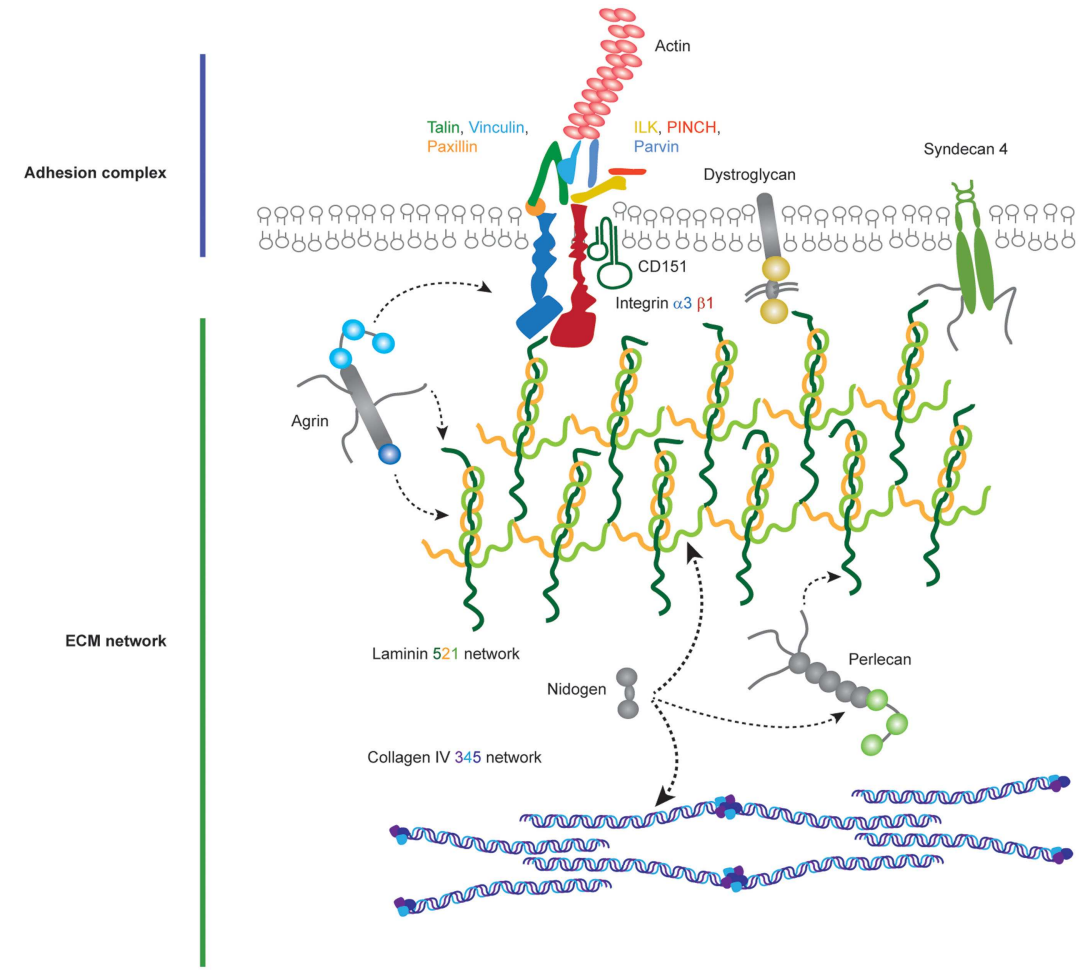

FIGURE 3 | Molecular components of the podocyte cell–matirx interface. Podocytes adhere to the underlying GBM using transmembrane adhesion receptors. The laminin-binding integrin α3β1 and the associated tetraspannin CD151 are highly expressed on the podocyte cell surface, in addition to adhesion receptors for other ECM ligands. Adhesion complexes form when activated integrins recruit adaptor, scaffold, and signaling proteins to their cytoplasmic tails. Integrins link to the actin cytoskeleton via two major axes; talin, vinculin, paxillin and integrin-linked kinase (ILK), PINCH, parvin. Podocytes attach to an ECM network containing laminin-521 networks, collagen IV α3α4α5 networks, agrin, perlecan, and nidogen.

in a severe phenotype of proteinuria from birth and renal failure by 3 weeks featuring both glomerular and tubular pathologies (62, 63).

The tetraspannin CD151 binds tightly to integrin α3β1 (64) and humans with mutations in *CD151* develop hematuria and proteinuria progressing to end-stage kidney disease in addition to pretibial epidermolysis bullosa, sensorineural deafness, and β-thalassemia minor (65). In mice, deletion of *Cd151*, both globally and specifically in podocytes, caused early proteinuria with abnormalities of the GBM loss of podocyte foot processes, glomerulosclerosis, loss of podocytes, and renal failure. This phenotype, however, is dependent on the genetic background of the mice, with *Cd151*-knockout mice on the FVB background displaying the pathological phenotype (61, 66, 67). It is hypothesized that CD151 increases the strength of podocyte adhesion to the GBM via integrin α3β1 engagement with laminin-521. *Cd151*-knockout mice on the C57BL/6 background do not spontaneously develop renal failure but when challenged with induced hypertension, they develop significant proteinuria. Furthermore, treatment of the susceptible *Cd151*-knockout FVB strain with ACE inhibitors ameliorated progression of renal failure. In addition to *in vivo* experiments, *in vitro* experiments showed that podocytes lacking CD151 lose their resistance to shear stress when cultured on laminin (67). This evidence supports a crucial role for integrin α3β1 as a major adhesion receptor, and in combination with CD151, a complex necessary to withstand mechanical forces within the glomerulus.

The laminin-binding integrin α6β4 may also be the key for the development and maintenance of the glomerular filtration barrier. Human mutations in *ITGB4* have been described and associated with junctional epidermolysis bullosa and pyloric atresia (68, 69). In one of these patients, there was coincident nephrotic range proteinuria and the study demonstrated reduced expression of integrin-β4 in podocytes; however, the possibility of an alternative genetic explanation for the glomerular dysfunction in this

case remains possible. Integrin αvβ3 has also been implicated in glomerular dysfunction (70, 71). This fibronectin receptor was shown to be activated following the induction of urokinase receptor (uPAR) signaling leading to increased podocyte motility and activation of GTPases. In a subsequent study, the same team identified soluble uPAR as a potential pathogenic mediator of disease in nephrotic syndrome associated with focal segmental glomerulosclerosis (FSGS) where there was also activation of integrin-β3 (72). These studies raise the possibility that abnormal integrin activation in the podocyte alters cell motility and this signaling pathway could potentially be targeted therapeutically.

In addition to the integrin family of adhesion receptors, transmembrane heparan sulfate proteoglycan receptors, such as the Syndecan family, are key regulators of cell-ECM interactions (54). Cooperation of integrins and syndecans in adhesion formation has been shown on a variety of ECM ligands including fibronectin, vitronectin, and laminin (73–76). Syndecans regulate intergrin trafficking to the cell surface (77), a process used by cells to regulate adhesion formation and disassembly (78–80). In addition to modulating integrin dynamics, syndecans facilitate growth factor binding to their receptors (81, 82). In podocytes null for EXT1, a key molecule in herparan sulfate glycosaminoglycan assembly, adhesion complexes were reduced in size, the actin cytoskeleton was rearranged, and cell surface syndecan 4 upregulated (83). However, mice null for EXT1 specifically in podocytes do not develop significant proteinuria, despite some podocyte abnormalities, including a degree of foot process effacement (84). In podocytes, autocrine signaling by the soluble vascular endothelial growth factor receptor, sFLT1, also causes actin rearrangements and this is associated with phosphorylation of both syndecan 1 and 4 within their EFYA motifs (85). Thus, there is accumulating evidence that syndecans contribute to cell–matrix adhesion and signaling in podocytes.

Dystroglycan is a cell surface adhesion receptor and comprises a highly glycosylated extracellular α-dystroglycan subunit, which can bind to laminins, and a non-covalently linked intracellular β-dystroglygan subunit that links to the actin cytoskeleton via an interaction with utrophin (86, 87). Dystroglycan is expressed by podocytes (88) and the expression pattern is altered in glomerular pathologies (89, 90). Therefore, it seemed likely that dystroglycan was important for podocyte adhesion; however, defective glycosylation of α-dystroglycan, which abrogates α-dystroglycan-laminin interactions does not cause proteinuria, only mild podocyte foot process effacement (91). Furthermore, podocyte-specific deletion of dystroglycan in mice caused only mild GBM thickening (92). These data suggest that dystroglycan is not a critical adhesion receptor in podocytes.

FOCAL ADHESION COMPLEXES

A number of proteins link integrins to the actin cytoskeleton and form focal adhesions (**Figure 3**). One such is talin-1, a 270 kDa protein comprising an N-terminal globular head and flexible rod domain. The head domain contains a FERM domain with binding sites for the integrin-β subunit cytoplasmic tail, F-actin, focal adhesion kinase (FAK), and PIPK1γ90. The rod domain contains an additional binding site for integrin, actin binding sites, and multiple vinculin binding sites, and this domain can also bind to RIAM (93). Finally, the C-terminal domain contains helices responsible for talin dimerization. Binding of talin to the cytoplasmic tail of β-integrins triggers a conformational change in the extracellular domain of integrins, which amplifies the affinity of the integrin for the ECM. Talin dependent recruitment of further proteins to active integrins causes the consequent formation of focal adhesions (94). Talin-1 expression in podocytes is required for the specialized actin morphology of foot processes. Podocyte-specific *Tln1*-knockout mice develop proteinuria and die within 10 weeks. These mice, however, did not have major defects in integrin β1 activation or podocyte adhesion. Nevertheless, the actin cytoskeleton was perturbed, and there was podocyte foot process effacement. These data show that talin-1, a protein known to be important in adhesion formation and linkage to the actin cytoskeleton *in vitro*, is a key player in relaying signals from integrins to the actin cytoskeleton in podocytes *in vivo* (95).

Another key adaptor protein involved in integrin-mediated adhesion complex formation is vinculin, a 123 kDa protein recruited by talin to focal adhesions and capable of binding to the actin cytoskeleton (96). Vinculin comprises an N-terminal head, proline rich neck, and a C-terminal tail domain (97, 98). Cytoplasmic vinculin assumes an autoinhibited inactive conformation (99) and following talin recruitment, vinculin undergoes a conformational change revealing an open active state (100). Vinculin is a force regulator and when extended by forces applied through actin, there is subsequent recruitment and release of focal adhesion proteins (101). This conformational change allows vinculin to directly interact with a number of proteins including α-actinin, Arp2/3, actin, and paxillin (102, 103). The vinculin head domain modulates integrin clustering, whereas the tail domain links to actin. Considering vinculin is an important link between integrins and the actin machinery, this protein may have a key role in the force sensing by podocytes via integrin α3β1.

Paxillin is another component of focal adhesions and it acts as a scaffolding protein. It contains multiple protein-binding modules, many of which are regulated by phosphorylation. It localizes to focal adhesions through phosphorylation of its C-terminal LIM domains (104, 105). Paxillin is an important molecular adaptor; its N-terminus controls most of its signaling activity that provides docking sites for vinculin, FAK, Src, and Crk. Paxillin is recruited to focal adhesion by talin (106) and brings about spatiotemporal control of Rho family small GTPases by recruiting numerous GEFs and GAPs (107).

Another highly studied focal adhesion protein is FAK. FAK is non-receptor tyrosine kinase, recruited to focal adhesions by talin and paxillin (108). FAK has a number of roles at focal adhesion sites, including recruitment of p130Cas, Crk1/2, and Src family kinases (109). Global deletion of FAK in mice is lethal in embryogenesis, causing a profound migration defect (110). The importance of FAK in podocytes was highlighted by the observation that FAK is phosphorylated upon podocyte injury (111). Surprisingly, podocyte-specific deletion of FAK in mice leads to a normal phenotype; however, these mice are protected from proteinuria and podocyte injury after experimental podocyte insults (111). Additionally, podocyte injury was reduced when a FAK inhibitor was administered in a mouse model of glomerular injury (111). A role for FAK has also been found in Alport syndrome where ectopic

laminins, α1 and α2, accumulate in the GBM. Laminin α2 caused phosphorylation of FAK at Y397, and this phosphorylation was associated with upregulation of the proteases MMP 9 and 10 and GBM defects (112). FAK inhibition reduced proteinuria, MMP levels, and GBM defects (112). These data, therefore, support a role for FAK in glomerular dysfunction.

Integrin-linked kinase binds directly to the integrin β1 cytoplasmic tail and is important for signal transduction at adhesion sites (113). ILK was originally identified as a kinase, but increasing evidence suggests that is a pseudokinase (114–119). In fact, the C-terminal kinase homology domain of ILK mediates multiple protein–protein interactions at adhesion sites, including interactions with α/β/γ-Parvin (120, 121). ILK also contains five ankyrin domains that mediate interactions with PINCH-1/2 (122–124). Kindlin 2 is another ILK interacting protein, which is expressed in podocytes, localizes to focal adhesions and through either ILK/PINCH/parvin or migfilin–filaminin interactions binds to the actin cytoskeleton (125–127). It is through this scaffolding role that ILK orchestrates focal adhesion signaling. The ILK/PINCH/parvin complex influences the actin cytoskeleton (128), in addition to negatively regulating cell contractility (129). Total loss of ILK or PINCH in mice is lethal in embryogenesis, due to failure in epiblast polarization (128, 130). The interaction between ILK and α-parvin is required for kidney development. Mutations in ILK K220 disrupt α-parvin binding and cause renal agenesis (131). Furthermore, a similar phenotype is observed when α-parvin is genetically deleted in mice (131). Podocyte-specific loss of ILK in mice causes GBM defects, loss of slit diaphragms, and podocyte foot process effacement (132, 133). Moreover, ILK interacts with nephrin at podocyte cell junctions, suggesting that ILK is a potential link between cell-cell and cell-ECM adhesion signaling (132). Finally, increased expression of ILK is observed in a variety of glomerular diseases (134, 135). This evidence strongly supports an important role for ILK in adhesion signaling in podocytes.

Overall cell adhesion to the GBM occurs at a complex cell–matrix interface (**Figure 4**). A wide range of scaffolding proteins localize to focal adhesions and transmit information regarding the extracellular environment via recruitment of effectors including kinases, and GTPases. The cellular adhesome has been predominantly investigated in the context non-adherent cells or fibroblasts but less so for epithelial cells, and similar analyses in glomerular cells will help to build our understanding about the key cellular components that are involved in cell–matrix adhesion in the glomerulus. These studies have the capacity to identify unexpected and novel proteins at adhesion sites, which are considerably more complex than previously thought.

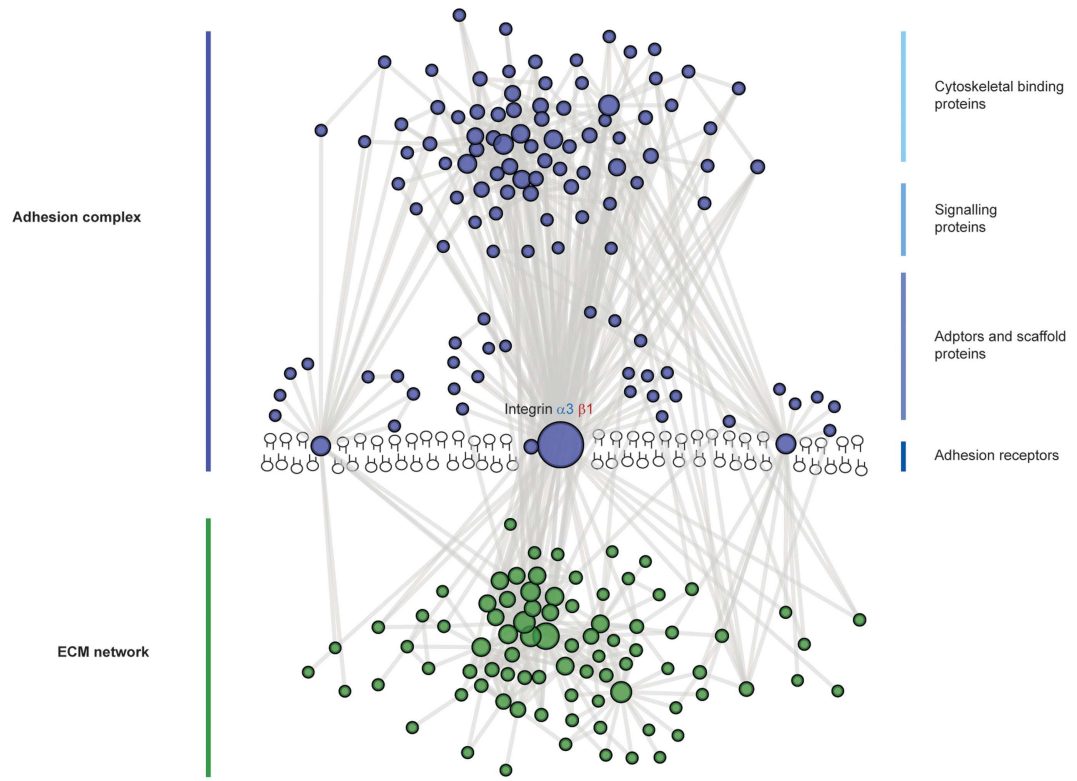

FIGURE 4 | The complexity of adhesion signaling, a protein–protein interaction view. A predicted protein–protein interaction network of adhesion signaling complexes in podocytes. Nodes represent proteins and lines between nodes represent experimentally derived protein–protein interactions. Extracellular matrix (ECM) proteins are green in color and adhesion signaling proteins are blue in color. Integrins do not have intrinsic enzymatic activity; however, through conformational changes, integrins can recruit tens to hundreds of different proteins to adhesion sites. These adhesion sites are hubs for cellular signaling, controlling all aspects of cell fate. Adhesion signaling is determined by the composition and physical properties of the extracellular matrix.

ADHESION AT THE PODOCYTE SLIT DIAPHRAGM

The junction between adjacent podocyte foot processes is termed the slit diaphragm and it is visible by electron microscopy as an electron dense structure close to the GBM. This specialized junction is thought to connect the entire length of adjacent foot processes providing a structural component to the filtration barrier. From early ultrastructural studies, a zipper-like substructure was described where protein bridges emanating from the podocyte plasma membrane link to a central filament in a lattice arrangement with rectangular pores (136). The calculated cross-sectional dimensions of these pores was 4 × 14 nm, approximately the size of an albumin molecule, and therefore, consistent with the observations from tracer studies using ferritin and dextrans that the slit diaphragm contributed significantly to the retention of macromolecules within the circulation (137, 138). Following these seminal ultrastructural and tracer studies was the discovery of the first unique slit diaphragm protein nephrin by positional cloning of the gene in congenital nephrotic syndrome of the Finnish type and leading, to further refinement of the zipper-like model of the podocyte cell junction (139, 140). More recent ultrastructural studies have used scanning electron microscopy to describe circular and ellipsoidal pores in the central region of the slit diaphragm, with a mean diameter of 12.1 nm (141). Interestingly, the same study demonstrated an increase in the size of some of these pores with proteinuria, perhaps providing an explanation for the increased transit of macromolecules across a defective filtration barrier.

Many studies have also shown that the architecture of the podocyte changes dramatically in human glomerular disease with flattening of the actin-rich foot processes and loss of slit diaphragms. Similar changes are observed in animal studies of puromycin aminonucleoside (PAN)-induced nephrotic syndrome or nephrosis (142). While these dramatic morphological changes are associated with a profound barrier defect, remarkably these changes seem to completely reverse especially in the subset of patients with nephrotic syndrome who respond to treatment with glucocorticoids.

COMPONENTS OF THE PODOCYTE SLIT DIAPHRAGM

The first junctions to form in podocytes are apical and have been described as tight junctions (143). During glomerular development, the junctional complexes descend toward the GBM and widen to become the mature slit diaphragm. These are highly specialized and unique junctions and many studies have identified components associated with more classical types of cell junctions. The zona occludens protein (ZO-1) was one of the first proteins found to localize to podocyte foot processes using immunogold labeling and electron microscopy (144). Using immunostaining of murine podocytes in culture and rat glomeruli, podocyte cell junctions were also shown to contain classical components of adherens junctions including cadherin-3 and α-, β-, and γ-catenins (145). The tight junction components JAM-A, occludin, and cingulin were also found to be associated with slit diaphragms (146) and the same study reported that PAN nephrosis increased the expression of these tight junction components. There is also a report of the gap junction protein connexin-43 localizing to the podocyte slit diaphragm, and it was found to be upregulated in the early phase of PAN nephrosis (147). Taken together, these findings suggest, not surprisingly, that there is context-dependent composition of these junctions.

In addition to components associated with other cell junctions, the slit diaphragm also contains unique proteins (**Figure 5**). Nephrin and the homologs Neph-1, Neph-2, and Neph-3 are known members of this cell junction and are comprehensively reviewed elsewhere (148). They are members of the immunoglobulin superfamily of cell-adhesion receptors and are involved in the development of specialized junctions in neurons and at the slit diaphragm (148). Orthologs of these proteins are expressed in *Drosophila* nephrocytes (149), which have nephrocyte diaphragms structures with very similar composition to the mammalian slit diaphragm (150). Interestingly, birds lack nephrin and Neph-3 but do form slit diaphragm-like structures (151). Further back in evolution, *Caenorhabditis elegans* expresses the orthologs SYG-1 (Neph1) and SYG-2 (nephrin), and these are required for synapse formation and specificity. Investigation of the crystal structures of these orthologs revealed SYG-1 homodimers with a conserved binding interface and an unusual, angled geometry in the heterophillic SYG1/2 complex (152). The crystal structures of nephrin and Neph homologs remain unresolved; however, there is some evidence for homophilic nephrin interactions. These interactions were detected using recombinant nephrin protein and surface plasmon resonance, and they were increased in the presence of calcium (153).

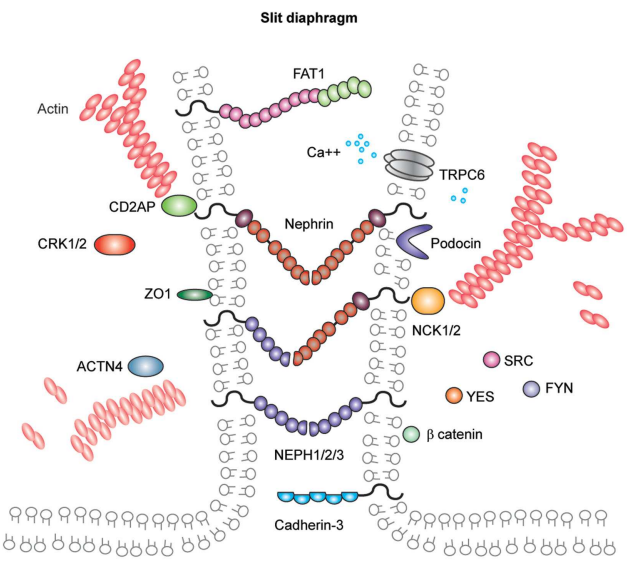

FIGURE 5 | Molecular components of the podocyte slit diaphragm. Podocyte slit diaphragms contain unique components, including nephrin, Neph family proteins, and podocin in addition to components of tight, gap, and adherens junctions, such as the cadherin-3 and FAT1. The adaptor proteins Nck1/2, CD2AP, and Crk1/2, Src family kinases, in addition to many more kinases and actin binding proteins localize to the podocyte slit diaphragm. The interactions between nephrin and other Neph proteins are thought to be homo- and heterophilic and recent structural analysis of the orthologs SYG1/2 has suggested an angled conformation (152) as depicted in this schematic.

Nephrin and Neph-1 are requisite components of the slit diaphragm. Mutations in nephrin cause human congenital nephrotic syndrome (139), which is most common in Finland, although many mutations have now been described in individuals with later onset of disease and from a diverse ethnic background (154). Infants require albumin infusions to maintain intravascular volume and ultimately proceed to removal of their kidneys prior to dialysis and transplantation. This disease phenotype is mimicked in the mouse where deletion of *Nphs1* leads to early massive proteinuria and the mice die within 24 h. Ultrastructural analysis of their glomeruli has revealed the absence of slit diaphragms and foot process effacement (155). *Neph1* deletion in mice is also associated with perinatal lethality with proteinuria and podocyte foot process effacement (156). As yet, no human mutations in *NEPH1* have been described but it is more widely expressed than nephrin, and therefore, mutations may be incompatible with life.

Other notable members of the slit diaphragm complex include podocin, a stomatin family protein that is also mutated in patients with early onset nephrotic syndrome (157). Podocin is important for the recruitment of proteins to the slit diaphragm complex and for facilitating signaling (158). CD2AP is an adaptor protein and its role in maintaining the integrity of the filtration barrier was first described in mice (159) and more recently in human disease (160). CD2AP and CIN85 appear to be important for the balance of receptor tyrosine kinase signaling in podocytes (161). FAT atypical cadherin-1 has also been shown to regulate barrier formation and mice lacking this component have significant glomerular defects, in addition to eye and brain abnormalities (144).

The protein complex at the slit diaphragm includes the components that make the connections between adjacent podocyte foot processes in addition to the more dynamic network of proteins that assemble intracellularly (**Figure 6**). To identify novel components of this complex, unbiased approaches with mass spectrometry have led to the discovery of proteins including IQGAP (162). These global analyses will continue to assist in the identification of more unexpected components of these junctions as methods to isolate and analyze the junctions improve. To give an indication of the scale of the components, a recent bioinformatic analysis of the cadhesome has predicted an assembly of 170 components many of which may be cell type and context specific (163).

SIGNAL TRANSDUCTION AT THE SLIT DIAPHRAGM

Over the past 16 years since the discovery of nephrin, a growing number of proteins have been linked to signaling at the slit diaphragm and there are several recent, comprehensive reviews (164, 165). Phosphorylation of nephrin and Neph family proteins is the key to signal transduction. Tyrosine phosphorylation by Src family kinases initiates a signal cascade and indeed the deletion of Fyn resulted in barrier dysfunction (166). Phosphorylation by Fyn leads to the recruitment of a number of proteins including the adaptor proteins Nck1/2 (167, 168), Crk1/2 (169), CrkL (170), and Grb2 (171, 172) in addition to PI3-kinase (173, 174). Following recruitment, Nck binds phosphorylated nephrin and leads to actin reorganization via the actin nucleation factor N-WASP (175). The receptor Robo2 also links to Nck and is expressed in podocytes (176). This receptor was found to inhibit actin reorganization and it appears to negatively regulate signaling via nephrin and Nck therefore to reduce podocyte foot process effacement. Crk is recruited to phosphorylated nephrin via p130Cas and deletion of Crk1/2 attenuated podocyte foot process effacement in a glomerular injury model (169). The p85 regulatory subunit of P13-kinase also interacts with nephrin leading to downstream activation of

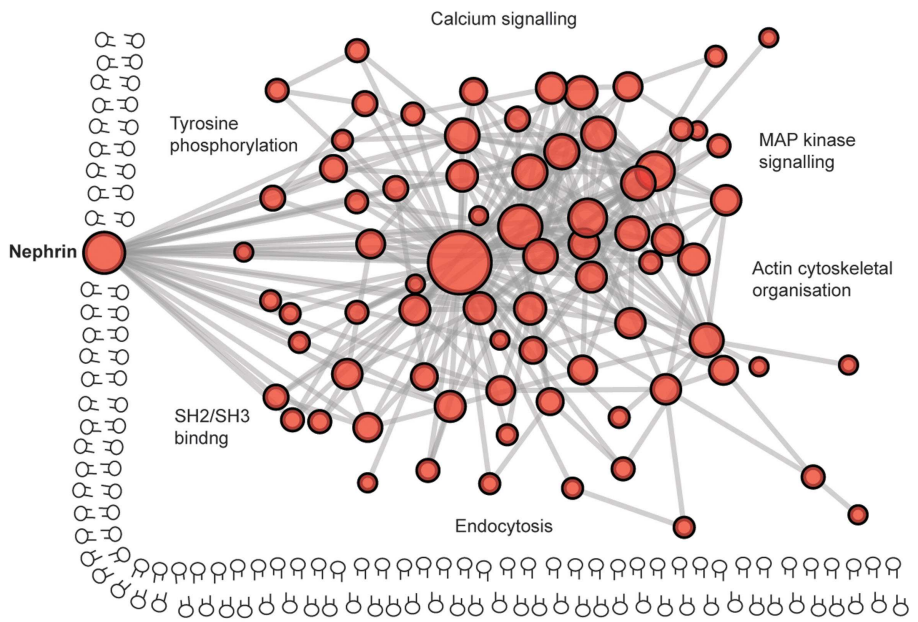

FIGURE 6 | The complexity of the podocyte slit diaphragm – a network view. A predicted protein–protein interaction network based on interactions of proteins localized to the slit diaphragm. Nodes represent proteins and lines between nodes represent experimentally derived protein–protein interactions. Labels represent Gene Ontology vocabularies significantly ($P < 0.05$) enriched in proteins localized to the slit diaphragm.

Akt (173) and subsequently to actin reorganization (174). Demonstrating the importance of signaling via Akt, deletion of the Akt2 isoform was associated with barrier dysfunction (177) and Akt phosphorylation in podocytes follows insulin signaling (178) and is also linked to mTOR signaling (177).

Regulation of the podocyte actin cytoskeleton is, therefore, the key to maintaining barrier integrity and actin reorganization in podocytes is likely to relate directly to the dramatic podocyte foot process effacement, which is seen across the spectrum of disease. The actin crosslinking protein alpha-actinin-4 has been associated with human nephrotic syndrome and mutations in *ACTN4* are associated with adult onset FSGS (179). Here, the mutated alpha-actinin-4 protein binds filamentous actin more strongly than the wild type protein, indicating that actin regulation is important for normal podocyte function. Another class of actin regulators is the GTPases, which in turn are regulated by GEFs, GAPs, and GDIs. Podocyte-specific deletion of the GTPase RhoA did not result in a barrier defect (180); however, activation of RhoA has been described in a number of glomerular injury models in addition to human disease including mutations in the formin INF2, leading to the commonest cause of adult onset FSGS (181). A number of studies have now investigated the role of Rac1 in podocytes, which is required for lamellipodia formation. Rac1 is not essential for glomerular development but overexpression leads to barrier dysfunction either with constitutive activation of Rac1 (182) or RhoGDI-alpha knockout (183). However, a number of studies have also shown that Rac1 is protective (170, 184) and it is likely that a fine balance is required. The GTPase CDC42 is linked to the formation of filopodia and its absence results in early barrier dysfunction (180, 185) and this may be due to links with apical-basal polarity proteins, which are also required for slit diaphragm formation (186).

The dynamic regulation of this specialized cell junction undoubtedly requires quality control and recycling of components, and it was recently shown using rat glomeruli that the turnover rates of slit diaphragm proteins was high and was regulated by atypical protein kinase C (aPKC) (187). Accordingly, the endocytic pathway components dynamin, synaptojanin, and endophilin have been shown to be important for maintaining barrier function (188). The correct localization of proteins is also the key, and nephrin localization at the plasma membrane requires the endocytic protein Myo1c (189). The long-tailed myosin, Myo1E, may also contribute to endocytosis in podocytes (190, 191). The role of calcium signaling at the slit diaphragm is also important and mutations in the transient receptor potential cation channel *TRPC6* have been associated with adult onset FSGS (192, 193).

There are still many research questions to address in order to understand how the specialized slit diaphragm is formed during development, regulated in health, and disrupted in disease. Chemical and mechanical factors in the microenvironment are likely to be the key, and although the barrier regulation by growth factors has not been discussed here, there is growing evidence for the important roles of vascular endothelial growth factor (VEGF-A) (85) and insulin (178). Regarding mechanical cues, it would seem likely that filtration forces contribute to the regulation of the barrier. The molecules spanning the slit diaphragm are directly exposed to force and it would be intriguing to determine whether some of these components respond to force in a similar manner to VE cadherin, which was recently investigated using a tension biosensor and shown to stretch in endothelial cells exposed to sheer stress (194). Understanding some of these basic mechanisms of regulation will be required to identify specific therapeutic strategies to maintain or restore glomerular function.

PROSPECTS FOR ADHESION-BASED THERAPY FOR GLOMERULAR DISEASE

Cell adhesion is clearly important to maintain normal barrier function but what are the prospects for adhesion-based therapies? Current therapies for glomerular disease include both immunomodulation and inhibition of the renin-angiotensin-aldosterone (RAAS) signaling pathway. RAAS pathway inhibition with ACE inhibitors and angiotensin receptor blockers (ARBs) are thought to act primarily by reducing glomerular hydrostatic pressure. Immunomodulatory drugs such as glucocorticoids and calcineurin inhibitors were initially thought to act via immune cells; however, these agents may also directly target the podocyte (195). The effects of existing and efficacious therapies on podocyte adhesion have not been formally tested, although there are some intriguing observations. Spironolactone (an inhibitor of aldosterone) was shown to reduce the urinary excretion of podocytes in a rat model of diabetic nephropathy (196), and this presumed reduction in podocyte detachment was associated with upregulation of integrin α3. Furthermore, in a study of human podocytes in culture, the glucocorticoid dexamethasone was shown to increase nephrin expression (197).

The manipulation of integrin activation as a possible route to therapy has been suggested in a series of recent studies. Activation of integrin-β3 has been implicated in the pathogenesis of nephrotic syndrome associated with FSGS. In a subset of patients with FSGS, there is strong evidence for the role of a circulating and disease-causing factor, which can lead to recurrence of primary disease in transplanted kidneys. Soluble urokinase receptor (suPAR) was identified as a pathogenic factor leading to activation of integrin-β3 in mouse models and human disease (72) leading to the suggestion that therapy for this condition could involve inhibition of the suPAR-integrin-β3 interaction by the use of small molecule inhibitors. Indeed, a beneficial effect of such an inhibitor has been shown in experimental glomerulonephritis (71).

Inactivation of integrin-β1 subunit has also been proposed, a mechanism of disease in patients with FSGS (198). Five patients with FSGS and positive B7-1 (CD80) immunostaining in glomeruli were treated with the B7-1 inhibitor Abatacept. This treatment was associated with a significant reduction in proteinuria in patients, otherwise resistant to standard therapies. While these discoveries suggest a role for manipulating signaling by adhesion receptors, given the myriad roles of these receptors, the challenge will be to target the treatment appropriately (199).

Disease-associated changes in ECM are likely to trigger a number of intracellular signaling events to influence cell adhesion. By understanding more about the pathways involved, it may be possible to target these therapeutically. In Alport syndrome, the primary molecular defect in the ECM is absence of the collagen IV–α3α4α5 network. Therefore, delivery of the wild type gene could be a potential future therapy and proof of principle was

recently shown by the induction *Col4a3* in *Col4 a3−/−* mice (200). Induced expression of collagen IV-α3 slowed disease progression and improved survival providing significant optimism for the prospects of gene therapy; however, the major issue will be gene delivery to the podocyte. Abnormal outside–in signaling from the ECM could also be targeted, and in another study of Alport syndrome, inhibition of FAK in mice led to partial restoration of the GBM and a reduction in proteinuria (112).

Manipulating signaling from slit diaphragm adhesion receptors may also be a future therapeutic strategy. In patients with *NPHS1* mutations, nephrin is thought to accumulate in the endoplasmic reticulum and the hypothesis that chemical chaperones would export nephrin to the plasma membrane was tested with sodium-4 phenylbutyrate in HEK293 cells. Several mutant nephrin proteins were rescued using this strategy, and there was evidence to suggest that the mutant proteins were functional (201). More recently, inhibition of deleterious Neph-1 signaling was demonstrated with a protein transduction approach involving the introduction of the Neph-1 cytoplasmic tail, which attenuated the mislocalization of Neph-1 in two models of podocyte injury (202).

Overall, these are a selection of many encouraging observations, which indicate that adhesion-based therapy may be a prospect for glomerular disease, although there is likely to be a long journey for some of these therapies to achieve ultimate patient benefit.

SUMMARY

Podocyte adhesion is evidently important for glomerular barrier integrity. Similarly, glomerular endothelial adhesion will be the key but has not yet been investigated in detail. The genetic investigations of human nephrotic syndrome have already demonstrated the range of molecular function associated with podocytopathies and severe glomerular barrier dysfunction (**Figure 7**) and these

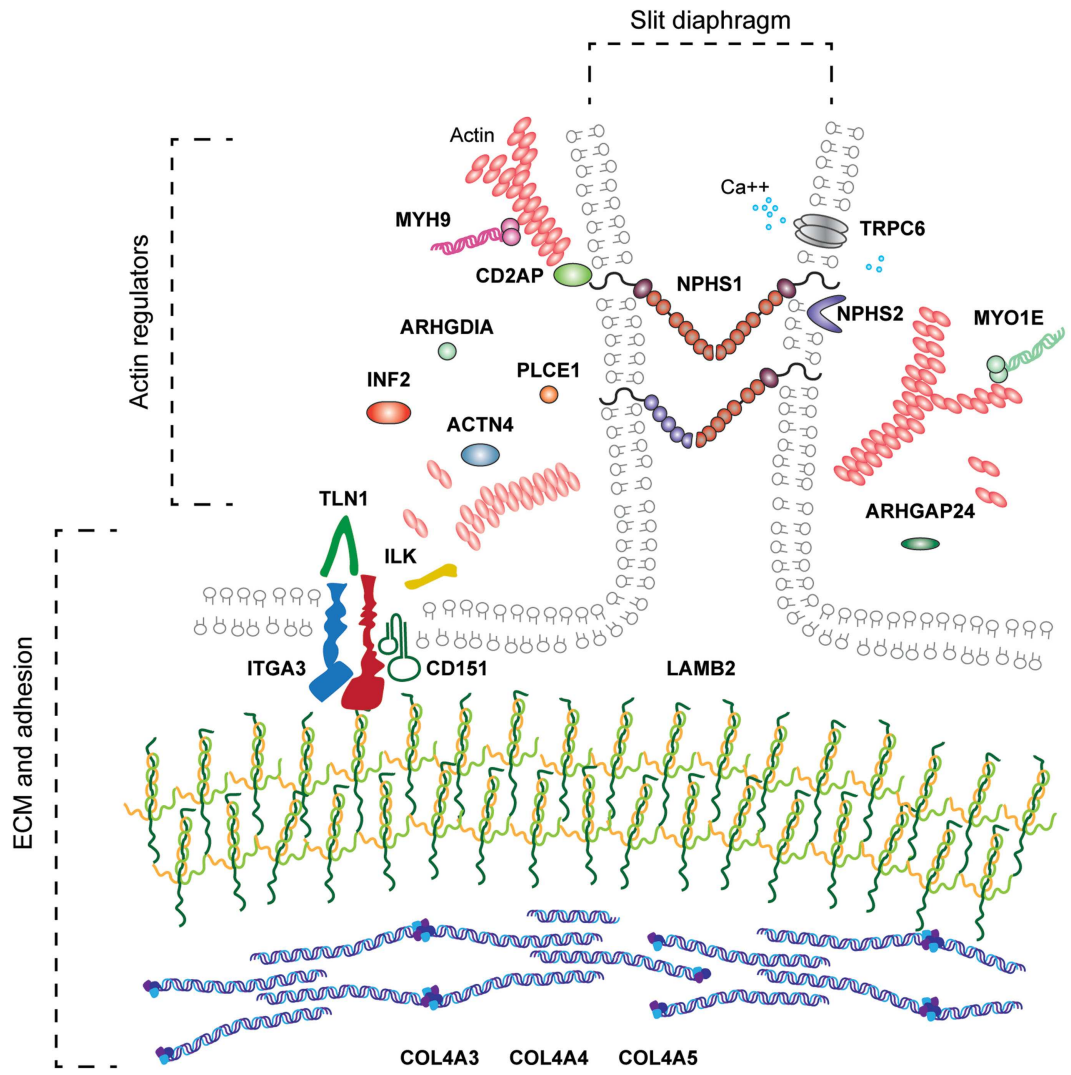

FIGURE 7 | The range of molecular functions associated with human podocytopathy. Genetic investigations in families with nephrotic syndrome have led to the discovery of molecules required for the development and or maintenance of the glomerular filter. These include molecules associated with basement membranes, ECM adhesion, cytoskeleton, slit diaphragm, mitochondrial function, and transcription factors. This review has focused on the molecules indicated in bold.

include defects in adhesion. Networks of signaling proteins at the cell–matrix interface and the cell–cell junctions are required to maintain barrier function, and cross talk between these adhesion complexes is likely to occur in response to mechanical and chemical signals from within the glomerular microenvironment. Improved understanding about cell adhesion in the glomerulus may lead to the identification of therapies to prevent or repair injury to this highly sophisticated filter.

KEY POINTS 1: MATRIX

- Extracellular matrix controls cell fate decisions and growth factor signaling.
- The GBM is the ECM compartment of the glomerular filtration barrier.
- The GBM contains at least 73 components including laminin 521 and collagen IV α3α4α5.
- Laminins are essential for basement membrane assembly.
- Collagen IV is required for structural strength of basement membranes.
- The GBM contains two laminin networks separated by a collagen IV α3α4α5 network along the centre of the GBM.
- Mutations in *LAMB2* cause Pierson syndrome in humans, and affected individuals have severe congenital nephrotic syndrome.
- Mutations leading to a reduction of the collagen IV α3α4α5 networks cause Alport syndrome humans, affected individuals have progressive renal disease.

KEY POINTS 2: CELL-MATRIX ADHESION

- Integrins link the extracellular environment to the actin cytoskeleton.
- Integrins do not have intrinsic enzymatic activity; therefore, they recruit effector proteins, which mediate adhesion signaling.
- The laminin receptor integrin α3β1 is the most highly expressed integrin on the podocyte cell surface.
- Homozygous mutations in *ITGA3* in humans lead to congenital nephrotic syndrome.
- The tetraspanin CD151 binds tightly to integrin α3β1 and individuals with mutations in *CD151* develop nephritis.
- Other podocyte cell surface receptors include syndecans and dystroglycan, but the importance of these receptors in the podocyte has yet to be fully determined.

KEY POINTS 3: ADHESION COMPLEXES

- Adhesion complexes contain over 232 components, which are dependent on cell type and context.
- Talin is an important linkage from integrins to the actin cytoskeleton; podocyte-specific talin 1 knockout mice develop proteinuria and die within 10 weeks.
- Focal adhesion kinase (FAK) is activated in podocytes during glomerular injury.
- Use of FAK inhibitors in mouse models of glomerular disease protects podocyte from injury and the animals from proteinuria.
- The ILK, PINCH, parvin axis is another key linkage from integrins to the actin cytoskeleton.
- Mice, which express ILK, that cannot bind to α-parvin display renal agenesis.
- Mice with podocyte-specific deletion of ILK express GBM defects, loss of slit diaphragms, and podocyte foot process effacement.

KEY POINTS 4: CELL-CELL ADHESION

- The junction between adjacent podocyte foot processes, termed the slit diaphragm, contains both adherens and tight junction components, in addition to unique components.
- Nephrin and the other NEPH family proteins are key members of this cell junction.
- Mutations in slit diaphrgam proteins cause nephrotic syndrome.
- Slit diaphragm signalling has a major influence on the actin cytoskeleton; adaptors such as NCK1/2, CD2AP, and CRK1/2 are involved in slit diaphragm actin linkage.

AUTHOR CONTRIBUTIONS

Rachel Lennon and Michael J. Randles researched the literature for this review; Michael J. Randles prepared the figures; and Rachel Lennon, Michael J. Randles, and Martin J. Humphries contributed to the preparation of the manuscript.

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REFERENCES

1. Samuel T, Hoy WE, Douglas-Denton R, Hughson MD, Bertram JF. Applicability of the glomerular size distribution coefficient in assessing human glomerular volume: the Weibel and Gomez method revisited. *J Anat* (2007) **210**:578–82. doi:10.1111/j.1469-7580.2007.00715.x
2. Singh A, Satchell SC, Neal CR, McKenzie EA, Tooke JE, Mathieson PW. Glomerular endothelial glycocalyx constitutes a barrier to protein permeability. *J Am Soc Nephrol* (2007) **18**:2885–93. doi:10.1681/ASN.2007010119
3. Keller G, Zimmer G, Mall G, Ritz E, Amann K. Nephron number in patients with primary hypertension. *N Engl J Med* (2003) **348**:101–8. doi:10.1056/NEJMoa020549
4. Couser W. Recurrent glomerulonephritis in the renal allograft: an update of selected areas. *Exp Clin Transplant* (2005) **3**:283–8.
5. Hynes RO. The extracellular matrix: not just pretty fibrils. *Science* (2009) **326**:1216–9. doi:10.1126/science.1176009
6. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* (2002) **110**:673–87. doi:10.1016/S0092-8674(02)00971-6
7. Tryggvason K, Patrakka J, Wartiovaara J. Hereditary proteinuria syndromes and mechanisms of proteinuria. *N Engl J Med* (2006) **354**:1387–401. doi:10.1056/NEJMra052131
8. Miner JH, Sanes JR. Collagen IV alpha 3, alpha 4, and alpha 5 chains in rodent basal laminae: sequence, distribution, association with laminins, and developmental switches. *J Cell Biol* (1994) **127**:879–91. doi:10.1083/jcb.127.3.879
9. Miner JH, Patton BL, Lentz SI, Gilbert DJ, Snider WD, Jenkins NA, et al. The laminin alpha chains: expression, developmental transitions, and chromosomal locations of alpha1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel alpha3 isoform. *J Cell Biol* (1997) **137**:685–701. doi:10.1083/jcb.137.3.685
10. Yurchenco PD, Tsilibary EC, Charonis AS, Furthmayr H. Laminin polymerization in vitro. Evidence for a two-step assembly with domain specificity. *J Biol Chem* (1985) **260**:7636–44.

11. Paulsson M. The role of Ca2+ binding in the self-aggregation of laminin-nidogen complexes. *J Biol Chem* (1988) **263**:5425–30.
12. Smyth N, Vatansever HS, Murray P, Meyer M, Frie C, Paulsson M, et al. Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. *J Cell Biol* (1999) **144**:151–60. doi:10.1083/jcb.144.1.151
13. Miner JH, Li C, Mudd JL, Go G, Sutherland AE. Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. *Development* (2004) **131**:2247–56. doi:10.1242/dev.01112
14. Schittny JC, Yurchenco PD. Terminal short arm domains of basement membrane laminin are critical for its self-assembly. *J Cell Biol* (1990) **110**:825–32. doi:10.1083/jcb.110.3.825
15. Yurchenco PD, Cheng YS. Self-assembly and calcium-binding sites in laminin. A three-arm interaction model. *J Biol Chem* (1993) **268**:17286–99.
16. Purvis A, Hohenester E. Laminin network formation studied by reconstitution of ternary nodes in solution. *J Biol Chem* (2012) **287**:44270–7. doi:10.1074/jbc.M112.418426
17. Colognato-Pyke H, O'Rear JJ, Yamada Y, Carbonetto S, Cheng YS, Yurchenco PD. Mapping of network-forming, heparin-binding, and alpha 1 beta 1 integrin-recognition sites within the alpha-chain short arm of laminin-1. *J Biol Chem* (1995) **270**:9398–406. doi:10.1074/jbc.270.16.9398
18. Colognato H, Maccarrick M, O'Rear JJ, Yurchenco PD. The laminin alpha2-chain short arm mediates cell adhesion through both the alpha1beta1 and alpha2beta1 integrins. *J Biol Chem* (1997) **272**:29330–6. doi:10.1074/jbc.272.46.29330
19. Nielsen PK, Yamada Y. Identification of cell-binding sites on the laminin alpha 5 N-terminal domain by site-directed mutagenesis. *J Biol Chem* (2001) **276**:10906–12. doi:10.1074/jbc.M008743200
20. Garbe JH, Gohring W, Mann K, Timpl R, Sasaki T. Complete sequence, recombinant analysis and binding to laminins and sulphated ligands of the N-terminal domains of laminin alpha3B and alpha5 chains. *Biochem J* (2002) **362**:213–21. doi:10.1042/0264-6021:3620213
21. Nishiuchi R, Takagi J, Hayashi M, Ido H, Yagi Y, Sanzen N, et al. Ligand-binding specificities of laminin-binding integrins: a comprehensive survey of laminin-integrin interactions using recombinant alpha3beta1, alpha6beta1, alpha7beta1 and alpha6beta4 integrins. *Matrix Biol* (2006) **25**:189–97. doi:10.1016/j.matbio.2005.12.001
22. Pierson M, Cordier J, Hervouuet F, Rauber G. An unusual congenital and familial congenital malformative combination involving the eye and kidney. *J Genet Hum* (1963) **12**:184–213.
23. Matejas V, Hinkes B, Alkandari F, Al-Gazali L, Annexstad E, Aytac MB, et al. Mutations in the human laminin beta2 (LAMB2) gene and the associated phenotypic spectrum. *Hum Mutat* (2010) **31**:992–1002. doi:10.1002/humu.21304
24. Noakes PG, Miner JH, Gautam M, Cunningham JM, Sanes JR, Merlie JP. The renal glomerulus of mice lacking s-laminin/laminin beta 2: nephrosis despite molecular compensation by laminin beta 1. *Nat Genet* (1995) **10**:400–6. doi:10.1038/ng0895-400
25. Jarad G, Cunningham J, Shaw AS, Miner JH. Proteinuria precedes podocyte abnormalities inLamb2-/- mice, implicating the glomerular basement membrane as an albumin barrier. *J Clin Invest* (2006) **116**:2272–9. doi:10.1172/JCI28414
26. Suh JH, Jarad G, Vandevoorde RG, Miner JH. Forced expression of laminin beta1 in podocytes prevents nephrotic syndrome in mice lacking laminin beta2, a model for Pierson syndrome. *Proc Natl Acad Sci U S A* (2011) **108**:15348–53. doi:10.1073/pnas.1108269108
27. Poschl E, Schlotzer-Schrehardt U, Brachvogel B, Saito K, Ninomiya Y, Mayer U. Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. *Development* (2004) **131**:1619–28. doi:10.1242/dev.01037
28. Hudson BG, Tryggvason K, Sundaramoorthy M, Neilson EG. Alport's syndrome, Goodpasture's syndrome, and type IV collagen. *N Engl J Med* (2003) **348**:2543–56. doi:10.1056/NEJMra022296
29. Vanacore R, Ham AJ, Voehler M, Sanders CR, Conrads TP, Veenstra TD, et al. A sulfilimine bond identified in collagen IV. *Science* (2009) **325**:1230–4. doi:10.1126/science.1176811
30. Bhave G, Cummings CF, Vanacore RM, Kumagai-Cresse C, Ero-Tolliver IA, Rafi M, et al. Peroxidasin forms sulfilimine chemical bonds using hypohalous acids in tissue genesis. *Nat Chem Biol* (2012) **8**:784–90. doi:10.1038/nchembio.1038
31. McCall AS, Cummings CF, Bhave G, Vanacore R, Page-McCaw A, Hudson BG. Bromine is an essential trace element for assembly of collagen IV scaffolds in tissue development and architecture. *Cell* (2014) **157**:1380–92. doi:10.1016/j.cell.2014.05.009
32. Hudson BG. The molecular basis of Goodpasture and Alport syndromes: beacons for the discovery of the collagen IV family. *J Am Soc Nephrol* (2004) **15**:2514–27. doi:10.1097/01.ASN.0000141462.00630.76
33. Grodecki KM, Gains MJ, Baumal R, Osmond DH, Cotter B, Valli VE, et al. Treatment of X-linked hereditary nephritis in samoyed dogs with angiotensin converting enzyme (ACE) inhibitor. *J Comp Pathol* (1997) **117**:209–25. doi:10.1016/S0021-9975(97)80016-3
34. Gross O, Beirowski B, Koepke ML, Kuck J, Reiner M, Addicks K, et al. Preemptive ramipril therapy delays renal failure and reduces renal fibrosis in COL4A3-knockout mice with Alport syndrome. *Kidney Int* (2003) **63**:438–46. doi:10.1046/j.1523-1755.2003.00779.x
35. Gross O, Licht C, Anders HJ, Hoppe B, Beck B, Tonshoff B, et al. Early angiotensin-converting enzyme inhibition in Alport syndrome delays renal failure and improves life expectancy. *Kidney Int* (2012) **81**:494–501. doi:10.1038/ki.2011.407
36. Fox JW, Mayer U, Nischt R, Aumailley M, Reinhardt D, Wiedemann H, et al. Recombinant nidogen consists of three globular domains and mediates binding of laminin to collagen type IV. *EMBO J* (1991) **10**:3137–46.
37. Arikawa-Hirasawa E, Watanabe H, Takami H, Hassell JR, Yamada Y. Perlecan is essential for cartilage and cephalic development. *Nat Genet* (1999) **23**:354–8. doi:10.1038/15537
38. Costell M, Gustafsson E, Aszodi A, Morgelin M, Bloch W, Hunziker E, et al. Perlecan maintains the integrity of cartilage and some basement membranes. *J Cell Biol* (1999) **147**:1109–22. doi:10.1083/jcb.147.5.1109
39. Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, et al. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* (1996) **85**:525–35. doi:10.1016/S0092-8674(00)81253-2
40. Goldberg S, Harvey SJ, Cunningham J, Tryggvason K, Miner JH. Glomerular filtration is normal in the absence of both agrin and perlecan-heparan sulfate from the glomerular basement membrane. *Nephrol Dial Transplant* (2009) **24**:2044–51. doi:10.1093/ndt/gfn758
41. Bader BL, Smyth N, Nedbal S, Miosge N, Baranowsky A, Mokkapati S, et al. Compound genetic ablation of nidogen 1 and 2 causes basement membrane defects and perinatal lethality in mice. *Mol Cell Biol* (2005) **25**:6846–56. doi:10.1128/MCB.25.15.6846-6856.2005
42. Suleiman H, Zhang L, Roth R, Heuser JE, Miner JH, Shaw AS, et al. Nanoscale protein architecture of the kidney glomerular basement membrane. *Elife* (2013) **2**:e01149. doi:10.7554/eLife.01149
43. Lennon R, Byron A, Humphries JD, Randles MJ, Carisey A, Murphy S, et al. Global analysis reveals the complexity of the human glomerular extracellular matrix. *J Am Soc Nephrol* (2014) **25**(5):939–51. doi:10.1681/ASN.2013030233
44. Byron A, Randles MJ, Humphries JD, Mironov A, Hamidi H, Harris S, et al. Glomerular cell cross-talk influences composition and assembly of extracellular matrix. *J Am Soc Nephrol* (2014) **25**(5):953–66. doi:10.1681/ASN.2013070795
45. Sachs N, Sonnenberg A. Cell-matrix adhesion of podocytes in physiology and disease. *Nat Rev Nephrol* (2013) **9**:200–10. doi:10.1038/nrneph.2012.291
46. Askari JA, Buckley PA, Mould AP, Humphries MJ. Linking integrin conformation to function. *J Cell Sci* (2009) **122**:165–70. doi:10.1242/jcs.018556
47. Byron A, Humphries JD, Craig SE, Knight D, Humphries MJ. Proteomic analysis of alpha4beta1 integrin adhesion complexes reveals alpha-subunit-dependent protein recruitment. *Proteomics* (2012) **12**:2107–14. doi:10.1002/pmic.201100487
48. Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. *J Cell Sci* (2006) **119**:3901–3. doi:10.1242/jcs.03098
49. Winograd-Katz SE, Fassler R, Geiger B, Legate KR. The integrin adhesome: from genes and proteins to human disease. *Nat Rev Mol Cell Biol* (2014) **15**:273–88. doi:10.1038/nrm3769
50. Humphries JD, Byron A, Bass MD, Craig SE, Pinney JW, Knight D, et al. Proteomic analysis of integrin-associated complexes identifies RCC2 as a dual regulator of Rac1 and Arf6. *Sci Signal* (2009) **2**:ra51. doi:10.1126/scisignal.2000396
51. Kuo JC, Han X, Hsiao CT, Yates JR III, Waterman CM. Analysis of the myosin-II-responsive focal adhesion proteome reveals a role for beta-Pix in negative regulation of focal adhesion maturation. *Nat Cell Biol* (2011) **13**:383–93. doi:10.1038/ncb2216

52. Schiller HB, Hermann MR, Polleux J, Vignaud T, Zanivan S, Friedel CC, et al. Beta1- and alphav-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin-based microenvironments. *Nat Cell Biol* (2013) **15**:625–36. doi:10.1038/ncb2747
53. Woods A, Couchman JR. Integrin modulation by lateral association. *J Biol Chem* (2000) **275**:24233–6. doi:10.1074/jbc.R000001200
54. Morgan MR, Humphries MJ, Bass MD. Synergistic control of cell adhesion by integrins and syndecans. *Nat Rev Mol Cell Biol* (2007) **8**:957–69. doi:10.1038/nrm2289
55. Korhonen M, Ylanne J, Laitinen L, Virtanen I. The alpha 1-alpha 6 subunits of integrins are characteristically expressed in distinct segments of developing and adult human nephron. *J Cell Biol* (1990) **111**:1245–54. doi:10.1083/jcb.111.3.1245
56. Baraldi A, Furci L, Zambruno G, Rubbiani E, Annessi G, Lusvarghi E. Very late activation-3 integrin is the dominant beta 1-integrin on the glomerular capillary wall: an immunofluorescence study in nephrotic syndrome. *Nephron* (1992) **62**:382–8. doi:10.1159/000187085
57. Sterk LM, De Melker AA, Kramer D, Kuikman I, Chand A, Claessen N, et al. Glomerular extracellular matrix components and integrins. *Cell Adhes Commun* (1998) **5**:177–92. doi:10.3109/15419069809040290
58. Has C, Sparta G, Kiritsi D, Weibel L, Moeller A, Vega-Warner V, et al. Integrin alpha3 mutations with kidney, lung, and skin disease. *N Engl J Med* (2012) **366**:1508–14. doi:10.1056/NEJMoa1110813
59. Nicolaou N, Margadant C, Kevelam SH, Lilien MR, Oosterveld MJ, Kreft M, et al. Gain of glycosylation in integrin alpha3 causes lung disease and nephrotic syndrome. *J Clin Invest* (2012) **122**:4375–87. doi:10.1172/JCI64100
60. Kreidberg JA, Donovan MJ, Goldstein SL, Rennke H, Shepherd K, Jones RC, et al. Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development* (1996) **122**:3537–47.
61. Sachs N, Kreft M, Van Den Bergh Weerman MA, Beynon AJ, Peters TA, Weening JJ, et al. Kidney failure in mice lacking the tetraspanin CD151. *J Cell Biol* (2006) **175**:33–9. doi:10.1083/jcb.200603073
62. Kanasaki K, Kanda Y, Palmsten K, Tanjore H, Lee SB, Lebleu VS, et al. Integrin beta1-mediated matrix assembly and signaling are critical for the normal development and function of the kidney glomerulus. *Dev Biol* (2008) **313**:584–93. doi:10.1016/j.ydbio.2007.10.047
63. Pozzi A, Jarad G, Moeckel GW, Coffa S, Zhang X, Gewin L, et al. Beta1 integrin expression by podocytes is required to maintain glomerular structural integrity. *Dev Biol* (2008) **316**:288–301. doi:10.1016/j.ydbio.2008.01.022
64. Yauch RL, Berditchevski F, Harler MB, Reichner J, Hemler ME. Highly stoichiometric, stable, and specific association of integrin alpha3beta1 with CD151 provides a major link to phosphatidylinositol 4-kinase, and may regulate cell migration. *Mol Biol Cell* (1998) **9**:2751–65. doi:10.1091/mbc.9.10.2751
65. Karamatic Crew V, Burton N, Kagan A, Green CA, Levene C, Flinter F, et al. CD151, the first member of the tetraspanin (TM4) superfamily detected on erythrocytes, is essential for the correct assembly of human basement membranes in kidney and skin. *Blood* (2004) **104**:2217–23. doi:10.1182/blood-2004-04-1512
66. Baleato RM, Guthrie PL, Gubler MC, Ashman LK, Roselli S. Deletion of CD151 results in a strain-dependent glomerular disease due to severe alterations of the glomerular basement membrane. *Am J Pathol* (2008) **173**:927–37. doi:10.2353/ajpath.2008.071149
67. Sachs N, Claessen N, Aten J, Kreft M, Teske GJ, Koeman A, et al. Blood pressure influences end-stage renal disease of Cd151 knockout mice. *J Clin Invest* (2012) **122**:348–58. doi:10.1172/JCI58878
68. Vidal F, Aberdam D, Miquel C, Christiano AM, Pulkkinen L, Uitto J, et al. Integrin beta 4 mutations associated with junctional epidermolysis bullosa with pyloric atresia. *Nat Genet* (1995) **10**:229–34. doi:10.1038/ng0695-229
69. Kambham N, Tanji N, Seigle RL, Markowitz GS, Pulkkinen L, Uitto J, et al. Congenital focal segmental glomerulosclerosis associated with beta4 integrin mutation and epidermolysis bullosa. *Am J Kidney Dis* (2000) **36**:190–6. doi:10.1053/ajkd.2000.8293
70. Wei C, Moller CC, Altintas MM, Li J, Schwarz K, Zacchigna S, et al. Modification of kidney barrier function by the urokinase receptor. *Nat Med* (2008) **14**:55–63. doi:10.1038/nm1696
71. Amann K, Haas CS, Schussler J, Daniel C, Hartner A, Schocklmann HO. Beneficial effects of integrin alphavbeta3-blocking RGD peptides in early but not late phase of experimental glomerulonephritis. *Nephrol Dial Transplant* (2012) **27**:1755–68. doi:10.1093/ndt/gfr603
72. Wei C, El Hindi S, Li J, Fornoni A, Goes N, Sageshima J, et al. Circulating urokinase receptor as a cause of focal segmental glomerulosclerosis. *Nat Med* (2011) **17**:952–60. doi:10.1038/nm.2411
73. Beauvais DM, Burbach BJ, Rapraeger AC. The syndecan-1 ectodomain regulates alphavbeta3 integrin activity in human mammary carcinoma cells. *J Cell Biol* (2004) **167**:171–81. doi:10.1083/jcb.200404171
74. Hozumi K, Suzuki N, Nielsen PK, Nomizu M, Yamada Y. Laminin alpha1 chain LG4 module promotes cell attachment through syndecans and cell spreading through integrin alpha2beta1. *J Biol Chem* (2006) **281**:32929–40. doi:10.1074/jbc.M605708200
75. McQuade KJ, Beauvais DM, Burbach BJ, Rapraeger AC. Syndecan-1 regulates alphavbeta5 integrin activity in B82L fibroblasts. *J Cell Sci* (2006) **119**:2445–56. doi:10.1242/jcs.02970
76. Ogawa T, Tsubota Y, Hashimoto J, Kariya Y, Miyazaki K. The short arm of laminin gamma2 chain of laminin-5 (laminin-332) binds syndecan-1 and regulates cellular adhesion and migration by suppressing phosphorylation of integrin beta4 chain. *Mol Biol Cell* (2007) **18**:1621–33. doi:10.1091/mbc.E06-09-0806
77. Morgan MR, Hamidi H, Bass MD, Warwood S, Ballestrem C, Humphries MJ. Syndecan-4 phosphorylation is a control point for integrin recycling. *Dev Cell* (2013) **24**:472–85. doi:10.1016/j.devcel.2013.01.027
78. Caswell PT, Vadrevu S, Norman JC. Integrins: masters and slaves of endocytic transport. *Nat Rev Mol Cell Biol* (2009) **10**:843–53. doi:10.1038/nrm2799
79. Ezratty EJ, Bertaux C, Marcantonio EE, Gundersen GG. Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. *J Cell Biol* (2009) **187**:733–47. doi:10.1083/jcb.200904054
80. Huttenlocher A, Horwitz AR. Integrins in cell migration. *Cold Spring Harb Perspect Biol* (2011) **3**:a005074. doi:10.1101/cshperspect.a005074
81. Fears CY, Woods A. The role of syndecans in disease and wound healing. *Matrix Biol* (2006) **25**:443–56. doi:10.1016/j.matbio.2006.07.003
82. Jakobsson L, Kreuger J, Holmborn K, Lundin L, Eriksson I, Kjellen L, et al. Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. *Dev Cell* (2006) **10**:625–34. doi:10.1016/j.devcel.2006.03.009
83. Chen S, Wassenhove-McCarthy D, Yamaguchi Y, Holzman L, Van Kuppevelt TH, Orr AW, et al. Podocytes require the engagement of cell surface heparan sulfate proteoglycans for adhesion to extracellular matrices. *Kidney Int* (2010) **78**:1088–99. doi:10.1038/ki.2010.136
84. Chen S, Wassenhove-McCarthy DJ, Yamaguchi Y, Holzman LB, Van Kuppevelt TH, Jenniskens GJ, et al. Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria. *Kidney Int* (2008) **74**:289–99. doi:10.1038/ki.2008.159
85. Jin J, Sison K, Li C, Tian R, Wnuk M, Sung HK, et al. Soluble FLT1 binds lipid microdomains in podocytes to control cell morphology and glomerular barrier function. *Cell* (2012) **151**:384–99. doi:10.1016/j.cell.2012.08.037
86. Ervasti JM, Campbell KP. A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J Cell Biol* (1993) **122**:809–23. doi:10.1083/jcb.122.4.809
87. Gee SH, Blacher RW, Douville PJ, Provost PR, Yurchenco PD, Carbonetto S. Laminin-binding protein 120 from brain is closely related to the dystrophin-associated glycoprotein, dystroglycan, and binds with high affinity to the major heparin binding domain of laminin. *J Biol Chem* (1993) **268**:14972–80.
88. Durbeej M, Henry MD, Ferletta M, Campbell KP, Ekblom P. Distribution of dystroglycan in normal adult mouse tissues. *J Histochem Cytochem* (1998) **46**:449–57. doi:10.1177/002215549804600404
89. Raats CJ, Van Den Born J, Bakker MA, Oppers-Walgreen B, Pisa BJ, Dijkman HB, et al. Expression of agrin, dystroglycan, and utrophin in normal renal tissue and in experimental glomerulopathies. *Am J Pathol* (2000) **156**:1749–65. doi:10.1016/S0002-9440(10)65046-8
90. Kojima K, Davidovits A, Poczewski H, Langer B, Uchida S, Nagy-Bojarski K, et al. Podocyte flattening and disorder of glomerular basement membrane are associated with splitting of dystroglycan-matrix interaction. *J Am Soc Nephrol* (2004) **15**:2079–89. doi:10.1097/01.ASN.0000133531.43177.21
91. Kojima K, Nosaka H, Kishimoto Y, Nishiyama Y, Fukuda S, Shimada M, et al. Defective glycosylation of alpha-dystroglycan contributes to podocyte flattening. *Kidney Int* (2011) **79**:311–6. doi:10.1038/ki.2010.403
92. Jarad G, Pippin JW, Shankland SJ, Kreidberg JA, Miner JH. Dystroglycan does not contribute significantly to kidney development or function, in health or after injury. *Am J Physiol Renal Physiol* (2011) **300**:F811–20. doi:10.1152/ajprenal.00725.2010

93. Goult BT, Zacharchenko T, Bate N, Tsang R, Hey F, Gingras AR, et al. RIAM and vinculin binding to talin are mutually exclusive and regulate adhesion assembly and turnover. *J Biol Chem* (2013) **288**:8238–49. doi:10.1074/jbc.M112.438119
94. Critchley DR, Gingras AR. Talin at a glance. *J Cell Sci* (2008) **121**:1345–7. doi:10.1242/jcs.018085
95. Tian X, Kim JJ, Monkley SM, Gotoh N, Nandez R, Soda K, et al. Podocyte-associated talin1 is critical for glomerular filtration barrier maintenance. *J Clin Invest* (2014) **124**:1098–113. doi:10.1172/JCI69778
96. Humphries JD, Wang P, Streuli C, Geiger B, Humphries MJ, Ballestrem C. Vinculin controls focal adhesion formation by direct interactions with talin and actin. *J Cell Biol* (2007) **179**:1043–57. doi:10.1083/jcb.200703036
97. Eimer W, Niermann M, Eppe MA, Jockusch BM. Molecular shape of vinculin in aqueous solution. *J Mol Biol* (1993) **229**:146–52. doi:10.1006/jmbi.1993.1014
98. Winkler J, Lunsdorf H, Jockusch BM. The ultrastructure of chicken gizzard vinculin as visualized by high-resolution electron microscopy. *J Struct Biol* (1996) **116**:270–7. doi:10.1006/jsbi.1996.0042
99. Chen H, Cohen DM, Choudhury DM, Kioka N, Craig SW. Spatial distribution and functional significance of activated vinculin in living cells. *J Cell Biol* (2005) **169**:459–70. doi:10.1083/jcb.200410100
100. Bakolitsa C, Cohen DM, Bankston LA, Bobkov AA, Cadwell GW, Jennings L, et al. Structural basis for vinculin activation at sites of cell adhesion. *Nature* (2004) **430**:583–6. doi:10.1038/nature02610
101. Carisey A, Tsang R, Greiner AM, Nijenhuis N, Heath N, Nazgiewicz A, et al. Vinculin regulates the recruitment and release of core focal adhesion proteins in a force-dependent manner. *Curr Biol* (2013) **23**:271–81. doi:10.1016/j.cub.2013.01.009
102. Zamir E, Geiger B. Molecular complexity and dynamics of cell-matrix adhesions. *J Cell Sci* (2001) **114**:3583–90.
103. Ziegler WH, Liddington RC, Critchley DR. The structure and regulation of vinculin. *Trends Cell Biol* (2006) **16**:453–60. doi:10.1016/j.tcb.2006.07.004
104. Brown MC, Perrotta JA, Turner CE. Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J Cell Biol* (1996) **135**:1109–23. doi:10.1083/jcb.135.4.1109
105. Brown MC, Perrotta JA, Turner CE. Serine and threonine phosphorylation of the paxillin LIM domains regulates paxillin focal adhesion localization and cell adhesion to fibronectin. *Mol Biol Cell* (1998) **9**:1803–16. doi:10.1091/mbc.9.7.1803
106. Wang P, Ballestrem C, Streuli CH. The C terminus of talin links integrins to cell cycle progression. *J Cell Biol* (2011) **195**:499–513. doi:10.1083/jcb.201104128
107. Brown MC, Turner CE. Paxillin: adapting to change. *Physiol Rev* (2004) **84**:1315–39. doi:10.1152/physrev.00002.2004
108. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci U S A* (1992) **89**:5192–6. doi:10.1073/pnas.89.11.5192
109. Schaller MD. Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *J Cell Sci* (2010) **123**:1007–13. doi:10.1242/jcs.045112
110. Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, et al. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* (1995) **377**:539–44. doi:10.1038/377539a0
111. Ma H, Togawa A, Soda K, Zhang J, Lee S, Ma M, et al. Inhibition of podocyte FAK protects against proteinuria and foot process effacement. *J Am Soc Nephrol* (2010) **21**:1145–56. doi:10.1681/ASN.2009090991
112. Delimont D, Dufek BM, Meehan DT, Zallocchi M, Gratton MA, Phillips G, et al. Laminin alpha2-mediated focal adhesion kinase activation triggers Alport glomerular pathogenesis. *PLoS One* (2014) **9**:e99083. doi:10.1371/journal.pone.0099083
113. Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, et al. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. *Nature* (1996) **379**:91–6. doi:10.1038/379091a0
114. Zervas CG, Gregory SL, Brown NH. *Drosophila* integrin-linked kinase is required at sites of integrin adhesion to link the cytoskeleton to the plasma membrane. *J Cell Biol* (2001) **152**:1007–18. doi:10.1083/jcb.152.5.1007
115. Mackinnon AC, Qadota H, Norman KR, Moerman DG, Williams BD. *C. elegans* PAT-4/ILK functions as an adaptor protein within integrin adhesion complexes. *Curr Biol* (2002) **12**:787–97. doi:10.1016/S0960-9822(02)00810-2
116. Wickstrom SA, Lange A, Montanez E, Fassler R. The ILK/PINCH/parvin complex: the kinase is dead, long live the pseudokinase! *EMBO J* (2010) **29**:281–91. doi:10.1038/emboj.2009.376
117. Fukuda K, Knight JD, Piszczek G, Kothary R, Qin J. Biochemical, proteomic, structural, and thermodynamic characterizations of integrin-linked kinase (ILK): cross-validation of the pseudokinase. *J Biol Chem* (2011) **286**:21886–95. doi:10.1074/jbc.M111.240093
118. Hannigan GE, McDonald PC, Walsh MP, Dedhar S. Integrin-linked kinase: not so "pseudo" after all. *Oncogene* (2011) **30**:4375–85. doi:10.1038/onc.2011.177
119. Qin J, Wu C. ILK: a pseudokinase in the center stage of cell-matrix adhesion and signaling. *Curr Opin Cell Biol* (2012) **24**:607–13. doi:10.1016/j.ceb.2012.06.003
120. Nikolopoulos SN, Turner CE. Actopaxin, a new focal adhesion protein that binds paxillin LD motifs and actin and regulates cell adhesion. *J Cell Biol* (2000) **151**:1435–48. doi:10.1083/jcb.151.7.1435
121. Yamaji S, Suzuki A, Sugiyama Y, Koide Y, Yoshida M, Kanamori H, et al. A novel integrin-linked kinase-binding protein, affixin, is involved in the early stage of cell-substrate interaction. *J Cell Biol* (2001) **153**:1251–64. doi:10.1083/jcb.153.6.1251
122. Tu Y, Li F, Goicoechea S, Wu C. The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. *Mol Cell Biol* (1999) **19**:2425–34.
123. Tu Y, Huang Y, Zhang Y, Hua Y, Wu C. A new focal adhesion protein that interacts with integrin-linked kinase and regulates cell adhesion and spreading. *J Cell Biol* (2001) **153**:585–98. doi:10.1083/jcb.153.3.585
124. Chiswell BP, Zhang R, Murphy JW, Boggon TJ, Calderwood DA. The structural basis of integrin-linked kinase-PINCH interactions. *Proc Natl Acad Sci U S A* (2008) **105**:20677–82. doi:10.1073/pnas.0811415106
125. Tu Y, Wu S, Shi X, Chen K, Wu C. Migfilin and Mig-2 link focal adhesions to filamin and the actin cytoskeleton and function in cell shape modulation. *Cell* (2003) **113**:37–47. doi:10.1016/S0092-8674(03)00163-6
126. Larjava H, Plow EF, Wu C. Kindlins: essential regulators of integrin signalling and cell-matrix adhesion. *EMBO Rep* (2008) **9**:1203–8. doi:10.1038/embor.2008.202
127. Qu H, Tu Y, Shi X, Larjava H, Saleem MA, Shattil SJ, et al. Kindlin-2 regulates podocyte adhesion and fibronectin matrix deposition through interactions with phosphoinositides and integrins. *J Cell Sci* (2011) **124**:879–91. doi:10.1242/jcs.076976
128. Sakai T, Li S, Docheva D, Grashoff C, Sakai K, Kostka G, et al. Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. *Genes Dev* (2003) **17**:926–40. doi:10.1101/gad.255603
129. Montanez E, Wickstrom SA, Altstatter J, Chu H, Fassler R. Alpha-parvin controls vascular mural cell recruitment to vessel wall by regulating RhoA/ROCK signalling. *EMBO J* (2009) **28**:3132–44. doi:10.1038/emboj.2009.295
130. Li S, Bordoy R, Stanchi F, Moser M, Braun A, Kudlacek O, et al. PINCH1 regulates cell-matrix and cell-cell adhesions, cell polarity and cell survival during the peri-implantation stage. *J Cell Sci* (2005) **118**:2913–21. doi:10.1242/jcs.02422
131. Lange A, Wickstrom SA, Jakobson M, Zent R, Sainio K, Fassler R. Integrin-linked kinase is an adaptor with essential functions during mouse development. *Nature* (2009) **461**:1002–6. doi:10.1038/nature08468
132. Dai C, Stolz DB, Bastacky SI, St-Arnaud R, Wu C, Dedhar S, et al. Essential role of integrin-linked kinase in podocyte biology: bridging the integrin and slit diaphragm signaling. *J Am Soc Nephrol* (2006) **17**:2164–75. doi:10.1681/ASN.2006010033
133. El-Aouni C, Herbach N, Blattner SM, Henger A, Rastaldi MP, Jarad G, et al. Podocyte-specific deletion of integrin-linked kinase results in severe glomerular basement membrane alterations and progressive glomerulosclerosis. *J Am Soc Nephrol* (2006) **17**:1334–44. doi:10.1681/ASN.2005090921
134. Guo L, Sanders PW, Woods A, Wu C. The distribution and regulation of integrin-linked kinase in normal and diabetic kidneys. *Am J Pathol* (2001) **159**:1735–42. doi:10.1016/S0002-9440(10)63020-9
135. Kretzler M, Teixeira VP, Unschuld PG, Cohen CD, Wanke R, Edenhofer I, et al. Integrin-linked kinase as a candidate downstream effector in proteinuria. *FASEB J* (2001) **15**:1843–5.
136. Rodewald R, Karnovsky MJ. Porous substructure of the glomerular slit diaphragm in the rat and mouse. *J Cell Biol* (1974) **60**:423–33. doi:10.1083/jcb.60.2.423
137. Farquhar MG, Wissig SL, Palade GE. Glomerular permeability. I. Ferritin transfer across the normal glomerular capillary wall. *J Exp Med* (1961) **113**:47–66. doi:10.1084/jem.113.1.47

138. Caulfield JP, Farquhar MG. The permeability of glomerular capillaries to graded dextrans. Identification of the basement membrane as the primary filtration barrier. *J Cell Biol* (1974) **63**:883–903. doi:10.1083/jcb.63.3.883
139. Kestila M, Lenkkeri U, Mannikko M, Lamerdin J, McCready P, Putaala H, et al. Positionally cloned gene for a novel glomerular protein – nephrin – is mutated in congenital nephrotic syndrome. *Mol Cell* (1998) **1**:575–82. doi:10.1016/S1097-2765(00)80057-X
140. Ruotsalainen V, Ljungberg P, Wartiovaara J, Lenkkeri U, Kestila M, Jalanko H, et al. Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci U S A* (1999) **96**:7962–7. doi:10.1073/pnas.96.14.7962
141. Gagliardini E, Conti S, Benigni A, Remuzzi G, Remuzzi A. Imaging of the porous ultrastructure of the glomerular epithelial filtration slit. *J Am Soc Nephrol* (2010) **21**:2081–9. doi:10.1681/ASN.2010020199
142. Caulfield JP, Reid JJ, Farquhar MG. Alterations of the glomerular epithelium in acute aminonucleoside nephrosis. Evidence for formation of occluding junctions and epithelial cell detachment. *Lab Invest* (1976) **34**:43–59.
143. Reeves W, Caulfield JP, Farquhar MG. Differentiation of epithelial foot processes and filtration slits: sequential appearance of occluding junctions, epithelial polyanion, and slit membranes in developing glomeruli. *Lab Invest* (1978) **39**:90–100.
144. Ciani L, Patel A, Allen ND, Ffrench-Constant C. Mice lacking the giant protocadherin mFAT1 exhibit renal slit junction abnormalities and a partially penetrant cyclopia and anophthalmia phenotype. *Mol Cell Biol* (2003) **23**:3575–82. doi:10.1128/MCB.23.10.3575-3582.2003
145. Reiser J, Kriz W, Kretzler M, Mundel P. The glomerular slit diaphragm is a modified adherens junction. *J Am Soc Nephrol* (2000) **11**:1–8.
146. Fukasawa H, Bornheimer S, Kudlicka K, Farquhar MG. Slit diaphragms contain tight junction proteins. *J Am Soc Nephrol* (2009) **20**:1491–503. doi:10.1681/ASN.2008101117
147. Yaoita E, Yao J, Yoshida Y, Morioka T, Nameta M, Takata T, et al. Up-regulation of connexin43 in glomerular podocytes in response to injury. *Am J Pathol* (2002) **161**:1597–606. doi:10.1016/S0002-9440(10)64438-0
148. Helmstadter M, Hohne M, Huber TB. A brief overview on IRM function across evolution. *J Neurogenet* (2014). doi:10.3109/01677063.2014.918976
149. Weavers H, Prieto-Sanchez S, Grawe F, Garcia-Lopez A, Artero R, Wilsch-Brauninger M, et al. The insect nephrocyte is a podocyte-like cell with a filtration slit diaphragm. *Nature* (2009) **457**:322–6. doi:10.1038/nature07526
150. Zhuang S, Shao H, Guo F, Trimble R, Pearce E, Abmayr SM. Sns and Kirre, the *Drosophila* orthologs of nephrin and Neph1, direct adhesion, fusion and formation of a slit diaphragm-like structure in insect nephrocytes. *Development* (2009) **136**:2335–44. doi:10.1242/dev.031609
151. Costa MS, Machado MC, Vieceli FM, Amista L, Baroneza JE, Yan CY, et al. The Rst-Neph family of cell adhesion molecules in *Gallus gallus*. *J Neurogenet* (2014). doi:10.3109/01677063.2014.933220
152. Ozkan E, Chia PH, Wang RR, Goriatcheva N, Borek D, Otwinowski Z, et al. Extracellular architecture of the SYG-1/SYG-2 adhesion complex instructs synaptogenesis. *Cell* (2014) **156**:482–94. doi:10.1016/j.cell.2014.01.004
153. Khoshnoodi J, Sigmundsson K, Ofverstedt LG, Skoglund U, Obrink B, Wartiovaara J, et al. Nephrin promotes cell-cell adhesion through homophilic interactions. *Am J Pathol* (2003) **163**:2337–46. doi:10.1016/S0002-9440(10)63590-0
154. Philippe A, Nevo F, Esquivel EL, Reklaityte D, Gribouval O, Tete MJ, et al. Nephrin mutations can cause childhood-onset steroid-resistant nephrotic syndrome. *J Am Soc Nephrol* (2008) **19**:1871–8. doi:10.1681/ASN.2008010059
155. Putaala H, Sainio K, Sariola H, Tryggvason K. Primary structure of mouse and rat nephrin cDNA and structure and expression of the mouse gene. *J Am Soc Nephrol* (2000) **11**:991–1001.
156. Donoviel DB, Freed DD, Vogel H, Potter DG, Hawkins E, Barrish JP, et al. Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. *Mol Cell Biol* (2001) **21**:4829–36. doi:10.1128/MCB.21.14.4829-4836.2001
157. Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, et al. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* (2000) **24**:349–54. doi:10.1038/74166
158. Huber TB, Simons M, Hartleben B, Sernetz L, Schmidts M, Gundlach E, et al. Molecular basis of the functional podocin-nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains. *Hum Mol Genet* (2003) **12**:3397–405. doi:10.1093/hmg/ddg360
159. Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, et al. Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* (1999) **286**:312–5. doi:10.1126/science.286.5438.312
160. Akilesh S, Koziell A, Shaw AS. Basic science meets clinical medicine: identification of a CD2AP-deficient patient. *Kidney Int* (2007) **72**:1181–3. doi:10.1038/sj.ki.5002575
161. Tossidou I, Kardinal C, Peters I, Kriz W, Shaw A, Dikic I, et al. CD2AP/CIN85 balance determines receptor tyrosine kinase signaling response in podocytes. *J Biol Chem* (2007) **282**:7457–64. doi:10.1074/jbc.M608519200
162. Lehtonen S, Ryan JJ, Kudlicka K, Iino N, Zhou H, Farquhar MG. Cell junction-associated proteins IQGAP1, MAGI-2, CASK, spectrins, and alpha-actinin are components of the nephrin multiprotein complex. *Proc Natl Acad Sci U S A* (2005) **102**:9814–9. doi:10.1073/pnas.0504166102
163. Zaidel-Bar R. Cadherin adhesome at a glance. *J Cell Sci* (2013) **126**:373–8. doi:10.1242/jcs.111559
164. Grahammer F, Schell C, Huber TB. The podocyte slit diaphragm – from a thin grey line to a complex signalling hub. *Nat Rev Nephrol* (2013) **9**:587–98. doi:10.1038/nrneph.2013.169
165. New LA, Martin CE, Jones N. Advances in slit diaphragm signaling. *Curr Opin Nephrol Hypertens* (2014) **23**:420–30. doi:10.1097/01.mnh.0000447018.28852.b6
166. Verma R, Wharram B, Kovari I, Kunkel R, Nihalani D, Wary KK, et al. Fyn binds to and phosphorylates the kidney slit diaphragm component nephrin. *J Biol Chem* (2003) **278**:20716–23. doi:10.1074/jbc.M301689200
167. Jones N, Blasutig IM, Eremina V, Ruston JM, Bladt F, Li H, et al. Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. *Nature* (2006) **440**:818–23. doi:10.1038/nature04662
168. Verma R, Kovari I, Soofi A, Nihalani D, Patrie K, Holzman LB. Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. *J Clin Invest* (2006) **116**:1346–59. doi:10.1172/JCI27414
169. George B, Verma R, Soofi AA, Garg P, Zhang J, Park TJ, et al. Crk1/2-dependent signaling is necessary for podocyte foot process spreading in mouse models of glomerular disease. *J Clin Invest* (2012) **122**:674–92. doi:10.1172/JCI60070
170. George B, Fan Q, Dlugos CP, Soofi AA, Zhang J, Verma R, et al. Crk1/2 and CrkL form a hetero-oligomer and functionally complement each other during podocyte morphogenesis. *Kidney Int* (2014) **85**:1382–94. doi:10.1038/ki.2013.556
171. Garg P, Verma R, Nihalani D, Johnstone DB, Holzman LB. Neph1 cooperates with nephrin to transduce a signal that induces actin polymerization. *Mol Cell Biol* (2007) **27**:8698–712. doi:10.1128/MCB.00948-07
172. Bisson N, Ruston J, Jeansson M, Vanderlaan R, Hardy WR, Du J, et al. The adaptor protein Grb2 is not essential for the establishment of the glomerular filtration barrier. *PLoS One* (2012) **7**:e50996. doi:10.1371/journal.pone.0050996
173. Huber TB, Hartleben B, Kim J, Schmidts M, Schermer B, Keil A, et al. Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Mol Cell Biol* (2003) **23**:4917–28. doi:10.1128/MCB.23.14.4917-4928.2003
174. Zhu J, Sun N, Aoudjit L, Li H, Kawachi H, Lemay S, et al. Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes. *Kidney Int* (2008) **73**:556–66. doi:10.1038/sj.ki.5002691
175. Schell C, Baumhakl L, Salou S, Conzelmann AC, Meyer C, Helmstadter M, et al. N-wasp is required for stabilization of podocyte foot processes. *J Am Soc Nephrol* (2013) **24**:713–21. doi:10.1681/ASN.2012080844
176. Fan X, Li Q, Pisarek-Horowitz A, Rasouly HM, Wang X, Bonegio RG, et al. Inhibitory effects of Robo2 on nephrin: a crosstalk between positive and negative signals regulating podocyte structure. *Cell Rep* (2012) **2**:52–61. doi:10.1016/j.celrep.2012.06.002
177. Canaud G, Bienaime F, Viau A, Treins C, Baron W, Nguyen C, et al. AKT2 is essential to maintain podocyte viability and function during chronic kidney disease. *Nat Med* (2013) **19**:1288–96. doi:10.1038/nm.3313
178. Welsh GI, Hale LJ, Eremina V, Jeansson M, Maezawa Y, Lennon R, et al. Insulin signaling to the glomerular podocyte is critical for normal kidney function. *Cell Metab* (2010) **12**:329–40. doi:10.1016/j.cmet.2010.08.015
179. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, et al. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* (2000) **24**:251–6. doi:10.1038/73456

180. Scott RP, Hawley SP, Ruston J, Du J, Brakebusch C, Jones N, et al. Podocyte-specific loss of Cdc42 leads to congenital nephropathy. *J Am Soc Nephrol* (2012) **23**:1149–54. doi:10.1681/ASN.2011121206
181. Sun H, Schlondorff J, Higgs HN, Pollak MR. Inverted formin 2 regulates actin dynamics by antagonizing Rho/diaphanous-related formin signaling. *J Am Soc Nephrol* (2013) **24**:917–29. doi:10.1681/ASN.2012080834
182. Yu H, Suleiman H, Kim AH, Miner JH, Dani A, Shaw AS, et al. Rac1 activation in podocytes induces rapid foot process effacement and proteinuria. *Mol Cell Biol* (2013) **33**:4755–64. doi:10.1128/MCB.00730-13
183. Shibata S, Nagase M, Yoshida S, Kawarazaki W, Kurihara H, Tanaka H, et al. Modification of mineralocorticoid receptor function by Rac1 GTPase: implication in proteinuric kidney disease. *Nat Med* (2008) **14**:1370–6. doi:10.1038/nm.1879
184. Schaldecker T, Kim S, Tarabanis C, Tian D, Hakroush S, Castonguay P, et al. Inhibition of the TRPC5 ion channel protects the kidney filter. *J Clin Invest* (2013) **123**:5298–309. doi:10.1172/JCI71165
185. Blattner SM, Hodgin JB, Nishio M, Wylie SA, Saha J, Soofi AA, et al. Divergent functions of the Rho GTPases Rac1 and Cdc42 in podocyte injury. *Kidney Int* (2013) **84**:920–30. doi:10.1038/ki.2013.175
186. Huber TB, Hartleben B, Winkelmann K, Schneider L, Becker JU, Leitges M, et al. Loss of podocyte aPKClambda/iota causes polarity defects and nephrotic syndrome. *J Am Soc Nephrol* (2009) **20**:798–806. doi:10.1681/ASN.2008080871
187. Satoh D, Hirose T, Harita Y, Daimon C, Harada T, Kurihara H, et al. aPKClambda maintains the integrity of the glomerular slit diaphragm through trafficking of nephrin to the cell surface. *J Biochem* (2014) **156**(2):115–28. doi:10.1093/jb/mvu022
188. Soda K, Balkin DM, Ferguson SM, Paradise S, Milosevic I, Giovedi S, et al. Role of dynamin, synaptojanin, and endophilin in podocyte foot processes. *J Clin Invest* (2012) **122**:4401–11. doi:10.1172/JCI65289
189. Arif E, Wagner MC, Johnstone DB, Wong HN, George B, Pruthi PA, et al. Motor protein Myo1c is a podocyte protein that facilitates the transport of slit diaphragm protein Neph1 to the podocyte membrane. *Mol Cell Biol* (2011) **31**:2134–50. doi:10.1128/MCB.05051-11
190. Krendel M, Osterweil EK, Mooseker MS. Myosin 1E interacts with synaptojanin-1 and dynamin and is involved in endocytosis. *FEBS Lett* (2007) **581**:644–50. doi:10.1016/j.febslet.2007.01.021
191. Krendel M, Kim SV, Willinger T, Wang T, Kashgarian M, Flavell RA, et al. Disruption of myosin 1e promotes podocyte injury. *J Am Soc Nephrol* (2009) **20**:86–94. doi:10.1681/ASN.2007111172
192. Reiser J, Polu KR, Moller CC, Kenlan P, Altintas MM, Wei C, et al. TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. *Nat Genet* (2005) **37**:739–44. doi:10.1038/ng1592
193. Winn MP, Conlon PJ, Lynn KL, Farrington MK, Creazzo T, Hawkins AF, et al. A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science* (2005) **308**:1801–4. doi:10.1126/science.1106215
194. Conway DE, Breckenridge MT, Hinde E, Gratton E, Chen CS, Schwartz MA. Fluid shear stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1. *Curr Biol* (2013) **23**:1024–30. doi:10.1016/j.cub.2013.04.049
195. Mathieson PW. The podocyte as a target for therapies – new and old. *Nat Rev Nephrol* (2012) **8**:52–6. doi:10.1038/nrneph.2011.171
196. Lin S, Li D, Jia J, Zheng Z, Jia Z, Shang W. Spironolactone ameliorates podocytic adhesive capacity via restoring integrin alpha 3 expression in streptozotocin-induced diabetic rats. *J Renin Angiotensin Aldosterone Syst* (2010) **11**:149–57. doi:10.1177/1470320310369603
197. Xing CY, Saleem MA, Coward RJ, Ni L, Witherden IR, Mathieson PW. Direct effects of dexamethasone on human podocytes. *Kidney Int* (2006) **70**:1038–45. doi:10.1038/sj.ki.5001655
198. Yu CC, Fornoni A, Weins A, Hakroush S, Maiguel D, Sageshima J, et al. Abatacept in B7-1-positive proteinuric kidney disease. *N Engl J Med* (2013) **369**:2416–23. doi:10.1056/NEJMoa1304572
199. Goodman SL, Picard M. Integrins as therapeutic targets. *Trends Pharmacol Sci* (2012) **33**:405–12. doi:10.1016/j.tips.2012.04.002
200. Lin X, Suh JH, Go G, Miner JH. Feasibility of repairing glomerular basement membrane defects in Alport syndrome. *J Am Soc Nephrol* (2014) **25**:687–92. doi:10.1681/ASN.2013070798
201. Liu XL, Done SC, Yan K, Kilpelainen P, Pikkarainen T, Tryggvason K. Defective trafficking of nephrin missense mutants rescued by a chemical chaperone. *J Am Soc Nephrol* (2004) **15**:1731–8. doi:10.1097/01.ASN.0000129826.28932.FD
202. Arif E, Rathore YS, Kumari B, Ashish F, Wong HN, Holzman LB, et al. Slit diaphragm protein Neph1 and its signaling: a novel therapeutic target for protection of podocytes against glomerular injury. *J Biol Chem* (2014) **289**:9502–18. doi:10.1074/jbc.M113.505743

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Structural analysis of how podocytes detach from the glomerular basement membrane under hypertrophic stress

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Podocytes are lost by detachment from the GBM as viable cells; details are largely unknown. We studied this process in the rat after growth stimulation with FGF-2. Endothelial and mesangial cells responded by hyperplasia, podocytes underwent hypertrophy, but, in the long run, developed various changes that could either be interpreted showing progressing stages in detachment from the GBM or stages leading to a tighter attachment by foot process effacement (FPE). This occurred in microdomains within the same podocyte; thus, features of detachment and of reinforced attachment may simultaneously be found in the same podocyte. (1) Initially, hypertrophied podocytes underwent cell body attenuation and formed large pseudocysts, i.e., expansions of the subpodocyte space. (2) Podocytes entered the process of FPE starting with the retraction of foot processes (FPs) and the replacement of the slit diaphragm by occluding junctions, thereby sealing the filtration slits. Successful completion of this process led to broad attachments of podocyte cell bodies to the GBM. (3) Failure of sealing the slits led to gaps of varying width between retracting FPs facilitating the outflow of the filtrate from the GBM. (4) Since those gaps are frequently overarched by broadened primary processes, the drainage of the filtrate into the Bowman's space may be hindered leading to the formation of small pseudocysts associated with bare areas of GBM. (5) The merging of pseudocysts created a system of communicating chambers through which the filtrate has to pass to reach Bowman's space. Multiple flow resistances in series likely generated an expansile force on podocytes contributing to detachment. (6) Such a situation appears to proceed to complete disconnection generally of a group of podocytes owing to the junctional connections between them. (7) Since such groups of detaching podocytes generally make contact to parietal cells, they start the formation of tuft adhesions to Bowman's capsule.

Keywords: podocyte hypertrophy, foot process effacement, sealing of filtration slits, pseudocysts, podocyte detachment, crescents

INTRODUCTION

Podocyte loss underlies the progression of glomerular diseases to end-stage renal failure, both in human beings (1) and in experimental animal models (2). How podocytes are lost is open to debate. Apoptosis is widely considered as a major mechanism, but this has been demonstrated largely in cell culture, and convincing evidence showing apoptosis of podocytes *in situ* by transmission electron microscopy (TEM) has never been presented. Also, necrosis of podocytes has rarely been shown and if it occurs, it is mostly in cells already detached from the GBM. On the other hand, viable podocytes in the urine have been found in several glomerular diseases (3, 4) and podocytes appearing to be viable detaching from the GBM in huge numbers were shown by TEM in various experimental models (5). This leads to the conclusion that detachment as viable cells is the major mechanism of podocyte loss and that the major causes of podocyte loss are likely to be mechanical forces challenging the attachment of podocytes to the GBM (6).

Recently, the hypothesis has been raised that the shear stress derived from filtrate flow through the filtration slits acting on the foot processes (FPs) may have a major impact on podocyte detachment (6, 7). Moreover, it has been suggested that glomerular hypertension, hyperfiltration, and excessive hypertrophy, which have previously been shown to underlie progression of glomerular diseases (8, 9), may account for increases in shear stress compromising the attachment of podocytes to the GBM.

Furthermore, it has been postulated that major changes of podocytes in glomerular diseases, such as foot process effacement (FPE) and cytoplasm shedding, which have been generally interpreted as pathological derangements of podocytes, actually show the "podocyte's fight" against detachment, and thus are changes

Abbreviations: CGP, collapsing glomerulopathy; FP, foot process; FPE, foot process effacement; FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane; PBM, parietal basement membrane.

undertaken to secure survival, i.e., continued attachment to the GBM (5).

The present study analyzes the individual steps of podocyte detachment in a model of excessive glomerular hypertrophy that had been previously studied by our group (10). Daily administration of FGF-2 led to a long-term growth stimulation of glomeruli followed by the development of widespread focal and segmental glomerulosclerosis (FSGS). At the time of this study, in 1995, the idea that podocytes are lost as viable cells in the urine was not yet born. The focus of the study lay in the question of how, subsequent to podocyte loss, tuft adhesions develop and lead to the degeneration of the entire nephron. In the present study, we used the same material to structurally analyze earlier stages to describe how podocytes undergo detachment. We show that the process of detachment from the GBM is extremely gradual and frequently complicated by the fact that the sequence of steps to detachment may be mixed up with changes locally reinforcing the attachment to the GBM.

MATERIALS AND METHODS

The material used in the present study was derived from previous experiments (10). Therefore, only a brief description of the methods is given here. The experiments complied with the "German law on the protection of animals."

A total of 36 Sprague-Dawley rats (7 weeks old; half males, half females) were studied. Half received daily subcutaneous injections of 320 μg FGF-2/kg body weight and the other half served as controls, receiving the vehicle only. Two-thirds of the rats were studied after total body perfusion at 8 weeks; the remaining one-third was studied after 13 weeks. After anesthesia with Nembutal® (1.2 ml/kg body weight), the kidneys were retrogradely perfused via the abdominal aorta without prior flushing for 3 min at a pressure of 220 mm Hg. In 24 rats intended for ultrastructural studies, the fixative contained 1.5% glutaraldehyde and 1.5% formaldehyde in phosphate buffered saline (260 mOsm, pH 7.4) supplemented with 0.5 g/l picric acid. Blocks of cortical tissue were processed by standard procedures (i) for paraffin sections (4 μm; stained with hematoxylin eosin) and (ii) after postfixation with OsO_4 or tannic acid (11), for Epon sections (1 μm, stained with methylene blue) to be studied by light micrograph and ultrathin sections stained with uranyl acetate and lead citrate to be studied by TEM. The remaining 12 rats were perfused with paraformaldehyde [see Ref. (10)].

STRUCTURAL STUDIES

In addition to the structural assessments in the previous study (10), a quantification of glomerular injuries as defined in the present study (pseudocysts, podocyte detachment associated with small pseudocysts, podocyte clusters, and tuft adhesions; **Figure 1A**) was done. In three independent 1 μm Epon sections of each experimental animal (6 males and 6 females; values from 8 and 13 weeks were pooled) and of six controls, the glomerular profiles and the number of the above listed lesions found in these glomeruli were counted, figured up for each animal, expressed as percentage of the total number of profiles of each animal, and compared. In order to receive an overall weighted damage index, we graded

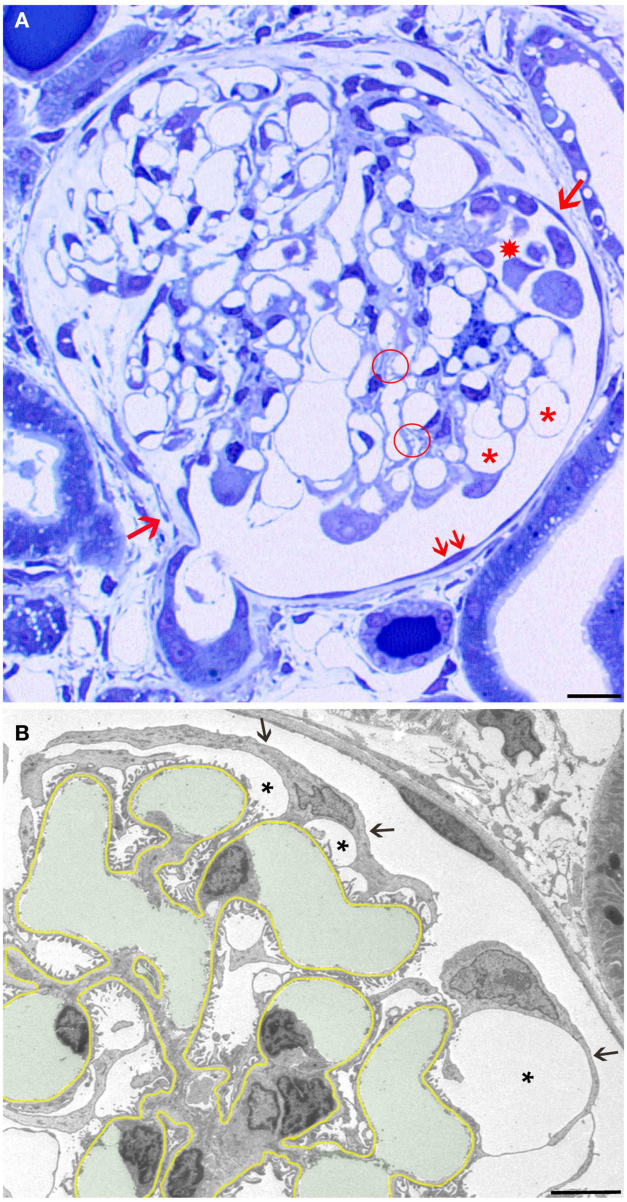

FIGURE 1 | Overview and early changes. (A) Light micrograph (LM) of a glomerular profile showing the crucial injuries presented later in more detail in transmission electron micrographs (TEMs) and quantified in **Table 1**. Hypertrophied podocytes forming large pseudocysts (asterisks), areas of small pseudocysts generally associated with local detachments (enclosed by a hatched circle), detachment of podocytes forming a cluster in Bowman's space (star), and a large tuft adhesion comprising almost two-thirds of the tuft (clockwise from arrow 1 to arrow 2) are seen. Note that outside the tuft adhesion, the parietal epithelium looks fully normal (two small arrows). **(B)** The GBM is highlighted in yellow, capillary lumens in green. The cell bodies of podocytes are attenuated (arrows) and pseudocysts (asterisks) have developed underneath them. Note that the foot process pattern is widely preserved including the floors of pseudocysts and, second, the parietal epithelium looks fully normal. Male rats, growth stimulation with FGF-2 for 13 weeks. **(A)** LM from 1 μm Epon section. Bar: 15 μm. **(B)** TEM. Bar: 5 μm.

large pseudocysts with 1, accumulations of small pseudocysts with 2, clusters of podocytes with 3, and adhesions with 4. Results are reported as mean ± SD; differences between the various groups were tested by one-way ANOVA using Sigma Plot.

RESULTS

Essential stereological results of the previous paper (10) are summarized as follows. Prolonged daily treatment of rats with FGF-2 for 8 weeks led to increasing albuminuria (0.30 ± 0.15 versus 141.5 ± 132.4 mg/18 h) in males and (0.11 ± 0.02 versus 79.8 ± 150.3 mg/18 h) in females; the respective numbers after 13 weeks read 0.21 ± 0.09 versus 303.4 ± 78.4 and 0.13 ± 0.03 versus 85.1 ± 31.5. Serum creatinine increased significantly, indicating the development of chronic renal failure. No increase in body weight or in kidney weight compared with controls was found. However, a prominent significant increase in glomerular tuft volume (1651 ± 209 versus 3035 ± 596 $\mu m^3 \times 10^3$ in males, 1138 ± 85 versus 1998 ± 392 $\mu m^3 \times 10^3$ in females), and a numerical increase in mesangial and endothelial cells but no increase in podocyte number were found, resulting in a dramatic decrease in podocyte density (N podo/$\mu m^2 \times 10^3$), more pronounced in males (0.86 ± 0.007 versus 0.48 ± 0.07) than females (1.05 ± 0.09 versus 0.67 ± 0.14).

The discrepant growth of glomeruli with podocytes growing by hypertrophy only, resulted in structural changes that terminated in the detachment of podocytes from the GBM. The present study focused on the structural details of how podocytes reinforce attachment to the GBM, undergo detachment from the GBM and on the fate of detached podocytes.

The first derangements of podocytes consisted of cell body attenuation and pseudocyst formation (**Figure 1B**), which, as shown previously in another model (12), were likely causally linked. Growing and expanding capillaries forced podocytes to cover increased surfaces, leading to stretching and broadening of podocytes' cell bodies. This, in turn, resulted in narrowing of the outflow clefts from the subpodocyte spaces, causing the focal bulging of the attenuated cytoplasmic sheets, usually called pseudocysts. Of note, at this stage, podocytes generally showed a normal FP pattern that also covered the floor of pseudocysts (**Figure 1B**).

The structural changes that finally led to the detachment of podocytes clearly started after this stage at the FPs (not necessarily as a consequence). The description is complicated by the fact that clear signs of detachment are mixed up with structural responses of podocytes taken to counteract detachment, which are generally subsumed under the term "FPE" (5).

Let us first consider the changes that may be suggested to be protective, i.e., to counteract detachment. FPs broadened making the filtration slits narrower; finally, the slit diaphragms were replaced by occluding junctions, thus sealing the filtration slits (**Figure 2A**). This was associated with a retraction of FPs into short, irregularly shaped cell projections (**Figure 2B**). Further on, the development led to the completion of FPE, changing podocyte processes into broad, flattened, disk-like projections that finally fused with the cell bodies resulting in podocytes adhering to the GBM along their total basal aspect. Thus, podocytes had become largely separated from each other, meaning that individual podocytes covered a clearly delimited capillary area, frequently the total circumference of one capillary profile (**Figure 2C**). Concomitantly, in the basal parts of these podocytes, a microfilament-based cytoskeletal mat developed (13) that has been interpreted as a way to achieve stronger adherence of podocytes to the GBM (5). This appearance

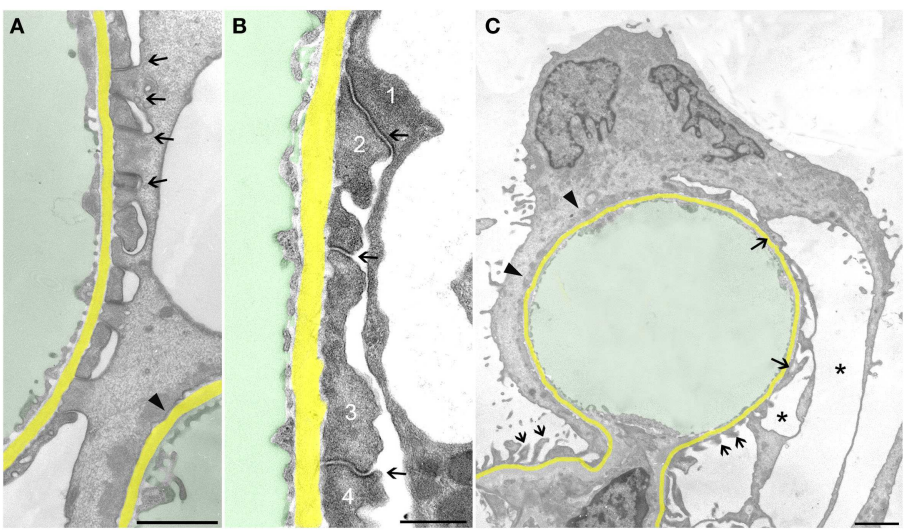

FIGURE 2 | Sealing of the slits may proceed to complete foot process effacement (FPE). The GBM is highlighted in yellow, capillary lumens in green. **(A)** Early stage of FPE with broadening of FPs and sealing of the slits by occluding junctions (some are labeled by arrows). In the right upper corner part of a capillary, profile with completed FPE (arrowhead) is seen. **(B)** "Foot processes" have changed into broad plaques (1,2,3,4) that closely stick to the GBM. The sealing of the filtration slits by occluding junctions between them is maintained (arrows). **(C)** Capillary profile covered by a podocyte that is closely attached to the GBM by "effaced" basal cell portions stuffed with a prominent cytoplasmic mat (arrowheads). At the opposite side, broadened FPs (arrows) are undergoing retraction, whereas at the transition to the mesangial region, FPs appear intact (two small arrows). Pseudocyst formation is also seen (asterisks). **(A)** and **(B)** male, **(C)** female rats, growth stimulation with FGF-2 for 13 weeks. TEMs. Bars: **(A)** 1 μm, **(B)** 0.5 μm, **(C)** 2 μm.

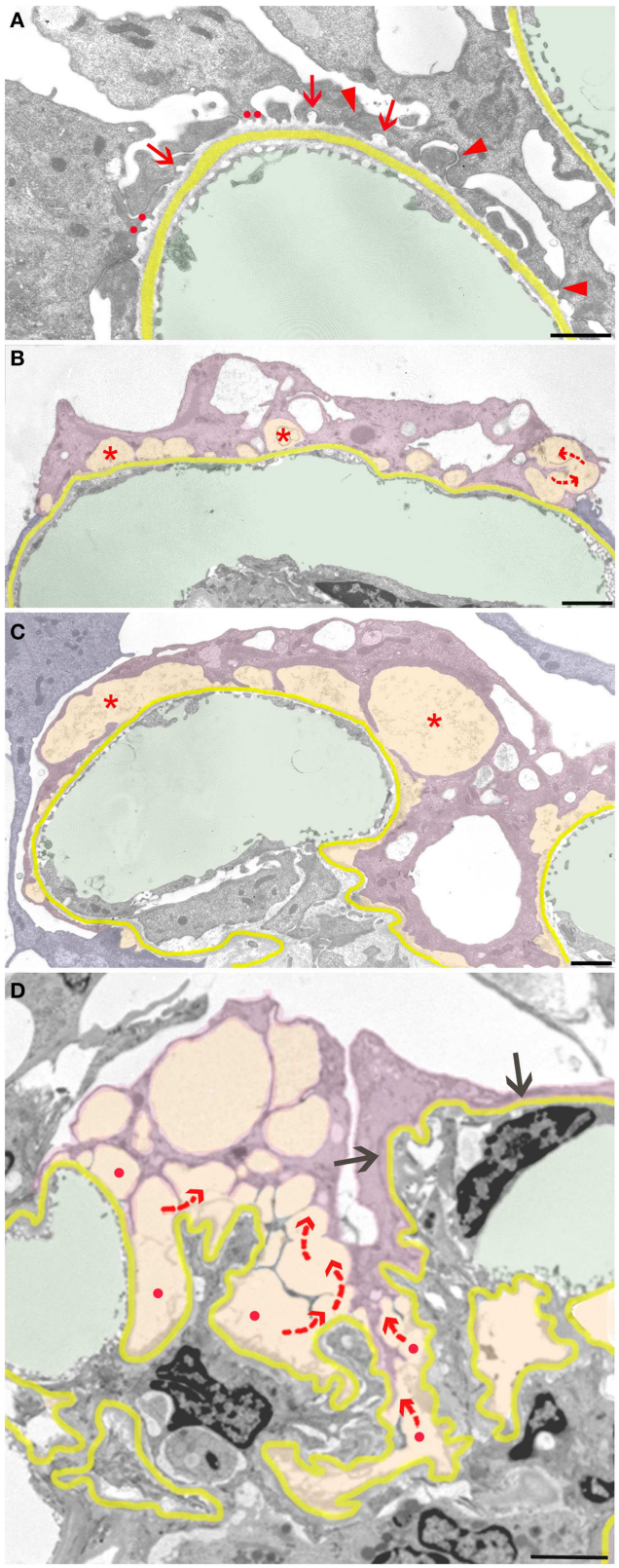

FIGURE 3 | Sequence of podocyte disconnection from the GBM.
(Continued)

FIGURE 3 | Continued
The GBM is highlighted in yellow, capillary lumens in green, and spaces considered as pseodocysts connected to bare GBM in yellow-orange. Individual podocytes are highlighted in different shades of violet. **(A)** Early stage of podocyte detachment from the GBM. A sealing of filtration slits by occluding junctions replacing slit membranes was only partially successful (arrowheads). At many other sites, retraction of FPs has left behind circumscribed areas of bare GBM (arrows) that start to merge (red dots). **(B)** Advanced stage of podocyte detachment from the GBM. The dome-shaped spaces (asterisks) above bare areas of GBM have enlarged bulging toward the urinary space. Merging of these spaces leads to a communicating system of pseudocysts (arrows). **(C)** The pseudocysts (asterisks) increase in size by merging and bulging; they are related to large stretches of bare GBM and overarched by broadened and attenuated primary processes. **(D)** The final detachment of a podocyte is frequently characterized by the merging of innumerous interconnected (red arrows) pseudocysts that start over bare GBM (red dots) and drain into Bowman's space (not seen in this section). Other portions of this podocyte may closely stick to the GBM with completed FPE (black arrows). Male rats, growth stimulation with FGF-2 for 13 weeks. TEMs. Bars: **(A)** 1 μm, **(B)** 2 μm, **(C)** 1 μm, **(D)** 2.5 μm.

may be regarded to underlie the successful prevention of podocyte detachment.

Neighboring such developments, even at other aspects of the same podocyte, these protective responses may fail, starting the focal detachment of podocytes at seemingly random sites. The complete detachment of an entire podocyte results from the final coalescence of numerous discrete disconnected areas (**Figure 3**). The smallest and probably first signs of detachment consist of circumscribed bare areas of GBM encountered in between two FPs of the same podocyte, thus the former interposed FP was missing (**Figure 3A**). This phenomenon frequently occurs in series and may be derived from the retraction of a series of FPs arising from a common larger process. In such cases, the co-ordinated retraction of adjacent FPs after formation of occluding junctions between them (as described above) seems to have failed.

Those areas of circumscribed detachments are frequently overarched by dome-shaped, flattened podocyte processes that are still attached to the GBM (**Figure 3B**). Thus, a new kind of small pseudocyst has developed that – in contrast to the above-described large pseudocysts – is underlain by sections of bare GBM. Progressive local detachment leads to the coalescence of these spaces (**Figure 3C**) and, finally, also to coalescence with large pseudocysts, creating a communicating system of extracellular spaces, through which the filtrate has to pass to reach Bowman's space (**Figure 3D**). Podocytes associated with such assemblies of pseudocysts finally undergo detachment.

In general, podocytes detach in groups (**Figure 4**) forming clusters of cells within Bowman's space, cells interconnected by occluding junctions. These interconnections are likely rooted in the early response to the danger of detachment, i.e., sealing the slits by replacing the slit diaphragms by occluding junctions. As mentioned above, sites of detachment from the GBM and sites of reinforced attachment including the attachment to adjacent podocytes may be found in the same podocyte early in the disease process. These interconnections were, at least partially, preserved when podocytes finally detached from the GBM as entire cells.

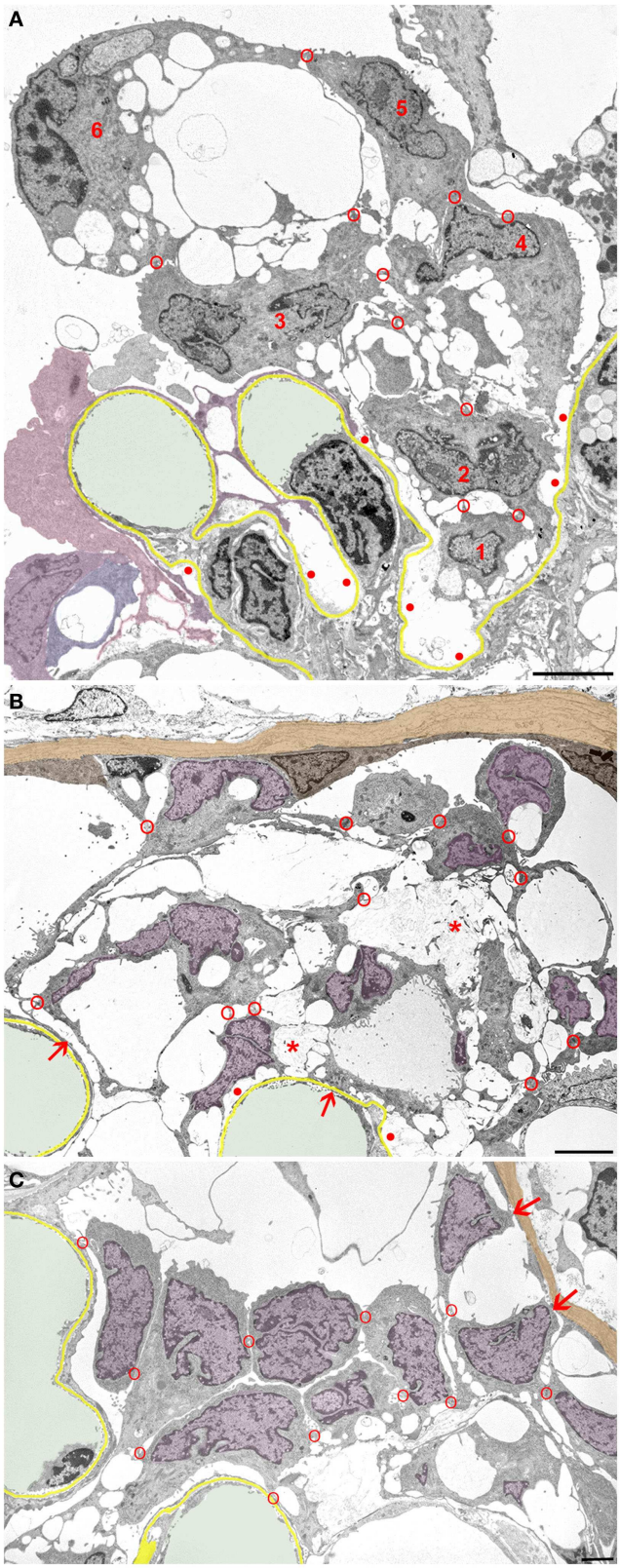

FIGURE 4 | Clusters of detaching podocytes in Bowman's space. *(Continued)*

FIGURE 4 | Continued
Capillary lumens are highlighted in green, the GBM in yellow. In B and C cell nuclei of podocytes are shown in violet, the PBM in light brown. **(A)** A group of 6 podocytes in the process of detachment. Only podocytes 1 to 4 still have contact with the GBM, whereas 5 and 6 are attached to the podocytes beneath by junctions (red circles). Within this cluster of podocytes, a communicating system of pseudocysts is seen that starts at bare areas of GBM (red dots). It may readily be suggested that the filtrate entering through bare GBM has to pass these spaces to reach Bowman's space; openings into Bowman's space are not hit in this section. Note that additional detaching podocytes (colored in various shades of violet) are gathered in another cluster. **(B)** Cluster of some 10 detaching or detached podocytes forming a cell bridge between the tuft and Bowman's capsule. The unlabeled cell within the parietal epithelium cannot be assigned by any plausible criterion to either parietal cells or to podocytes. The innermost cells are still partially attached to the GBM (arrows). The cells are interconnected to each other and still contain abundant pseudocysts that form a continuous chamber system starting from bare GBM (red dots) extending to the outermost layer. Note that the walls of many pseudocysts are undergoing shedding (asterisks). **(C)** Podocytes after almost complete detachment from the GBM are assembled as a cluster in Bowman's space interconnected to each other by cell contacts (red circles). The inner row of podocytes shows assemblies of small pseudocysts, the outer row appears as rounded cells. The outermost cells have contacts to Bowman's capsule (arrows). Male rats, growth stimulation with FGF-2 for 13 weeks. TEMs. Bars: **(A)** 5 μm, **(B)** 4 μm, **(C)** 2 μm.

Initially, those clusters of podocytes are connected to the GBM by the innermost cells (**Figure 4A**). In later stages, they made contact via the outermost cells with the parietal epithelium (**Figures 4B,C** and **5**), thereby forming multicellular "bridges" between the GBM and the parietal epithelium. During this process, podocytes change in shape. Apparently by shedding of the pseudocysts, i.e., of their thin walls, the detached podocytes turned into groups of rounded cells without any matrix in between (**Figures 4** and **5**). Mitotic figures were never encountered in these cells.

In even later stages, these bridging clusters of detached podocytes initiate the formation of tuft adhesions to Bowman's capsule, i.e., firm cell and matrix connections between the GBM and the parietal basement membrane, as described previously (10). Concomitantly, the tuft itself underwent considerable shrinkage, including wrinkling of the GBM, loss of capillaries, and mesangial cells (**Figure 5**; to be described in detail in another context).

The detachment of podocytes that have come to protrude into the urinary orifice has already been described in various models and interpreted as the consequence of high shear forces within the urinary orifice acting on the cell bodies (5, 14). Here, we found that the final changes accounting for detachment are likely also rooted in the destabilization of FP attachment to the GBM, as described above, and that these podocytes frequently detach in groups and travel in groups along the tubule (not shown).

QUANTIFICATION OF GLOMERULAR INJURIES

Crucial steps of the above-described sequence in damage progression were quantified in 1 μm sections. **Figure 1A** shows an entire glomerular profile indicating the injuries that were counted and compared. As seen in **Table 1** and in agreement with the clinical data of the former study (10), the damage was significantly more severe in males than in females.

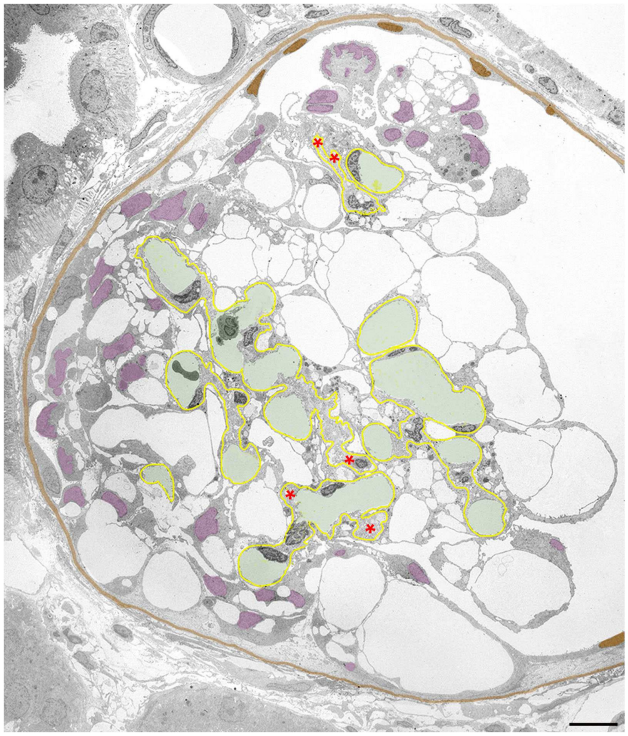

FIGURE 5 | Clustering of podocytes in Bowman's space is associated with shrinkage of the mesangio-capillary area. The GBM is highlighted in yellow, capillary lumens in green, the PBM and cell nuclei of parietal cells in brown, podocyte cell nuclei in violet. Glomerular profile with a tuft that is broadly connected to Bowman's capsule by a large cellular crescent that surrounds the tuft from three sides. The mesangio-capillary area (delimited by the GBM) is small compared to the area occupied by podocytes. Note that podocyte cell bodies (visualized by their cell nuclei) have almost completely disappeared from central tuft areas being all contained within the crescent. Most of the podocytes have retained pseudocysts. The cluster of podocytes on top of the tuft does not show any contacts to the tuft nor to the PBM, whereas the major part of the crescent displays extensive contacts to the GBM as well as to Bowman's capsule. Nuclei of cells, which by no plausible criterion can be assigned as podocytes or parietal cells are uncolored. Note that the GBM is heavily wrinkled at several sites and the mesangial area appears to be collapsed with disappearance of capillaries at several loci (asterisks). Male rat, growth stimulation with FGF-2 for 13 weeks. TEM. Bar: 10 μm.

Table 1 | Quantification of glomerular injuries.

	Experimental animals		Controls
	8 + 13 weeks, pooled		
	Female, $n = 6$	Male, $n = 6$	$n = 6$
No. of glomerular profiles	536	385	509
Large pseudocysts%	77 ± 11	87 ± 7	0
Small pseudocysts%	37 ± 8*	62 ± 16	0
Clusters of podocytes%	19 ± 9**	42 ± 7	0
Tuft adhesions%	4 ± 3*	18 ± 21	0
Weighted damage score	56 ± 30**	103 ± 48	0

A glomerular profile was considered positive for the respective injury if a minimum of (i) three large pseudocysts, (ii) two areas of small pseudocysts (indicative of local detachments), (iii) four podocytes forming a cluster of connected cells in Bowman's space, and (iv) one adhesion was encountered. The weighted damage score includes all changes in a graded way (see Materials and Methods).
$*p < 0.05$, $**p < 0.001$.

DISCUSSION

GENERAL

It is commonly agreed that the loss of podocytes underlies the development of progressive renal failure. Research of the past 20 years has shown that podocytes are lost by a unique mechanism, i.e., by detachment from the GBM as viable cells and excretion in the urine. This fact, in conjunction with their inability to replicate, puts podocytes in a precarious situation, unlike any other cell (5). Thus, the precise knowledge of why and how podocytes undergo detachment from the GBM is of great importance when searching for strategies to delay the progression to end-stage renal failure.

The present study has addressed this question in a model of excessive glomerular growth initiated by long-term stimulation with FGF-2 (10). Endothelial and mesangial cells proliferated in this condition; podocytes participating solely by hypertrophy could not cope with the overall glomerular growth, underwent structural derangements and, finally, detached from the GBM. This growth-stimulating rat model is similar to a recently published transgenic rat model (15) in which the ability of podocytes for hypertrophy was restricted resulting, after growth stimulation by uninephrectomy, in a mismatch of endothelial and mesangial cell proliferation on the one hand and podocyte hypertrophy on the other, followed by podocyte loss through detachment. Both models mirror what happens in degenerating progressive glomerular diseases including all forms of secondary FSGS.

THE DETACHMENT OF PODOCYTES FROM THE GBM

The difficulty in the interpretation of the changes encountered in conjunction with podocyte detachment consists of separating the sequence of changes progressing to detachment from the sequence terminating in complete FPE, thus reinforcing attachment to the GBM. Changes belonging to either of these developments may be found at different sites of the same podocyte. This emphasizes the most important insight of this study: podocyte damage is local, even in the sense that the same podocyte undergoes detachment in one part of the cell, whereas at another part, it successfully reinforced its attachment to the GBM. This fact supports the view that, at least in this model, overall derangements of podocyte cell function are not the crucial causes underlying detachment but local imbalances between the forces accounting for attachment and those for detachment appear to play the major role.

This triad of hypertrophy, hypertension, and hyperfiltration has been convincingly shown to account for the progression of glomerular diseases under a great variety of circumstances (8, 9). These three factors are causally interconnected with each other but it remains an open question whether one of them plays a dominant role. Likely all three are involved in a mutually reinforcing process (16, 17). An elegant study showing in two mouse models of glomerulosclerosis that the development of glomerular injuries was significantly curtailed after unilateral ligation of the ureter

emphasizes the effects of changes in filtration dynamics (18), whereas prevention of glomerular growth in a model with established hyperfiltration and glomerular hypertension also resulted in a marked decrease in sclerosis development (19), showing a major impact of growth. Also, in human beings, glomerular hypertrophy was found to be the decisive cofactor in minimal change disease accounting for progression to FSGS (20). The present study suggests that excessive hypertrophy of podocytes may be a major factor in progression to what extent hyperfiltration and glomerular hypertension develop in this model is unknown.

Therefore, damage development in the FGF-2 model may be taken to reflect the combined effects of all three factors.

Let us look in detail to a hypothetical local scenario that may start the process of detachment. A certain podocyte has reached its ultimate limit of hypertrophy that allowed it to cover a correspondingly increased area of GBM with normal FPs. In case the rheological situation with high filtrate flows overtaxes the available slit area, the only protective option remaining consists of sealing the slits by occluding junctions. This represents a most crucial event. The formation of cell–cell junctions between FPs in glomerular disease models has been described previously by several groups, characterizing them as an "occluding" type (21–27). More recent work has shown that they are a certain type of tight junctions (28, 29). Moreover, it was shown that tight junctional proteins increase several-fold in puromycin-induced nephrosis (29).

If sealing the slits is successful, compensation by adjacent podocytes is required, with these being exposed to higher local filtrate flows. If these podocytes have some reserve capacity to hypertrophy, they may adequately manage the situation.

In the case that the sealing strategy fails, a fatal situation will develop. Damage to the slit membrane will increase the shear stress on the involved FPs and may initiate its local detachment, thereby increasing the bare area of GBM. This will lead to local increases in flow (local hyperfiltration) and worsen the unstable situation. Thus, as soon as any detachment has started locally, the final struggle against the detachment of the entire cell has begun. If there is any chance to reverse this situation, it probably consists of spreading of neighboring, uninvolved podocytes on the GBM covering the denuded area. If this maneuver also fails, detachment of the first podocyte will proceed and may include adjacent podocytes.

The process of detachment seems to follow a rather stereotyped structural sequence. Circumscribed bare areas of GBM in between two adherent FPs of the adjacent podocyte (as the result of initial local detachments) frequently form the floors of dome-like cavities overarched by attenuated cytoplasm of broadened primary processes. These spaces may be considered as a second type of pseudocyst associated, in contrast to the large pseudocysts, with bare GBM. They merge with each other, later with the large pseudocysts, finally forming a communicating system of extracellular spaces that starts above scattered areas of bare GBM and opens into Bowman's space through the narrow openings of the subpodocyte spaces. In many previous studies, this system of empty spaces has been termed "podocyte vacuolation," considered to consist of intracellular vacuoles ("blebs") (23, 30–34), indicating a prenecrotic stage. Here, we show that for the most part, these are extracellular spaces bordered by intact cell membranes of viable podocytes.

It may readily be suggested that sites of bare GBM allow increased filtrate flows and are the sites of protein leakage, as frequently suggested and shown previously (22, 35–37). The filtrate has to find a way out of these spaces along routes offering some amount of resistance to the outflow. The resulting expansile forces on the walls of these spaces induce progressive bulging and detachment, leaving behind increasing areas of bare GBM. Consequently, the flow of the filtrate through the system of connected pseudocysts likely exerts expansile forces on the concerned podocyte, tending to lift it off the GBM (**Figures 4** and **5**).

Even if started locally at a single podocyte, the process of detachment generally comprises a group of podocytes. This may be another explanation for the observation by Ichikawa et al. (38) that damage to a podocyte always damages neighboring podocytes. In our view, the detachment of a podocyte, once started, will often take others along and may be difficult to arrest.

The interpretation of the sealing of filtration slits by "FPE" as a protective measure of podocytes against detachment conflicts with the widely held view that FPE represents the common initial derangement of podocytes leading to proteinuria potentially progressing to more severe glomerular damage (39). As discussed previously (5), FPE is necessarily accompanied by transient protein leakage as a consequence of the motility associated with the extensive local rearrangements. The transient character of this kind of proteinuria has recently been found in studies after Rac-1 stimulation (40–42) that is known to elicit FPE (39, 43). Progression of the damage to FSGS has not been found in these studies supporting our view of a protective impact.

THE FATE OF DETACHED PODOCYTES

In static structural studies, it is impossible to follow individual podocytes as they are translocated from one site to another. However, we can observe that along the course of the disease, podocytes are regularly encountered at other sites than where they are found under normal conditions. From such comparisons, we may deduce the routes how they came from site A to site B. We do not think that such movements of podocytes are effected by any active motility of the podocytes themselves, We think that detachment from the GBM exposes them to the rheological forces of filtrate flow pushing them toward Bowman's capsule and the urinary orifice.

The fraction of podocytes that detach directly in front of the urinary orifice generally will be swept into the tubular lumen (5, 10, 14). Surprisingly, during their passage along the tubule, they did not undergo anoikosis but, as known from several studies (e.g., 3 and 4), may have reached the renal pelvis as living cells.

Podocytes detaching from most other sites of the tuft may form massive cell accumulations within Bowman's space interconnected by occluding junctions with the innermost cells keeping contact to the GBM. During this gradual process of detachment, podocytes change in shape; they shed all the peripheral material forming the thin walls of pseudocysts, finally appearing as rounded cells. Frequently, such cell clusters establish, with their outermost cells, contacts to parietal epithelial cells, thus forming purely cellular crescents connecting the tuft and Bowman's capsule.

These observations are in full agreement with recent *in vivo* studies by multiphoton microscopy in two other models with

podocyte detachment, i.e., unilateral ureter ligation (UUO) and Adriamycin nephropathy (44). In these studies, the formation of multicellular clusters of podocytes in Bowman's space was observed that made contact to Bowman's capsule; also, podocytes in single file moving down the tubule were seen. The authors also observed that the podocyte clusters made contacts to parietal cells by cell projections and nano-tubes. We found by TEM abundant occluding junctions between podocytes as well as between podocytes and parietal epithelial cells suggesting that they are responsible for the mechanical cohesion. As also verified in serial sections, the outermost cells of the podocyte clusters are fully separated from the GBM but fixed to inner cells of the cluster by occluding junctions. It appears that as soon as they come into contact with parietal cells, they establish junctional contacts to parietal cells, as well.

The further evolution of such clusters of detached podocytes attaching to Bowman's capsule, finally turning into tuft adhesions and crescents has been shown and discussed previously (10, 45, 46).

Relationships to pathologies seen in human renal diseases

It is quite obvious that the clustering of podocytes within Bowman's space and the shrinkage and collapse of the mesangio-capillary area have equivalents in human renal diseases, known as "cellular crescent," "cellular lesion," and "collapsing glomerulopathy" (CGP). The origin of the abnormal cells in these pathologies is under debate (47–50). In the present degenerative animal model, the cells clustering on the outer aspect of the tuft within Bowman's space are clearly derived from podocytes and do not proliferate. CGP combines the collapse of the mesangio-capillary compartment with hypertrophy and seemingly hyperplasia of the cells that take the place of podocytes on the outside of the GBM, assembled in clusters looking like crescents (51–54). The shrinkage of the mesangio-capillary area with wrinkling of the GBM and collapse of capillaries in CGP look identical to what is seen in the present animal model. Also, the clustering of cells in Bowman's space in CGP may look very similar to the clustering of detached podocytes in our animal model.

The great number of such cells seen in a cross-sectional profile of CGP seems incompatible with detachment as the only mechanism. However, as far as we know, the total number of podocytes on the surface of a collapsed tuft in CGP has never been estimated by stereological methods. Though a fully reliable calculation is presently not possible, we tried the following approach applying the recently published method by Venkatareddy et al. (55) to estimate podocyte number from a single glomerular profile in a single section. Since we do not have own biopsy material from a case of CGP, we took the glomerulus shown in **Figure 3** from the paper by Jhaveri et al. (56) and made the following calculation. In this glomerular profile, we counted 90 podocyte cell nuclei. From the listed magnification, the tuft diameter is about 100 μm (healthy tufts are usually in the range 180–220 μm, glomerular tufts are shrunken in CGP). The cross-sectional area is, therefore, 7854 μm^2 and the area density of nuclei is $90/7854 = 0.01146$. Using the estimated caliper diameter for human podocyte nuclei of 8.2 μm (given in 55), we obtain the volumetric podocyte density of $0.01146/8.2 = 0.001397$ podocytes/μm^3. The 100 μm tuft diameter yields an estimated tuft volume of 523,585 μm^3 (much smaller than a typical tuft volume of 3,000,000 μm^3). Multiplying the density by the tuft volume gives an estimate of 731 podocytes in the tuft. To the degree that any podocytes are binucleate, this will be an overestimate of cell number.

Thus, it seems possible that the seemingly great number of podocytes on a shrunken tuft in CGP is all derived from detachment, gathering on the shrunken surface without any cell proliferation. This is supported by the fact that mitotic figures are generally not found in these cell assemblies in CGP. Major support in favor of detachment comes from the work of Bariety et al. (57) and also from findings in a transgenic mouse model of HIV-associated nephropathy (58).

CONCLUSION

Loss of podocytes represents the central event underlying the glomerular disease progression (4, 46). Here, in a model of continuous growth stimulation by FGF-2, we show that loss of podocytes occurs by detachment from the GBM as viable cells. This is a gradual process that starts focally at randomly distributed sites. The local origin strongly supports the view that the forces starting detachment consists of local rheological disturbances. The detachment of a podocyte, once started, may be difficult to arrest. However, podocytes have developed protective mechanisms against detachment, most important the sealing of the filtration slits followed by FPE.

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REFERENCES

1. Pagtalunan ME, Miller PL, Jumping-Eagle S, Nelson RG, Myers BD, Rennke HG, et al. Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* (1997) **99**:342–8. doi:10.1172/JCI119163
2. Wharram BL, Goyal M, Wiggins JE, Sanden SK, Hussain S, Filipiak WE, et al. Podocyte depletion causes glomerulosclerosis: diphteria toxin-induced podocyte depletion in rats expressing human diphteria toxin receptor transgene. *J Am Soc Nephrol* (2005) **16**:2941–52. doi:10.1681/ASN.2005010055
3. Vogelmann SU, Nelson WJ, Myers BD, Lemley KV. Urinary excretion of viable podocytes in health and renal disease. *Am J Physiol Renal Physiol* (2003) **285**:F40–8. doi:10.1152/ajprenal.00404.2002
4. Wickman L, Afshinnia F, Wang SQ, Yang Y, Wang F, Chowdhury M, et al. Urine podocyte mRNAs, proteinuria, and progression in human glomerular diseases. *J Am Soc Nephrol* (2013) **24**:2081–95. doi:10.1681/ASN.2013020173
5. Kriz W, Shirato I, Nagata M, Le Hir M, Lemley KV. The podocyte's response to stress: the enigma of foot process effacement. *Am J Physiol Renal Physiol* (2013) **304**:F333–47. doi:10.1152/ajprenal.00478.2012
6. Kriz W, Lemley KV. A potential role for mechanical forces in the detachment of podocytes and the progression of CKD. *J Am Soc Nephrol* (2014). doi:10.1681/ASN.2014030278
7. Endlich N, Endlich KH. The challenge and response of podocytes to glomerular hypertension. *Semin Nephrol* (2012) **32**:327–41. doi:10.1016/j.semnephrol.2012.06.004
8. Hostetter TH, Olson JL, Rennke HG, Venkatachalam MA, Brenner BM. Hyperfiltration in remnant nephrons: a potentially adverse response to renal ablation. *Am J Physiol* (1981) **241**:F85–93.
9. Brenner BM, Lawler EV, Mackenzie HS. The hyperfiltration theory: a paradigma shift in nephrology. *Kidney Int* (1996) **49**:1774–7. doi:10.1038/ki.1996.265

10. Kriz W, Hähnel B, Rösener S, Elger M. Long-term treatment of rats with FGF-2 results in focal segmental glomerulosclerosis. *Kidney Int* (1995) **48**:1435–50. doi:10.1038/ki.1995.433
11. Sakai T, Kriz W. The structural relationship between mesangial cells and basement membrane of the renal glomerulus. *Anat Embryol* (1987) **176**:373–86. doi:10.1007/BF00310191
12. Nagata M, Kriz W. Glomerular damage after uninephrectomy in young rats. II. Mechanical stress on podocytes as a pathway to sclerosis. *Kidney Int* (1992) **42**:148–60. doi:10.1038/ki.1992.272
13. Shirato I, Sakai T, Kimura K, Tomino Y, Kriz W. Cytoskeletal changes in podocytes associated with foot process effacement in Masugi nephritis. *Am J Pathol* (1996) **148**:1283–96.
14. Friedrich C, Endlich N, Kriz W, Endlich KH. Podocytes are sensitive to fluid shear stress in vitro. *Am J Physiol Renal Physiol* (2006) **291**:F856–65. doi:10.1152/ajprenal.00196.2005
15. Fukuda A, Chowdhury MA, Venkatareddy MP, Wang SQ, Nishizono R, Suzuki T, et al. Growth-dependent podocyte failure causes glomerulosclerosis. *J Am Soc Nephrol* (2012) **23**:1351–63. doi:10.1681/ASN.2012030271
16. Hostetter TH. Progression of renal disease and renal hypertrophy. *Annu Rev Physiol* (1995) **57**:263–78. doi:10.1146/annurev.ph.57.030195.001403
17. Hostetter TH. Hypertrophy and hyperfunction of the diabetic kidney. *J Clin Invest* (2001) **107**:161–2. doi:10.1172/JCI12066
18. Matsusaka T, Kobayashi K, Kon V, Pastan I, Fogo AB, Ichikawa I. Glomerular sclerosis is prevented during urinary tract obstruction due to podocyte protection. *Am J Physiol Renal Physiol* (2011) **300**:F792–800. doi:10.1152/ajprenal.00570.2010
19. Yoshida Y, Fogo AB, Ichikawa I. Glomerular hemodynamic changes vs. hypertrophy in experimental glomerular sclerosis. *Kidney Int* (1989) **35**:654–60. doi:10.1038/ki.1989.35
20. Fogo AB, Hawkins EP, Berry PL, Glick AD, Chiang ML, MacDonell RC Jr, et al. Glomerular hypertrophy in minimal change disease predicts subsequent progression to focal glomerular sclerosis. *Kidney Int* (1990) **38**:115–23. doi:10.1038/ki.1990.175
21. Seiler MW, Rennke HG, Venkatachalam MA, Cotran RS. Pathogenesis of polycation-induced alterations ("fusion") of glomerular epithelium. *Lab Invest* (1977) **36**:48–62.
22. Venkatachalam MA, Karnovsky MJ, Cotran RS. Glomerular permeability – ultrastructural studies in experimental nephrosis using horseradish peroxidase as a tracer. *J Exp Med* (1969) **130**:381–99. doi:10.1084/jem.130.2.381
23. Caulfield JP, Reid JJ, Farquhar MG. Alterations of the glomerular epithelium in acute aminonucleoside nephrosis. Evidence for formation of occluding junctions and epithelial cell detachment. *Lab Invest* (1976) **34**: 43–59.
24. Kerjaschki D. Polycation-induced dislocation of slit diaphragms and formation of cell junctions in rat kidney glomeruli. The effects of low temperature, divalent cations, colchicine, and cytochalasin B. *Lab Invest* (1978) **39**:430–40.
25. Pricam C, Humbert F, Perrelet A, Amherdt M, Orci L. Intercellular junctions in podocytes of the nephrotic glomerulus as seen with freeze-fracture. *Lab Invest* (1975) **33**:209–18.
26. Inokuchi S, Sakai T, Shirato I, Tomino Y, Koide H. Ultrastructural changes in glomerular epithelial cells in acute puromycin aminonucleoside nephrosis: a study by high-resolution scanning electron microscopy. *Virchows Arch A Pathol Anat Histopathol* (1993) **423**:111–9. doi:10.1007/BF01606585
27. Kurihara H, Anderson JM, Kerjaschki D, Farquhar MG. The altered glomerular filtration slits seen in puromycin aminonucleoside nephrosis and protamine sulfate-treated rats contain the tight junction protein ZO-1. *Am J Pathol* (1992) **141**:805–15.
28. Shono A, Tsukaguchi H, Yaoita E, Nameta M, Kurihara H, Qin XS, et al. Podocin participates in the assembly of tight junctions between foot processes in nephrotic podocytes. *J Am Soc Nephrol* (2007) **18**:2525–33. doi:10.1681/ASN.2006101084
29. Fukasawa H, Bornheimer S, Kudlicka K, Farquhar MG. Slit diaphragms contain tight junction proteins. *J Am Soc Nephrol* (2009) **20**:1491–503. doi:10.1681/ASN.2008101117
30. Wheeler J, Robertson H, Morley AR, Appleton DR. Anti-glomerular basement membrane glomerulonephritis (anti-GBM GN) in the mouse: BrdU-labelling indices and histological damage. *Int J Exp Pathol* (1993) **74**:9–19.
31. Yoshikawa N, Hiroshi I, Akamatsu R, Hazikano H, Okada S, Matsuo T. Glomerular podocyte vacuolation in focal segmental glomerulosclerosis. *Arch Pathol Lab Med* (1986) **110**:394–8.
32. Aizawa K, Takeda S, Tashiro Y, Yorozu K, Hirata M, Kanada H, et al. Renoprotection by continuous erythropoietin receptor activator in puromycin aminonucleoside-induced nephrotic syndrome. *Am J Nephrol* (2013) **36**:419–26. doi:10.1159/000343493
33. Toth T, Takebayashi S. Glomerular podocyte vacuolation in idiopathic membranous glomerulonephritis. *Nephron* (1992) **61**:16–20. doi:10.1159/000186828
34. Yamazaki T. Podocytic degeneration and regeneration in puromycin aminonucleoside nephropathy in the rat. *Pathol Int* (1995) **45**:465–7. doi:10.1111/j.1440-1827.1995.tb03487.x
35. Whiteside C, Prutis K, Cameron R, Thompson J. Glomerular epithelial detachment, not reduced charge density, correlates with proteinuria in adriamycin and puromycin nephrosis. *Lab Invest* (1989) **61**:650–60.
36. Fries JW, Sandstrom DJ, Meyer TW, Rennke HG. Glomerular hypertrophy and epithelial cell injury modulate progressive glomerulosclerosis in the rat. *Lab Invest* (1989) **60**:205–18.
37. Olson JL, Hostetter TH, Rennke HG, Brenner BM, Venkatachalam MA. Altered glomerular permselectivity and progressive sclerosis following extreme ablation of renal mass. *Kidney Int* (1982) **22**:112–26. doi:10.1038/ki.1982.143
38. Ichikawa I, Ma J, Motojima M, Matsusaka T. Podocyte damage damages podocytes: autonomous vicious cycle that drives local spread of glomerular sclerosis. *Curr Opin Nephrol Hypertens* (2005) **14**:205–10.
39. Greka A, Mundel P. Cell biology and pathology of podocytes. *Annu Rev Physiol* (2012) **74**:299–323. doi:10.1146/annurev-physiol-020911-153238
40. Blattner SM, Hodgin JB, Nishio M, Wylie SA, Saha J, Soofi AA, et al. Divergent functions of the Rho GTPases Rac1 and Cdc42 in podocyte injury. *Kidney Int* (2013) **84**:920–30. doi:10.1038/ki.2013.175
41. Yu H, Suleiman H, Kim AH, Miner JH, Dani A, Shaw AS, et al. Rac1 activation in podocytes induces rapid foot process effacement and proteinuria. *Mol Cell Biol* (2013) **33**:4755–64. doi:10.1128/MCB.00730-13
42. Asao R, Asanuma K, Kodama F, Takagi M, Hosoe Y, Tanaka E, et al. Rac1 in podocytes protects glomeruli against the formation of sclerosis. *J Am Soc Nephrol FR-PO851.* (2013).
43. Schaldecker T, Kim S, Tarabanis C, Tian D, Hakroush S, Castonguay P, et al. Inhibition of the TRPC5 ion channel protects the kidney filter. *J Clin Invest* (2013) **123**:5298–309. doi:10.1172/JCI71165
44. Hackl M, Burford JL, Villanueva K, Lam L, Susztak K, Schermer B, et al. Tracking the fate of glomerular epithelial cells in vivo using serial multiphoton imaging in new mouse models with fluorescent lineage tags. *Nat Med* (2013) **19**:1661–6. doi:10.1038/nm.3405
45. Le Hir M, Keller C, Eschmann V, Hähnel B, Hosser H, Kriz W. Podocyte bridges between the tuft and Bowman's capsule: an early event in experimental crescentic glomerulonephritis. *J Am Soc Nephrol* (2001) **12**:2060–71.
46. Kriz W, Le Hir M. Pathways to nephron loss starting from glomerular diseases – insights from animal models. *Kidney Int* (2005) **67**:404–19. doi:10.1111/j.1523-1755.2005.67097.x
47. Schwartz MM, Lewis EJ. Focal segmental glomerular sclerosis: the cellular lesion. *Kidney Int* (1985) **28**:968–74. doi:10.1038/ki.1985.225
48. Schwartz MM, Evans J, Bain RP, Korbet SM. Focal segmental glomerulosclerosis: prognostic implications of the cellular lesion. *J Am Soc Nephrol* (1999) **10**:1900–7.
49. Wang S, Kim JH, Moon KC, Hong HK, Lee HS. Cell-cycle mechanisms involved in podocyte proliferation in cellular lesion of focal segmental glomerulosclerosis. *Am J Kidney Dis* (2004) **43**:19–27. doi:10.1053/j.ajkd.2003.09.010
50. Taneda S, Honda K, Uchida K, Nitta K, Yumura W, Oda H, et al. Histological heterogeneity of glomerular segmental lesions in focal segmental glomerulosclerosis. *Int Urol Nephrol* (2012) **44**:183–96. doi:10.1007/s11255-011-9932-y
51. Barisoni L, Schnaper HW. A proposed taxonomy for the podocytopathies: a reassessment of the primary nephrotic diseases. *Clin J Am Soc Nephrol* (2007) **2**:529–42. doi:10.2215/CJN.04121206
52. Stokes MB, Valeri AM, Markowitz GS, d'Agati VD. Cellular focal segmental glomerulosclerosis: clinical and pathologic features. *Kidney Int* (2006) **70**:1783–92. doi:10.1038/sj.ki.5001903

53. Laurinavicius A, Rennke HG. Collapsing glomerulopathy – a new pattern of renal injury. *Semin Diagn Pathol* (2002) **19**:106–15.
54. Markowitz GS, Appel GB, Fine PL, Fenves AZ, Loon NR, Jagannath S, et al. Collapsing focal segmental glomerulosclerosis following treatment with high-dose pamidronate. *J Am Soc Nephrol* (2001) **12**:1164–72.
55. Venkatareddy M, Wang S, Yang Y, Patel S, Wickman L, Nishizono R, et al. Estimating podocyte number and density using a single histologic section. *J Am Soc Nephrol* (2014) **25**:1118–29. doi:10.1681/ASN.2013080859
56. Jhaveri KD, Shah HH, Calderon K, Campenot ES, Radhakrishnan J. Glomerular diseases seen with cancer and chemotherapy: a narrative review. *Kidney Int* (2013) **84**:34–44. doi:10.1038/ki.2012.484
57. Bariety J, Nochy D, Mandet C, Jacquot C, Glotz D, Meyrier A. Podocytes undergo phenotypic changes and express macrophagic-associated markers in idiopathic collapsing glomerulopathy. *Kidney Int* (1998) **53**:918–25. doi:10.1111/j.1523-1755.1998.00845.x
58. Barisoni L, Bruggeman LA, Mundel P, d'Agati VD, Klotman PE. HIV-1 induces renal epithelial dedifferentiation in a transgenic model of HIV-associated nephropathy. *Kidney Int* (2000) **58**:173–81. doi:10.1046/j.1523-1755.2000.00152.x

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Podocyte dedifferentiation: a specialized process for a specialized cell

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The podocyte is one of the two cell types that contribute to the formation of the glomerular filtration barrier (GFB). It is a highly specialized cell with a unique structure. The key feature of the podocyte is its foot processes that regularly interdigitate. A structure known as the slit diaphragm can be found bridging the interdigitations. This molecular sieve comprises the final layer of the GFB. It is well accepted that the podocyte is the target cell in the pathogenesis of nephrotic syndrome. In nephrotic syndrome, the GFB no longer restricts the passage of macromolecules and protein is lost into the urine. A number of phenotypic and morphological changes are seen in the diseased podocyte and in the literature these have been described as an epithelial–mesenchymal transition (EMT). However, there is a growing appreciation that this term does not accurately describe the changes that are seen. Definitions of type-2 EMT are based on typical epithelial cells. While the podocyte is known as a visceral epithelial cell, it is not a typical epithelial cell. Moreover, podocytes have several features that are more consistent with mesenchymal cells. Therefore, we suggest that the term podocyte disease transformation is more appropriate.

Keywords: nephrotic syndrome, podocytes, proteinuria, epithelial–mesenchymal transition, dedifferentiation

INTRODUCTION

The podocyte is thought to be the target cell in the pathogenesis of nephrotic syndrome. The term "podocytopathy" is being increasingly used to describe disease that has arisen due to insult or injury to the podocyte. Minimal change nephropathy (MCN), focal segmental glomerulosclerosis (FSGS), diffuse mesangial sclerosis (DMS), and collapsing glomerulopathy (CG) are all thought to be podocytopathic nephrotic diseases (1). Irrespective of the cause, podocytes demonstrate dramatic morphological differences when there is nephrotic range proteinuria (2). Actin cytoskeleton rearrangement, slit diaphragm loss, and more cuboidal morphology are all hallmark features of the diseased podocyte (2). The field has tended to refer to this loss of typical phenotype as an epithelial–mesenchymal transition (EMT) event (3–5).

However, EMT is a highly plastic and reversible process: indeed, the word "transition" was chosen deliberately to reflect this transient nature. The phenotypic and morphological changes seen in diseased podocytes are only reversible if the insult is not very severe. In minimal change disease, the disease phenotype is reversible following glucocorticoid therapy. However, in FSGS, the changes in morphology and phenotype are not only irreversible but progressive (6). Therefore, using the term EMT implies an innate reversibility, which is not strictly accurate in the case of the podocyte. Therefore, it is important to reassess the extent to which the term EMT accurately describes the transposition from the healthy to the diseased podocyte morphology. Additionally, the podocyte is not a typical epithelial cell. Despite podocytes also being known as visceral epithelial cells, they retain several mesenchymal features (spindle shaped morphology and high levels of matrix interaction) and lack archetypal epithelial markers (cell–cell contacts that are based predominantly on P rather than E-cadherin). Therefore, the podocyte fails to demonstrate the emblematic features of EMT. If the change in podocyte morphology and phenotype seen in disease does not fulfill the accepted criteria for an EMT event and is not necessarily transient in nature, then the usefulness of this term in this context must be questioned.

Immortalized podocytes *in vitro* respond to the classic inducer of EMT, TGF-B1. Following TGF-B1 treatment, human podocytes *in vitro* demonstrate increased levels of α-SMA, cadherin switch from P-cadherin to N-cadherin, and expression of the main effector transcription factors of EMT: SNAIL and SLUG (7). Similar phenotypic changes are seen in mouse podocytes *in vitro* when exposed to TGF-B1. Again suppression of P-cadherin along with suppression of ZO-1 and nephrin with concomitant upregulation of desmin, fibronectin, and collagen I is observed (8). *In vitro*, following 24 h exposure to TGF-B1, human podocytes lose their highly arborized morphology and adopt a more cobblestone-like morphology. The existence of similar phenotypic changes in both mouse and human podocytes in response to TGF-B1 is indicative of an evolutionarily conserved disease mechanism.

The pathological effects of such phenotypic changes within a cell-type, such as the podocyte, are severe. Foot process effacement is linked to a diminished ability to restrict urinary protein loss. This leads to runaway proteinuria and nephrotic syndrome. Effaced podocytes have less contact with the glomerular basement membrane, making podocyte loss much more likely. Additionally, the increased synthesis of extracellular matrix (ECM) components such as fibronectin and collagen I may contribute to GBM thickening. These are all hallmark features of diabetic nephropathy (9).

FSP1 is a fibroblast (mesenchymal) marker, which has been found in podocytes from patients with diabetic nephropathy. Healthy podocytes do not express FSP1; therefore, *de novo*

expression of a mesenchymal marker is a pathological change in phenotype. Moreover, the frequency of $FSP1^+$ podocytes in the urine has been linked to disease severity (8). $FSP1^+$ podocytes have also been found in the glomeruli of FSGS patients (10).

The term EMT is over simplistic in this context, and does not encapsulate the process that is seen in the disease state *in vivo* and disease models *in vitro*. Broadly speaking, the morphological and phenotypic changes seen in diseased podocytes appear to be an EMT type event. However, in order for the assertion that this is an EMT event to be accurate, one must first consider to what extent the changes seen correlate with the definition of EMT.

THE PODOCYTE: A SPECIALIZED CELL

The podocyte is a highly specialized cell that is situated on the outer surface of the GBM. It comprises three structurally and functionally unique segments. These are known as the cell body, the major process, and the foot process (11). The cell body, the major processes, and the foot processes share a common actin cytoskeleton contractile apparatus similar to that found in smooth muscle cells or pericytes (11). The foot processes extend from the major processes and cover the GBM. Neighboring foot processes interdigitate, and where this occurs a modified cell-to-cell junction known as the slit diaphragm is formed (12). The slit diaphragm forms the final layer of the glomerular filtration barrier (GFB) and has both charge and size selective properties (13).

The foot processes are the main functional unit of the podocyte. These contain loops of filamentous actin (F-actin) that can be assembled, disassembled, and bundled together in response to the changing requirements of the foot process. The tensile strength of F-actin and its concentration in the foot processes enable the podocyte to withstand the pressure of glomerular flow (14). It is this ability of actin, to be soluble as a monomer and then rapidly polymerize to provide structure and support that allows the redistribution of the podocytes foot processes. Bivalent molecules, such as α-actinin-4 and dystrophin can link bundled actin fibers for added strength (15). There are more than 100 proteins that are involved in the regulation of actin filament formation and breakdown indicating that these are essential procedures (16). The importance of the actin cytoskeletal regulation in the proper functioning of the podocyte is indicated by the number of so-called "nephrotic" genes whose protein products act on the actin cytoskeleton (17) (Table S1 in the Supplementary Material).

Such is the importance of the podocyte and the slit diaphragm in particular that there are multiple monogenic mutations that cause nephrotic syndrome as shown in Table S1 of Supplementary Material.

Structural and functional defects in the GFB result in an inability to restrict urinary protein loss. Nephrotic syndrome is defined by the triad of proteinuria, hypoalbuminemia, and edema. Proteinuria is defined by presence of non-physiological levels of a mixture of proteins in the urine (>200 mg/l) (20). However, in clinical practice, the albumin–creatinine ratio is more likely to be employed as it accounts for differences in urine dilution, i.e., a level of >30 μg/mg demonstrates proteinuria (21). Structural and functional defects in the GFB result in an inability to restrict urinary protein loss.

Podocytes are terminally differentiated cells meaning that they are unable to proliferate. This lack of podocyte proliferation limits their capacity to recover from any damage. Therefore, podocyte injury is thought to be central to nephrotic syndrome pathogenesis. The range of podocyte injuries that can play a role in nephrotic syndrome pathogenesis is collectively referred to as podocytopathies. Many of these podocytopathies are caused by mutations in key genes. These genes are central to podocyte function; encoding either slit diaphragm proteins, transcription factors, or signaling mediators. The genetic causes of nephrotic syndrome are discussed in detail in an excellent review by Hildebrandt (17). In addition to genetic podocytopathies, there are also reactive podocytopathies. In these reactive podocytopathies, the podocyte is damaged by mediators in the microenvironment (1). These extrinsic podocyte stressors can be from several sources. They can be viral, toxic, immune-mediated, mechanical, or metabolic disorder derived.

EPITHELIAL–MESENCHYMAL TRANSITION

It is well accepted that a loss of the highly specialized podocyte structure limits the capacity of the final layer of the GFB to restrict urinary protein loss. The changes seen in diseased podocytes could be due to an EMT event.

It has been posited in the literature that since podocytes develop from mesenchymal cells via a mesenchymal–epithelial-transition (MET) event, it is reasonable to assume that the loss of mature podocyte characteristics is the reverse of this process (22). Glomerular development consists of four stages as follows: the vesicle stage, the *S*-shaped body stage, the capillary loop stage, and the maturation stage (23). The early podocytes can first be distinguished as a layer of cells at the proximal end of the *S*-shaped body. During this stage, the podocytes develop from the columnar epithelial cells (24). In turn, the columnar epithelial cells arise from the metanephric mesenchyme via a MET event (25). At this point, the podocytes express specific podocyte markers such as Wilms' tumor suppressor (WT1) and nephrin (26, 27). As the capillary loops form, the podocyte progenitors lose their lateral cell–cell contacts and begin to migrate. As the capillary loop stage progresses, the podocyte foot processes form. At this point, expression of the slit diaphragm proteins, nephrin, podocin, and CD2AP can be seen (28–30).

Epithelial-to-mesenchymal transition is a tightly regulated process by which epithelial cells lose their hallmark epithelial characteristics and gain the features of mesenchymal cells. EMT can be initiated in response to circulating mediators such as TGF-B1 (31, 32). During the process of EMT, the podocytes should lose their epithelial polarity, the cell-to-cell junctions (the slit diaphragm) will be altered, and the actin cytoskeleton will be rearranged (33). Following stimulation with TGF-B1, podocytes lose expression of nephrin and ZO-1 (34). As important slit diaphragm proteins, the loss of nephrin and ZO-1 expression is detrimental to the function of the podocyte. Not only does TGF-B1 lead to loss of epithelial characteristics in podocytes (as evidenced by the loss of nephrin expression) but also an increase in mesenchymal characteristics. Desmin is one such mesenchymal marker that is upregulated by TGF-B1, moreover, desmin upregulation by podocytes is seen in glomerular diseases where podocyte damage is a key feature (35).

These changes have severe consequences for the structure and function of the podocyte cell. The loss of epithelial cell–cell junctions during EMT is best represented by a loss of the slit diaphragm in the diseased podocyte. As previously described, podocytes demonstrate a clear change in their morphology and phenotype in the diseased state. Expression of the slit diaphragm is lost and the foot processes are effaced. However, does the loss of essential podocyte features seen in disease and disease models, both *in vivo* and *in vitro*, respectively, represent a typical EMT event?

Epithelial–mesenchymal transition has been categorized into three types: type 1, associated with implantation, embryo formation, and organ development, type 2, associated with wound healing tissue regeneration and organ fibrosis, and type 3, which is associated with cancer metastasis and progression (36). The distinctions between these subtypes of EMT are outside the scope of this review but are extensively covered in a review by Kalluri and Weinberg (36). For the purposes of this review, EMT will now be a reference to type-2 EMT. The criteria for type-2 EMT are listed in **Table 1**.

The changes in morphology and phenotype between healthy and diseased podocytes only partially satisfies most of the criteria for type-2 EMT and does not fit at all with the rest. The table demonstrates quite clearly the inaccuracies of describing this process as EMT. For instance, one of the criteria for type-2 EMT is a cadherin switch from E-cadherin to N-cadherin. While the podocytes do undergo a cadherin switch in the diseased state, this is from P-cadherin to N-cadherin. No evidence could be found in the literature for podocytes expressing DDR2 or HSP47. Neither there is clear evidence for the nuclear translocation of TWIST. Otherwise, it is the lack of typical epithelial phenotype in podocytes that precludes them from matching this definition of EMT.

First of all, podocytes are atypical epithelial cells. While it is the case that podocytes demonstrate epithelial features, such as clear apical–basal cell polarity, they also exhibit mesenchymal features such as vimentin and intermediate filament expression (16). They also express features of differentiated mesenchymal cells, in particular smooth muscle actin, akin to a pericyte phenotype (42).

High migration capacity is a mesenchymal feature, while low invasive capacity is an epithelial feature, again demonstrating the dichotomous nature of the podocyte. The healthy podocyte maintains a dynamic range within which motility is regulated (43). It has been shown that plasma from patients with active FSGS significantly increases podocyte motility *in vitro* (44). The insult caused by the disease is increasing the mesenchymal characteristic of the podocyte. These hypermotile podocytes are not invasive due to the location of the podocytes on the outside of the GBM. The flux of hyperfiltrate through the GFB leads to a loss of podocytes in the urine. Indeed, as mentioned previously, viable urinary podocytes have been found with mesenchymal fibroblastic markers such as FSP1 (25).

Table 1 | Hallmark characteristics of EMT.

Criteria	Evidence in the literature	Criteria met?
Novel FSP1 and DDR2 expression associated with basement membrane disruption	When podocytes are exposed to high glucose concentration *in vitro*, they demonstrate a clear upregulation of FSP1 (37). Urinary podocytes from diabetic nephropathy patients are $FSP1^+$ (5). Moreover, ectopic overexpression of the known EMT inducer TGF-B1 in the glomerulus stimulates FSP1 in the podocyte (38)	Partially
Increased expression of HSP47, collagen 1 (α1), collagen 2 (α2), or vimentin	HSP47 is a marker of collagen producing cells and has been found in crescentic cells but not in podocytes *in vivo* or *in vitro*. TGF-B1, a potent EMT inducer stimulates collagen 1 expression in mouse podocytes (8). Although mature podocytes express vimentin, the expression increases following TGF-B1 treatment *in vitro* (39)	Partially
Cadherin switch from E-cadherin to N-cadherin	The typical switch from E- to N-cadherin expression is not seen since mature podocytes do not express E-cadherin (40). They do, however, express P-cadherin. A switch from P-cadherin to N-cadherin is seen following TGF-B1 treatment (7)	No
Nuclear relocalization of CBF-A or B-catenin or new expression of SNAIL, SLUG, or TWIST	Nuclear translocation of beta-catenin is seen in experimental models of nephrotic syndrome both *in vitro* and *in vivo* and also in diabetic nephropathy (41). Wnt signaling is responsible for the translocation of beta-catenin and plays an important role in podocyte injury and proteinuria (41). TGF-B1 treatment stimulates SNAIL expression *in vitro*. Additionally, ectopic expression of SNAIL induces changes in podocyte phenotype consistent with EMT (8). SLUG is also expressed following TGF-B1 treatment *in vitro* (7)	Partially
Loss or reduction of epithelial cell markers	The podocyte dedifferentiation seen in response to TGF-B1 treatment is associated with a reduction in epithelial markers such as ZO-1 and P-cadherin (8, 39)	Partially
Spindle shape morphology with redistribution of stress fibers and loss of polarity	Podocytes have a spindle-like arborized morphology when fully differentiated. This morphology is lost following insult. A loss of apical–basal polarity leads to the mislocalization of nephrin and concomitant proteinuria. This loss of polarity has not been seen in models of EMT either *in vitro* or *in vivo*	No

The dedifferentiation of podocytes seen in podocytopathic nephrotic syndromes only partially satisfies some of the criteria for a type-2 EMT transition. Clearly, the morphological and phenotypic changes seen in diseased podocytes are not obtained via a type-2 EMT event.

Podocytes express P-cadherin instead of E-cadherin; again this is atypical for an epithelial cell (7, 45). The generation of a spindle-like morphology is indicative of a mesenchymal phenotype. However, normal podocytes both *in vitro* and *in vivo* have a highly arborized structure that is consistent with the aforementioned spindle-like morphology. Following dedifferentiation, the podocyte actin cytoskeleton rearrangement causes foot process effacement leading to the morphology seen in **Figure 1**. This effaced morphology could be described as analogous to the typical epithelial "cobblestone" morphology. In this way, following dedifferentiation, podocyte morphology goes from the mesenchymal-like highly arborized morphology to the epithelial-like "cobblestone" morphology. The epithelial and mesenchymal characteristics of the podocyte are summarized in **Figure 1**.

Therefore, when describing podocyte dedifferentiation, one must be careful to consider to what differential state the podocyte is reverting. A partial reversal of the mature, partially mesenchymal state to a more immature epithelial state seen during the *S*-shaped body, and capillary loop stages of development could be termed as MET. However, complete dedifferentiation to the "mesenchymal rest" state of development could also conceivably occur, and be more accurately described as EMT, though in neither case is the terminology adequately nuanced.

The terminology "EMT" was adapted from the original term "epithelial–mesenchymal transformation" in order to emphasize the transient nature of this mechanism and the potential for its reversal (46). Clearly, the podocyte is not the average epithelial cell and its dedifferentiation does not entirely fit with the definition of EMT outlined in this article. This is more than simply a semantic argument. Evidently, mediators such as TGF-B1 can instigate EMT and induce podocyte dedifferentiation that is at least reminiscent of EMT. However, by constraining the conceptualization of this process by only thinking in terms of EMT, novel mediators and disease processes causing podocyte dedifferentiation may be missed. In light of the criticisms of this term to describe podocyte dedifferentiation in disease as EMT, perhaps the term podocyte disease transformation (PDT) is more appropriate. With transformation reflecting that PDT is less transient than EMT.

GENETIC FORMS OF NEPHROTIC SYNDROME AND EMT

Single gene defects causing NS tend to cause irreversible podocyte damage. This is to be expected since many of the genes that are known to cause nephrotic syndrome express proteins that are key components of the slit diaphragm (nephrin and podocin) or are essential to the specialized architecture of the podocyte (such as alpha actinin IV). However, the incorporation of transition in this term is deliberate. The diseased podocyte phenotype can be transient. Nephrotic syndrome is often treated with glucocorticoids and/or calcineurin inhibitors both of which have been shown to have direct effects on the actin cytoskeleton of the podocyte (47–49). One key renal developmental gene, known to cause NS when mutated, has been shown to result in features of podocyte dedifferentiation. This is WT1, a transcription factor both highly expressed and necessary for renal development (50), yet only expressed in the podocyte in the mature kidney. Mouse models of the human disease resulting from WT1 mutations, Denys–Drash Syndrome (DDS), reveal loss of podocyte ZO-1 expression and upregulation of podocyte TGF-B (51). Furthermore, loss of WT1 and re-expression of PAX2 and cytokeratin in podocytes have been described in cellular lesions in FSGS biopsies, implying an epithelial switch during acquired disease (52). Our own data examining the phenotype of conditionally immortalized podocytes from children with DDS confirms re-expression of PAX2, but also clear mesenchymal features suggestive of complete dedifferentiation (unpublished results). Thus, WT1 may be a key regulator of podocyte EMT both developmentally and as a target in disease.

The extracellular domain of nephrin forms the protein scaffold of the slit diaphragm (53). There are eight IgG-like motifs in the extracellular domain each of which contains two cysteine residues that are bound to each other via disulfide bridges. In addition, there are three "free" cysteine residues per nephrin molecule that are available to form disulfide bridges with bordering nephrin molecules (54). In this way, nephrin forms the scaffold for the slit diaphragm. The extracellular domain of nephrin interacts with other proteins in order to maintain the integrity of the barrier. Two such proteins are Neph 1 and Neph 2 (55). Clearly, the loss of most of the extracellular domain, as in patients with the Fin-major mutation, has massive implications for the formation of the slit diaphragm (56). The cytoplasmic domain of nephrin, of which part is missing in patients with the Fin-minor mutation, plays a role in the maintenance of the structural and functional capabilities of nephrin (56). The cytoplasmic domain also connects nephrin, and hence the slit diaphragm to the actin cytoskeleton of the foot process (57). It has been suggested that nephrin can bind directly to the actin cytoskeleton of the foot process via its cytoplasmic domain, while Yuan and colleagues have shown that nephrin is at least capable of binding to actin (58).

Podocin comprises 383 amino acids and has a hairpin structure such that both the C and N terminus are cytoplasmic (59). It is a raft-associated constituent of the foot process membrane that is localized at the insertion of the slit diaphragm itself (29). Within the raft, podocin can form oligomers, which lead to invagination of the foot process membrane, to which CD2AP and nephrin are recruited (60). In fact, a fully functioning podocin is required for nephrin transport to the membrane (61). This is the foundation of the slit diaphragm assembly (62). Between 10 and 28% of all non-familial childhood, SRNS cases are caused by recessive podocin mutations, such is the importance of this protein (62).

CD2AP is an adaptor molecule that possesses a coiled coil domain and 3 Src homology 3 (SH3) domains (59, 63). It also has an actin binding site at its NH_2 terminus and is believed to contribute to dynamic actin assembly (64, 65). CD2AP is capable of interacting with nephrin and in complex with nephrin and podocin is able to recruit PI3K to the plasma membrane (63).

CONCLUSION

In reactive podocytopathies, the podocyte is injured by a circulating factor (66). It is clear, however, that the podocytes undergo a set of phenotypic and morphological changes during nephrotic syndrome. This process has been likened to EMT. TGF-B1 is a potent inducer of EMT. The research described in this review has relied heavily on *in vitro* work centered on the response of

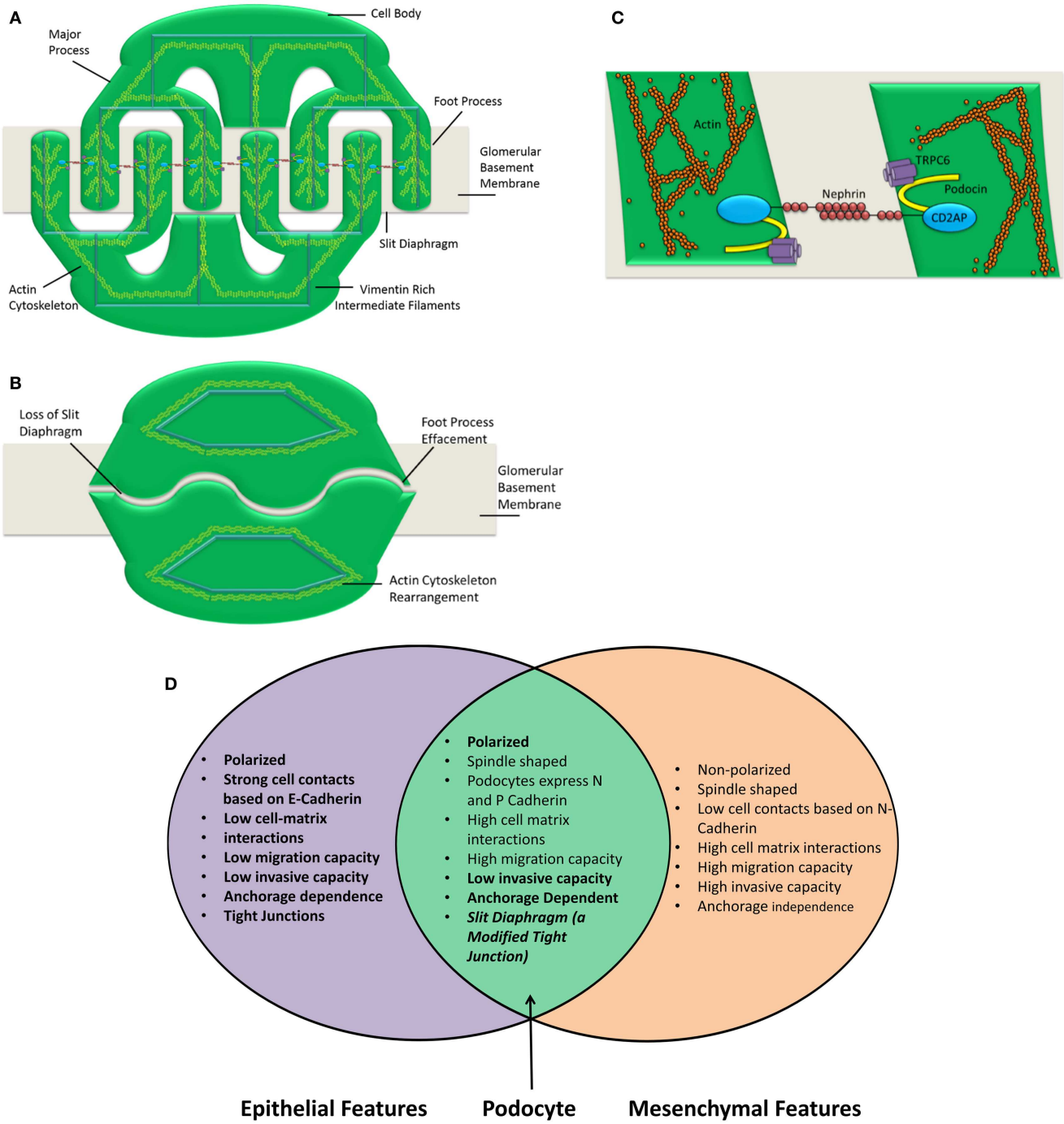

FIGURE 1 | Podocyte structure and characteristics are shown. The podocyte comprises three main compartments, the cell body, the major processes, and the foot processes **(A)**. Each of these segments shares a common actin cytoskeleton. Neighboring foot processes regularly interdigitate. The gap between these interdigitations is bridged by a specialized cell–cell junction known as the slit diaphragm **(C)**. Following insult, the podocyte foot processes are effaced **(B)**. This concomitantly causes a loss of the slit diaphragm. The actin cytoskeleton is rearranged and the podocyte is no longer able to restrict urinary protein loss. A number of insults can cause the podocyte to lose its essential morphology rendering it unable to perform as the final layer of the glomerular filtration barrier. The number of slit diaphragms is reduced and the neat morphology of the podocyte is lost in nephrotic syndrome. This is clearly demonstrated by Patrakka et al. (68). **(D)** Epithelial and mesenchymal features of the podocyte are shown. Epithelial and mesenchymal characteristics as defined by Voulgari and Pintzas (40). The podocyte clearly possesses features that are both epithelial and mesenchymal. Based on this observation, it is not accurate to describe the podocyte as an epithelial cell. Hence, describing podocyte dedifferentiation as EMT is also an oversimplification.

podocytes to TGF-B1 treatment. As demonstrated, the podocytes do not undergo a typical type-2 EMT in response to TGF-B1. The new term "podocyte disease transformation" has been coined in order to distinguish this process from EMT.

A new technique known as transcriptome *in vivo* analysis (TIVA), has been developed to analyze the transcriptome of single cell populations *in vivo* without destroying the tissue (67). This technique could be employed to further study the changes in podocytes in disease models.

The traditional definition of a podocyte as an epithelial cell is clearly simplistic, and in fact, this is a uniquely differentiated cell, fit for a specific functional purpose that fulfills features of a partial mesenchymal and partial epithelial cell. It reaches this mature phenotype via immature mesenchymal and then epithelial stages, and therefore, dedifferentiation in disease could result in regression to either of these states. It will be important in understanding podocyte disease to understand the drivers of these changes, and recognizing the developmental features that are correlated with specific clinical conditions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fendo.2014.00148/abstract

REFERENCES

1. Barisoni L, Schnaper HW, Kopp JB. A proposed taxonomy for the podocytopathies: a reassessment of the primary nephrotic diseases. *Clin J Am Soc Nephrol* (2007) **2**(3):529–42. doi:10.2215/CJN.04121206
2. Sedor JR, Madhavan SM, Kim JH, Konieczkowski M. Out on a LIM: chronic kidney disease, podocyte phenotype and the Wilm's tumor interacting protein (WTIP). *Trans Am Clin Climatol Assoc* (2011) **122**:184–97.
3. Liu Y. New insights into epithelial-mesenchymal transition in kidney fibrosis. *J Am Soc Nephrol* (2010) **21**(2):212–22. doi:10.1681/ASN.2008121226
4. Lee HS. Mechanisms and consequences of TGF-ss overexpression by podocytes in progressive podocyte disease. *Cell Tissue Res* (2012) **347**(1):129–40. doi:10.1007/s00441-011-1169-7
5. Yamaguchi Y, Iwano M, Suzuki D, Nakatani K, Kimura K, Harada K, et al. Epithelial-mesenchymal transition as a potential explanation for podocyte depletion in diabetic nephropathy. *Am J Kidney Dis* (2009) **54**(4):653–64. doi:10.1053/j.ajkd.2009.05.009
6. D'Agati VD, Kaskel FJ, Falk RJ. Focal segmental glomerulosclerosis. *N Engl J Med* (2011) **365**(25):2398–411. doi:10.1056/NEJMra1106556
7. Ghiggeri GM, Gigante M, Di Donato A. Constitutional nephrin deficiency in conditionally immortalized human podocytes induced epithelial-mesenchymal transition, supported by beta-catenin/nf-kappa b activation: a consequence of cell junction impairment? *Int J Nephrol* (2013) **2013**:457490. doi:10.1155/2013/457490
8. Li Y, Kang YS, Dai C, Kiss LP, Wen X, Liu Y. Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria. *Am J Pathol* (2008) **172**(2):299–308. doi:10.2353/ajpath.2008.070057
9. Jefferson JA, Shankland SJ, Pichler RH. Proteinuria in diabetic kidney disease: a mechanistic viewpoint. *Kidney Int* (2008) **74**(1):22–36. doi:10.1038/ki.2008.128
10. Samejima K, Nakatani K, Suzuki D, Asai O, Sakan H, Yoshimoto S, et al. Clinical significance of fibroblast-specific protein-1 expression on podocytes in patients with focal segmental glomerulosclerosis. *Nephron Clin Pract* (2012) **120**(1):c1–7. doi:10.1159/000334184
11. Faul C, Asanuma K, Yanagida-Asanuma E, Kim K, Mundel P. Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. *Trends Cell Biol* (2007) **17**(9):428–37. doi:10.1016/j.tcb.2007.06.006
12. Mundel P, Shankland SJ. Podocyte biology and response to injury. *J Am Soc Nephrol* (2002) **13**(12):3005–15. doi:10.1097/01.ASN.0000039661.06947.FD
13. Tryggvason K. Unraveling the mechanisms of glomerular ultrafiltration: nephrin, a key component of the slit diaphragm. *J Am Soc Nephrol* (1999) **10**(11):2440–5.
14. Pavenstadt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* (2003) **83**(1):253–307.
15. Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle. *Physiol Rev* (2000) **80**(2):853–924.
16. Welsh GI, Saleem MA. The podocyte cytoskeleton – key to a functioning glomerulus in health and disease. *Nat Rev Nephrol* (2012) **8**(1):14–21. doi:10.1038/nrneph.2011.151
17. Hildebrandt F. Genetic kidney diseases. *Lancet* (2010) **375**(9722):1287–95. doi:10.1016/S0140-6736(10)60236-X
18. Brinkkoetter PT, Ising C, Benzing T. The role of the podocyte in albumin filtration. *Nat Rev Nephrol* (2013) **9**(6):328–36. doi:10.1038/nrneph.2013.78
19. McCarthy HJ, Bierzynska A, Wherlock M, Ognjanovic M, Kerecuk L, Hegde S, et al. Simultaneous sequencing of 24 genes associated with steroid-resistant nephrotic syndrome. *Clin J Am Soc Nephrol* (2013) **8**(4):637–48. doi:10.2215/CJN.07200712
20. Nielsen R, Christensen EI. Proteinuria and events beyond the slit. *Pediatr Nephrol* (2010) **25**(5):813–22. doi:10.1007/s00467-009-1381-9
21. Hutchison AS, O'Reilly DS, MacCuish AC. Albumin excretion rate, albumin concentration, and albumin/creatinine ratio compared for screening diabetics for slight albuminuria. *Clin Chem* (1988) **34**(10):2019–21.
22. Little MH, McMahon AP. Mammalian kidney development: principles, progress, and projections. *Cold Spring Harb Perspect Biol* (2012) **4**(5). doi:10.1101/cshperspect.a008300
23. Takano K, Kawasaki Y, Imaizumi T, Matsuura H, Nozawa R, Tannji M, et al. Development of glomerular endothelial cells, podocytes and mesangial cells in the human fetus and infant. *Tohoku J Exp Med* (2007) **212**(1):81–90. doi:10.1620/tjem.212.81
24. Kreidberg JA. Podocyte differentiation and glomerulogenesis. *J Am Soc Nephrol* (2003) **14**(3):806–14. doi:10.1097/01.ASN.0000054887.42550.14
25. Reidy K, Susztak K. Epithelial-mesenchymal transition and podocyte loss in diabetic kidney disease. *Am J Kidney Dis* (2009) **54**(4):590–3. doi:10.1053/j.ajkd.2009.07.003
26. Armstrong JF, Pritchard-Jones K, Bickmore WA, Hastie ND, Bard JB. The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. *Mech Dev* (1993) **40**(1–2):85–97. doi:10.1016/0925-4773(93)90090-K
27. Putaala H, Sainio K, Sariola H, Tryggvason K. Primary structure of mouse and rat nephrin cDNA and structure and expression of the mouse gene. *J Am Soc Nephrol* (2000) **11**(6):991–1001.
28. Kawachi H, Koike H, Kurihara H, Yaoita E, Orikasa M, Shia MA, et al. Cloning of rat nephrin: expression in developing glomeruli and in proteinuric states. *Kidney Int* (2000) **57**(5):1949–61. doi:10.1046/j.1523-1755.2000.00044.x
29. Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, et al. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* (2000) **24**(4):349–54. doi:10.1038/74166
30. Li C, Ruotsalainen V, Tryggvason K, Shaw AS, Miner JH. CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere. *Am J Physiol Renal Physiol* (2000) **279**(4):F785–92.
31. Bottinger EP, Bitzer M. TGF-beta signaling in renal disease. *J Am Soc Nephrol* (2002) **13**(10):2600–10. doi:10.1097/01.ASN.0000033611.79556.AE
32. Chanan-Khan A, Miller KC, Musial L, Padmanabhan S, Yu J, Ailawadhi S, et al. Bortezomib in combination with pegylated liposomal doxorubicin and thalidomide is an effective steroid independent salvage regimen for patients with relapsed or refractory multiple myeloma: results of a phase II clinical trial. *Leuk Lymphoma* (2009) **50**(7):1096–101. doi:10.1080/10428190902912460
33. Valcourt U, Kowanetz M, Niimi H, Heldin CH, Moustakas A. TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. *Mol Biol Cell* (2005) **16**(4):1987–2002. doi:10.1091/mbc.E04-08-0658
34. Shankland SJ. The podocyte's response to injury: role in proteinuria and glomerulosclerosis. *Kidney Int* (2006) **69**(12):2131–47. doi:10.1038/sj.ki.5000410
35. Zou J, Yaoita E, Watanabe Y, Yoshida Y, Nameta M, Li H, et al. Upregulation of nestin, vimentin, and desmin in rat podocytes in response to injury. *Virchows Arch* (2006) **448**(4):485–92. doi:10.1007/s00428-005-0134-9

36. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* (2009) **119**(6):1420–8. doi:10.1172/JCI39104
37. Lv Z, Hu M, Zhen J, Lin J, Wang Q, Wang R. Rac1/PAK1 signaling promotes epithelial-mesenchymal transition of podocytes in vitro via triggering beta-catenin transcriptional activity under high glucose conditions. *Int J Biochem Cell Biol* (2013) **45**(2):255–64. doi:10.1016/j.biocel.2012.11.003
38. Wang D, Dai C, Li Y, Liu Y. Canonical Wnt/beta-catenin signaling mediates transforming growth factor-beta1-driven podocyte injury and proteinuria. *Kidney Int* (2011) **80**(11):1159–69. doi:10.1038/ki.2011.255
39. Herman-Edelstein M, Thomas MC, Thallas-Bonke V, Saleem M, Cooper ME, Kantharidis P. Dedifferentiation of immortalized human podocytes in response to transforming growth factor-beta: a model for diabetic podocytopathy. *Diabetes* (2011) **60**(6):1779–88. doi:10.2337/db10-1110
40. Voulgari A, Pintzas A. Epithelial-mesenchymal transition in cancer metastasis: mechanisms, markers and strategies to overcome drug resistance in the clinic. *Biochim Biophys Acta* (2009) **1796**(2):75–90. doi:10.1016/j.bbcan.2009.03.002
41. Dai C, Stolz DB, Kiss LP, Monga SP, Holzman LB, Liu Y. Wnt/beta-catenin signaling promotes podocyte dysfunction and albuminuria. *J Am Soc Nephrol* (2009) **20**(9):1997–2008. doi:10.1681/ASN.2009010019
42. Saleem MA, Zavadil J, Bailly M, McGee K, Witherden IR, Pavenstadt H, et al. The molecular and functional phenotype of glomerular podocytes reveals key features of contractile smooth muscle cells. *Am J Physiol Renal Physiol* (2008) **295**(4):F959–70. doi:10.1152/ajprenal.00559.2007
43. Kistler AD, Altintas MM, Reiser J. Podocyte GTPases regulate kidney filter dynamics. *Kidney Int* (2012) **81**(11):1053–5. doi:10.1038/ki.2012.12
44. Harris JJ, McCarthy HJ, Ni L, Wherlock M, Kang H, Wetzels JF, et al. Active proteases in nephrotic plasma lead to a podocin-dependent phosphorylation of VASP in podocytes via protease activated receptor-1. *J Pathol* (2013) **229**(5):660–71. doi:10.1002/path.4149
45. Lehtonen S, Lehtonen E, Kudlicka K, Holthöfer H, Farquhar MG. Nephrin forms a complex with adherens junction proteins and CASK in podocytes and in Madin-Darby canine kidney cells expressing nephrin. *Am J Pathol* (2004) **165**(3):923–36. doi:10.1016/S0002-9440(10)63354-8
46. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* (2014) **15**(3):178–96. doi:10.1038/nrm3758
47. Ransom RF, Lam NG, Hallett MA, Atkinson SJ, Smoyer WE. Glucocorticoids protect and enhance recovery of cultured murine podocytes via actin filament stabilization. *Kidney Int* (2005) **68**(6):2473–83. doi:10.1111/j.1523-1755.2005.00723.x
48. Xing CY, Saleem MA, Coward RJ, Ni L, Witherden IR, Mathieson PW. Direct effects of dexamethasone on human podocytes. *Kidney Int* (2006) **70**(6):1038–45. doi:10.1038/sj.ki.5001655
49. Faul C, Donnelly M, Merscher-Gomez S, Chang YH, Franz S, Delfgaauw J, et al. The actin cytoskeleton of kidney podocytes is a direct target of the antiproteinuric effect of cyclosporine A. *Nat Med* (2008) **14**(9):931–8. doi:10.1038/nm.1857
50. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, et al. WT-1 is required for early kidney development. *Cell* (1993) **74**(4):679–91. doi:10.1016/0092-8674(93)90515-R
51. Patek CE, Fleming S, Miles CG, Bellamy CO, Ladomery M, Spraggon L, et al. Murine Denys-Drash syndrome: evidence of podocyte de-differentiation and systemic mediation of glomerulosclerosis. *Hum Mol Genet* (2003) **12**(18):2379–94. doi:10.1093/hmg/ddg240
52. Ohtaka A, Ootaka T, Sato H, Soma J, Sato T, Saito T, et al. Significance of early phenotypic change of glomerular podocytes detected by Pax2 in primary focal segmental glomerulosclerosis. *Am J Kidney Dis* (2002) **39**(3):475–85. doi:10.1053/ajkd.2002.31391
53. Qin XS, Tsukaguchi H, Shono A, Yamamoto A, Kurihara H, Doi T. Phosphorylation of nephrin triggers its internalization by raft-mediated endocytosis. *J Am Soc Nephrol* (2009) **20**(12):2534–45. doi:10.1681/ASN.2009010011
54. Ruotsalainen V, Ljungberg P, Wartiovaara J, Lenkkeri U, Kestilä M, Jalanko H, et al. Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci U S A* (1999) **96**(14):7962–7. doi:10.1073/pnas.96.14.7962
55. Gerke P, Huber TB, Sellin L, Benzing T, Walz G. Homodimerization and heterodimerization of the glomerular podocyte proteins nephrin and NEPH1. *J Am Soc Nephrol* (2003) **14**(4):918–26. doi:10.1097/01.ASN.0000057853.05686.89
56. Akhtar M, Al Mana H. Molecular basis of proteinuria. *Adv Anat Pathol* (2004) **11**(6):304–9. doi:10.1097/01.pap.0000146219.03058.ea
57. Shih NY, Li J, Cotran R, Mundel P, Miner JH, Shaw AS. CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. *Am J Pathol* (2001) **159**(6):2303–8. doi:10.1016/S0002-9440(10)63080-5
58. Salant J. Podocyte slit-diaphragm protein nephrin is linked to the actin cytoskeleton. *Am J Physiol Renal Physiol* (2002) **282**(4):F585–91.
59. Caridi G, Perfumo F, Ghiggeri GM. NPHS2 (podocin) mutations in nephrotic syndrome. Clinical spectrum and fine mechanisms. *Pediatr Res* (2005) **57**(5 Pt 2):54R–61R. doi:10.1203/01.PDR.0000160446.01907.B1
60. Schwarz K, Simons M, Reiser J, Saleem MA, Faul C, Kriz W, et al. Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. *J Clin Invest* (2001) **108**(11):1621–9. doi:10.1172/JCI200112849
61. Huber TB, Simons M, Hartleben B, Sernetz L, Schmidts M, Gundlach E, et al. Molecular basis of the functional podocin-nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains. *Hum Mol Genet* (2003) **12**(24):3397–405. doi:10.1093/hmg/ddg360
62. Karle SM, Uetz B, Ronner V, Glaeser L, Hildebrandt F, Fuchshuber A. Novel mutations in NPHS2 detected in both familial and sporadic steroid-resistant nephrotic syndrome. *J Am Soc Nephrol* (2002) **13**(2):388–93.
63. Huber TB, Hartleben B, Kim J, Schmidts M, Schermer B, Keil A, et al. Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Mol Cell Biol* (2003) **23**(14):4917–28. doi:10.1128/MCB.23.14.4917-4928.2003
64. Dustin ML, Olszowy MW, Holdorf AD, Li J, Bromley S, Desai N, et al. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* (1998) **94**(5):667–77. doi:10.1016/S0092-8674(00)81608-6
65. Welsch T, Endlich N, Kriz W, Endlich K. CD2AP and p130Cas localize to different F-actin structures in podocytes. *Am J Physiol Renal Physiol* (2001) **281**(4):F769–77.
66. Coppo R. Different targets for treating focal segmental glomerular sclerosis. *Contrib Nephrol* (2013) **181**:84–90. doi:10.1159/000348637
67. Lovatt D, Ruble BK, Lee J, Dueck H, Kim TK, Fisher S, et al. Transcriptome in vivo analysis (TIVA) of spatially defined single cells in live tissue. *Nat Methods* (2014) **11**(2):190–6. doi:10.1038/nmeth.2804
68. Patrakka J, Lahdenkari AT, Koskimies O, Holmberg C, Wartiovaara J, Jalanko H. The number of podocyte slit diaphragms is decreased in minimal change nephrotic syndrome. *Pediatr Res* (2002) **52**(3):349–55. doi:10.1203/00006450-200209000-00007

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Genes and podocytes – new insights into mechanisms of podocytopathy

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After decades of primarily morphological study, positional cloning of the *NPHS1* gene was the landmark event that established aberrant podocyte genetics as a pivotal cause of malfunction of the glomerular filter. This ended any uncertainty whether genetic mutation plays a significant role in hereditary nephrotic syndromes (NS) and confirmed podocytes as critical players in regulating glomerular protein filtration. Although subsequent sequencing of candidate genes chosen on the basis of podocyte biology had less success, unbiased analysis of genetically informative kindreds and syndromic disease has led to further gene discovery. However, the 45 genes currently associated with human NS explain not more than 20–30% of hereditary and only 10–20% of sporadic cases. It is becoming increasingly clear both from genetic analysis and phenotypic data – including occasional response to immunosuppressive agents and post-transplant disease recurrence in Mendelian disease – that monogenic inheritance of abnormalities in podocyte-specific genes disrupting filter function is only part of the story. Recent advances in genetic screening technology combined with increasingly robust bioinformatics are set to allow identification and characterization of novel disease causing variants and more importantly, disease modifying genes. Emerging data also support a significant but incompletely characterized immunoregulatory component.

Keywords: podocyte nephropathy, podocytes, genetic predisposition to disease, gene mutation, nephrotic syndrome, nephrotic genes

NEPHROTIC SYNDROME AND THE GLOMERULAR FILTRATION BARRIER

Nephrotic syndrome (NS) is one of the commonest kidney conditions to affect children and adults. It manifests as excessive leak of protein into the urine, with interstitial edema occurring through albumin loss, aggravated by salt and water retention. Moreover, secondary knock-on effects on lipid metabolism, hemostasis, and the endocrine system through loss of key binding proteins serve to augment morbidity and mortality. NS represents a heterogeneous group of conditions, some idiopathic, others with a clear genetic basis. It may be highly kidney specific, associated with extra-renal developmental malformations or occur as a complication of systemic disease. The mainstay of treatment is immunosuppression, also effective in about 8–10% of genetic cases. Non-responders generally progress to renal failure with kidney transplantation, the only life-saving treatment.

THE ROLE OF PODOCYTES

Despite the apparent heterogeneity, the unifying feature in NS is malfunction of the glomerular filtration barrier (GFB). This highly sophisticated macromolecular sieve with size and charge restricting characteristics is the primary ultra-filter of plasma by the kidney. It allows free flow of water and small solutes but restricts the passage of molecules >15 kDa; proteinuria occurs through loss of these normal permselective properties. The GFB comprises three layers: fenestrated endothelium, glomerular basement membrane (GBM) and podocytes, specialized terminally differentiated epithelial cells connected by slit diaphragms (SD), unique intercellular junctions interposed between interdigitating foot processes (1). Endothelial cells and podocytes have a negatively charged surface glycocalyx, which together with GBM sialoproteins and heparan sulfate gives the GFB an overall negative charge at physiological pH. Although size is the primary determinant of molecular filterability, recent detection of mutations in the main component of the podocyte glycocalyx, podocalyxin, in familial NS (2) supports additional charge selection through electrostatic repulsion.

Although damage to any of the three layers can result in significant proteinuria and kidney disease (3), podocytes are considered pivotal for maintaining barrier integrity. They encircle the glomerular capillaries creating a compact interdigitating network on the urinary side of the GFB. The connecting SDs integrate structural components of tight, adhesion, gap, and neuronal junctions to meet the diverse functional requirements of macromolecular filtering under high pressure while subjected to rapid changes in mechanical shear stress (4). SDs connect with the actin cytoskeleton to initiate signaling pathways that regulate podocyte function, namely plasticity of foot processes, mechanosensation, calcium flux, endocytosis, cell polarity, and cell survival. It is perhaps not surprising that podocyte gene mutations link to these key cellular functions. Podocytes also react very stereotypically to injury irrespective of whether this is acquired or resulting from an intrinsic

developmental defect with reorganization of the actin cytoskeleton, foot process effacement, molecular re-characterization of SDs, apoptosis, and detachment from the GBM (5). These dramatic morphological changes correlate with dysregulation of specific markers of podocyte differentiation including WT1, PAX2, and nephrin again signifying an underlying molecular basis. Additionally, de-differentiated podocytes can attempt healing sometimes by excessive proliferation, with the eventual outcome of repair or cell death attributable to complex interplay of poorly defined genetic and epigenetic mechanisms (6–8).

Clinical data support the hypothesis that the predominant cellular lesion targets podocytes; GBM gene defects usually result in insidious plasma protein leak whereas defects in podocyte/slit-diaphragm genes cause precipitous leak and NS. Moreover, there is evidence for a potential role for immune-regulation, particularly in childhood. Podocytes express cytokine and chemokine receptors as well as Toll Like receptors (TLRs) (9) and can respond to immune stimuli both *in vivo* and *in vitro*. Moreover, emerging evidence indicates that podocyte injury in NS may sometimes result from an unknown circulating factor, cytokine imbalance, or immune complex injury, with rare genomic variants potentially dictating susceptibility or resistance to immune triggers and the degree of subsequent response.

ADVANCES IN MOLECULAR CHARACTERIZATION OF PODOCYTES

Initial evidence supporting a molecular basis for NS came from positional cloning in familial cases and experimental animal models. Inherited diseases are frequently caused by mutations in genes with restricted expression patterns that generally do not cause early embryonic lethality but instead manifest at the time when gene function becomes critical for a specific tissue and subsequent survival (10). This is certainly the case for *NPHS1* and *NPHS2* (11, 12), the first podocyte genes cloned in NS encoding nephrin, an immunoglobulin superfamily member, and podocin an integral membrane protein both exclusively expressed at the podocyte SD supporting a key role in protein filtration. Mutations are associated with autosomal recessive NS manifesting at birth or early life during post-natal glomerular maturation and resultant increase in glomerular filtration.

Detection of *WT1* mutations in human syndromic NS (13), together with murine transgenic approaches using *WT1, CD2AP* (14), and *NEPH1* (15) underscored the contribution of developmentally regulated podocyte genes. Subsequent identification of mutations in *ACTN4* (16) and *INF2* (17) emphasized the central role of the actin cytoskeleton whereas gain of function mutations in *TRCP6* linked podocyte disease to abnormalities of cellular calcium flux and associated signaling pathways (18). Inherited defects of mitochondrial (19) and lysosomal components (20) have also been shown to lead to profound podocyte dysfunction, suggesting that they have high energy requirements and turn over. This also lead to the discovery that lysosomes participate in autophagy, increasingly recognized as protecting podocytes against injury (21, 22) Other genes such as *CD2AP* that participate in endocytosis and/or actin assembly have also been detected in glomerular disease providing further important clues about podocyte biology.

Familial and candidate gene studies have provided important information about the molecular basis of fundamental podocyte functions such as slit-diaphragm signaling, regulation of actin cytoskeleton dynamics, and cell–matrix interactions. However, until recently, only limited information about the overall genetic landscape was available, which posed a challenge to correct interpretation of genetic findings. Previously, podocytes could be extracted with high purity, but low cell yields during isolation hampered complete characterization of the transcriptome and proteome. Primary cells are difficult to grow in culture, so immortalized podocytes provided a useful tool to study gene function but incompletely mirrored their *in vivo* counterparts at molecular level. However, recent development of a double fluorescent reporter mouse model and optimized bead perfusion protocol, combined with FACS sorting, microarray, proteomics, and unbiased mathematical biology has yielded >5223 differentially regulated genes within the podocyte transcriptome and >1280 translated proteins (23). These studies have revealed that the podocyte proteome is enriched with plasma membrane, cytoskeleton, and neuronal-type proteins. Subsequent functional analysis of transcriptome and proteome data resulted in seven gene clusters: endoplasmatic reticulum, ubiquitination, cytoskeleton, nuclear elements, mitochondria, peroxisome and protein transport, and cell junction. Interestingly, nuclear and translational proteins were depleted, reflecting the post-mitotic nature of podocytes and alternative splicing was reduced, perhaps to ensure discreet cellular regulation. This has also enabled more meaningful RNA sequencing, which has identified eight miRNA's preferentially expressed in podocytes with significantly reduction fold change of miRNA target genes supporting miRNA-mediated mRNA degradation.

Improved description of the podocyte genome has also resulted in more effective interpretation of data from next generation sequencing (NGS) and to detect new variants or genes that cause or contribute to NS. NGS allows rapid sequencing of the whole exome or genome, and is becoming an increasingly powerful and cost effective method of analyzing the genetic contribution to any disorder. An unbiased approach can be taken to highlight new genes, new pathways, and new disease mechanisms through rigorous statistical genetics followed by functional experiments. This is especially relevant to NS where there is already known association with complex genetic determinants and this is likely to prove important mechanistically. However, as with any rare diseases, finding causal variants in NS suffers from twin problems of moderately low sample size and disease heterogeneity. In presumed Mendelian NS, the approach is still to search for genetic variants that segregate with disease within a family. In sporadic NS, we hypothesize that the condition will be caused by a rare variant in a small number of cases (limiting sample size and statistical power) and that the variant and, indeed the gene, may vary between cases. Added to this, although it is often assumed that there will be a heterozygous, homozygous, or compound heterozygous change that is causal, in common with related glomerular disorders, there is evidence for modifier effects in NS, at the genetic (24) and environmental level (25). This introduces the concept that NS is more complex than the Mendelian one podocyte gene, one disease situation as previously thought as variants are not always completely penetrant (26), synergistic activation between genes may

occur and in non-heritable disease, an environmental trigger is needed to cause disease. There is now clear evidence that in at least one podocyte gene, the pathogenicity of a particular allele depends on the trans-associated mutation (27). These concepts are supported by increasing evidence that expected phenotype–genotype correlations are not always adhered to and pathological changes may develop focally (not in all glomeruli) and segmentally (only in parts of a glomerulus), a pattern suggestive of an initial insult precipitated by an environmental stressor, perhaps a viral infection, that leads to localized cell injury (28).

Attempts to analyze NGS data in rare yet moderately complex diseases such as NS initially tend to focus on three main issues, namely how to best divide the genome for maximum signal, which statistical test to use and how to computationally assess the function of any variants found. Unlike common complex conditions, variants in conditions such as NS are too rare to provide meaningful sample sizes for statistical testing on their own and must be collapsed or aggregated to the level of gene, network, or other biologically meaningful unit. That is, testing for enrichment of variants in NS versus a control population will likely involve counting the number of rare protein altering variants in each gene or other biological unit in cases and controls and finding genes with an excess of these rare variants, rather than searching for an enrichment of any one variant as would be the case in a common complex Genome Wide Association Study (GWAS) approach. In addition to deciding on the biological unit by which to combine variants, decisions also need to be made about the statistical techniques used to combine and then analyze variants (29, 30). There are then several databases and algorithms by which the functional deleteriousness of potential variants can be assessed [(31): CADD; (32): ClinVar; (33): HGMD; (34): SIFT; (35): PolyPhen]. These might be used throughout the analysis to remove samples with known causal variants before statistical testing, or choose likely deleterious variants during testing. They can also be used to examine the likelihood that new variants might be causal prior to investing resources in replication studies or functional testing *in vitro* or *in vivo*. Nonetheless, understanding genotype/phenotype relationships without powerful systems biology tools in place has become increasingly complicated as emerging NGS data reveal not only considerable genetic heterogeneity but also evidence showing that factors such as epigenetic modifications, imprinting, non-coding RNAs, and RNA editing may play an important role in determining phenotype (36).

PODOCYTE-SPECIFIC GENES: THE OLD AND THE NEW

Mutations in 45 genes have been associated with familial and sporadic NS to date (**Figure 1**). These disrupt function either through SD disassembly, damaging cell architecture or metabolism, disturbing cell–matrix interactions, and/or impeding signaling pathways. All are expressed in podocytes, but there is increasing evidence to suggest that other systems, including immunoregulatory, play a role. Moreover, mutations in podocyte genes currently only explain 20–30% of familial and 10–20% of sporadic NS. From a clinical perspective, the renal phenotype rarely correlates absolutely with genotype as histology overlaps, suggesting a final common pathway to glomerular damage. Nonetheless, mode of inheritance and age of onset can give important clues. Gene mutations detected in early life are biased toward developmental genes, podocyte/SD/GBM malformation, and autosomal recessive inheritance whereas in later life, mutations are more frequently autosomal dominant (AD), preferentially affecting genes that participate directly or indirectly in regulation of the actin cytoskeleton. Moreover, combined gene defects in more than one podocyte gene may play a role in the development of NS, for example, mutations in both *NPHS1* and *NPHS2* can cause a tri-allelic hit modifying phenotype (37), and bi-allelic trans-heterozygosity has been described for *CD2AP* and *NPHS2* in sporadic NS (38). Additionally, *ADCK4* appears to modify CoQ10 (39) Furthermore, R229Q, a non-neutral *NPHS2* polymorphism that may predispose to NS in adults appears pathogenic only when associated with 3′ *NPHS2* mutations in trans- (27), or deleterious mutation in another podocyte gene (37). Another emerging layer of complexity is miRNAs, for example, miR-193a downregulates the expression of WT1 in transgenic mice resulting in rapid and progressive NS (40). Other genes regulate podocyte differentiation and SD function by interacting synergistically on common enhancers or repressors, e.g., LMX1B, which combinatorially regulates *NPHS2* with FoxC (41). This underlines the likely diversity of genetic interaction within podocytes, supporting a requirement for additional environmental factors and/or modifier genes for full phenotypic expression.

Table 1 summarizes all genes currently associated with human NS. Broadly speaking, these can be categorized on the basis of function substantiated by the predicted effect of mutations. SD associated and adaptor proteins normally communicate between the SD and the podocyte cytoskeleton, forming multimeric signaling complexes associated with lipid raft micro-domains coordinating actin remodeling, cell survival, and endocytosis. The prototypes are nephrin (*NPHS1*), and podocin (*NPHS2)*, which interact with proteins such as CD2AP (42) and TRPC6 to adapt between SD and cytoskeleton through a number of cytoskeletal linkers including F-actin and synaptopodin (43). More than 236 mutations in *NPHS1* have been described (HGMD® Professional 2014.2), and 171 mutations in *NPHS2* (www.lovd.nl/NPHS2, HGMD® Professional 2014.2). *CD2AP* haploinsufficiency causes NS, and is associated with trans-heterozygosity (38). Gain of function TRPC6 mutations cause AD NS mainly but not exclusively in adult life, by increasing calcium influx and dysregulating the actin cytoskeleton via aberrant signaling (18). Nuclear proteins regulate transcription, podocyte differentiation and homeostasis; mutations result in dysregulation of downstream targets often initiating catastrophic collapse of the entire podocyte-stabilizing system. *WT1* mutations cause Denys–Drash (intersex, Wilm's tumor, NS) and Frasier syndromes (intersex, NS, gonadoblastoma) and AD adult onset NS (44). *LMX1B*, a homeobox transcription factor, regulates SDs and is essential for the maintenance of the actin cytoskeleton. Haploinsufficiency causes Nail–Patella syndrome, a rare AD NS that can occur as just kidney specific disease (45). The most recent gene to be cloned is *WDR73,* its exact function in the podocyte is as yet unknown but mutations result in Galloway–Mowat syndrome, a rare association between microcephaly, diaphragmatic hernia, and SRNS (46). Proteins regulating the actin cytoskeleton are also targeted. *ACTN4* (α-actinin-4) causes AD adult onset NS with incomplete penetrance. Here, mutant proteins show stronger affinity for F-actin and impair cytoskeletal function. Another actin regulating gene, *INF2* belongs to the formin family and normally homodimerizes to inhibit actin depolymerization.

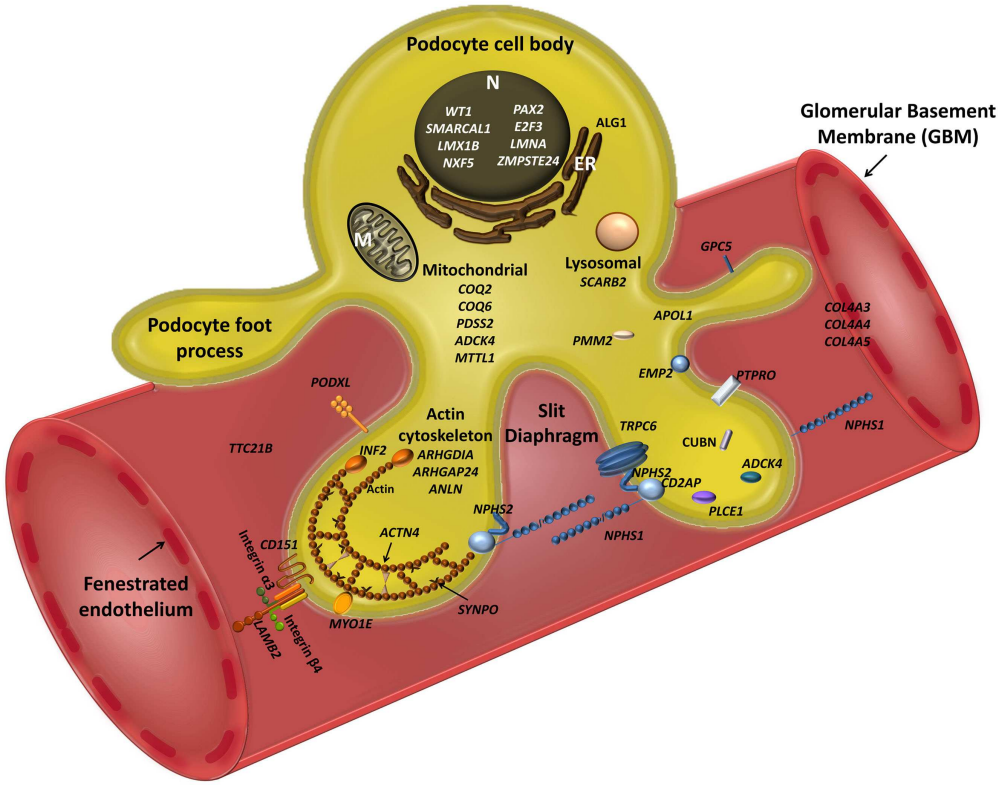

FIGURE 1 | Schematic view of podocyte genes associated with steroid resistant nephrotic syndrome (with reference to Table 1). Podocyte cell with foot processes, slit diaphragm, the glomerular basement membrane (GBM), and fenestrated endothelium are shown. *N*, podocyte nucleus; ER, endoplasmic reticulum; M, mitochondrion. Slit diaphragm associated and adaptor proteins: nephrin (*NPHS1*), podocin (*NPHS2*), CD2-associated protein (*CD2AP*), PLCε1 (*PLCE1*), transient receptor potential channel 6 (*TRPC6*), and protein tyrosine phosphatase receptor type O (*PTPRO*). Nuclear proteins and transcription factors: Wilm's tumor protein (*WT1*), SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 (*SMARCAL1*), LIM Homeobox Transcription Factor 1β (*LMX1B*), nuclear RNA export factor 5 (*NXF5*), paired box protein (*PAX2*) and transcription factor E2F3 (*E2F3*), lamin A/C (*LMNA*), zinc metallopeptidase STE24 (*ZMPSTE24*). Actin cytoskeleton and signalling: podocalyxin (*PODXL*), inverted formin 2 (*INF2*), α-actinin-4 (*ACTN4*), synaptopodin (*SYNPO*), myosin 1E (*MYO1E*), Rho-GDP-dissociation inhibitor 1 (*ARHGDIA*), and Rho-GTPase-activating protein 24 (*ARHGAP24*), and actin-binding protein anillin (*ANLN*), cubilin (*CUBN*), tetratricopeptide repeat protein 21B (IFT139, *TTC21B*); epithelial membrane protein 2 (*EMP2*). Mitochondria-associated proteins: parahydroxybenzoate-polyprenyl transferase (*COQ2*), ubiquinone biosynthesis monooxygenase (*COQ6*), decaprenyl-diphosphate synthase subunit 2 (*PDSS2*), AarF domain-containing kinase 4 (*ADCK4*), and mitochondrially encoded tRNA leucine 1 (*MTTL1*). Metabolic and lysosomal proteins: chitobiosyldiphosphodolichol β-mannosyltransferase (*ALG1*), phosphomannomutase 2 (*PMM2*), and scavenger receptor class B, member 2 (*SCARB2*). GBM associated proteins: collagen 4 – *COL4A3, 4, 5*; integrin-α3 (*ITGA3*), integrin-β4 (*ITGB4*), laminin-β2 (*LAMB2*); CD151 antigen (*CD151*).

Mutations are detected in a significant proportion of familial but not sporadic NS. Mutations in *PLCe1* (phospholipase C epsilon) mostly cause early-onset NS by dysregulating the cytoskeleton and signaling, and may be variably penetrant with some missense mutations resulting in later onset disease responding to immunosuppression (47). *PLCe1* mutations may also co-exist with trans-heterozygote mutations in other podocyte genes. *PTPRO* encodes a protein tyrosine phosphatase receptor located on the apical side of podocyte foot processes that controls the glomerular pressure/filtration rate (48). Only two splice-site mutations have been detected in *PTPRO* and cases partially responded to immunosuppression (49). Recently, mutations in *ANLN* (anillin), another F-Actin binding protein, were detected in familial NS (50), and other mutation screens of inherited disease have identified *ARHGDIA* (51) and *ARHGAP24* (52), underpinning a key role for RHO GTPases and Rho–Rac signaling in regulating the podocyte cytoskeleton. *MYO1E* (53) and *MYH9* (24), mutated in autosomal recessive NS regulate actin cytoskeletal function and cell shape. *APOL1* mutations also result in increased NS in African Americans but the mechanism remains unclear (54). Mutations affecting protein expressed in the GBM may also disrupt GFB functions sufficiently to cause NS. *LAMB2* mutations, encoding laminin β2, cause Pierson's syndrome characterized by early-onset NS with or without microcoria, depending on the severity of the mutation (55). *ITGA3* and *ITGB4* encode integrins thought to be mainly passive GBM stabilizers, with mutations causing hyperglycosylation that prevents formation of functional integrins manifesting as NS and skin defects (56). COL4A3, A4, and A5 are key GBM proteins that cause Alport's syndrome; recent data suggest caution in interpreting COL4A variants detected in apparent familial NS (57). Mitochondrial and other rare metabolic syndromes also present with NS, by disrupting podocyte metabolism.

Table 1 | Genes directly associated nephrotic syndrome.

Gene	Inheritance	Disease association and onset
SD ASSOCIATED AND ADAPTOR PROTEINS		
NPHS1	AR	CNS/NS
NPHS2	AR	CNS, NS – childhood and adult onset
CD2AP	?	Early-onset NS, HIV nephropathy
PLCe1	AR	Early-onset NS
TRPC6	AD	Adult onset NS
PTPRO	AR	Childhood-onset NS
NUCLEAR PROTEINS		
WT1	Sporadic; AD	Adult onset NS, Denys–Drash and Frasier Syndromes
LMX1B	AR	Nail–Patella Syndrome/NS only
SMARCAL1	AR	Schimke immuno-osseous dysplasia
E2F3	Chromosomal deletion	Early-onset NS and mental retardation
NXF5	X-linked recessive	NS with co-segregating heart block disorder
PAX2	AD	Adult onset NS
WDR73	AR	Galloway–Mowat Syndrome
ACTIN CYTOSKELETON AND SIGNALING		
ACTN4	AD	Adult onset NS
MYH9	Risk allele	Adult onset NS
INF2	AD	Familial/sporadic NS; Charcot-Marie-Tooth
SYNPO	?	Adult onset NS
APOL1	Complex; AR	Adult onset NS
MYO1E	AR	Early or adult onset NS
ARHGAP24	AD	Adult onset NS
ARHGDIA	AR	CNS
ANLN	AD	Adult onset NS
EMP2	AR	Childhood-onset NS
CUBN	AR	Intermittent nephrotic range proteinuria and epilepsy
GPC5	Risk allele	Adult onset NS
PODXL	AD	Early or adult onset NS
TTC21B	AR	NS with tubulointerstitial involvement
CLTA4	Risk allele	Sporadic NS
MITOCHONDRIAL		
MTTL1	?	MELAS syndrome; NS ± deafness and diabetes
tRNAIle	?	Deafness, NS, epilepsy, and dilated cardiomyopathy
tRNAAsn	?	Multiorgan failure and NS
tRNATyr	?	Mitochondrial cytopathy and NS
COQ2	AR	Mitochondrial disease/isolated nephropathy
COQ6	AR	NS with sensorineural deafness
ZMPSTE24	AR	Mandibuloacral dysplasia with NS
PDSS2	AR	Leigh syndrome
ADCK4	AR	NS
CYP11B2	Risk allele	C-344T SNP risk factor for IgA nephropathy, NS, proliferative glomerulopathy
GBM		
LAMB2	AR	Pierson syndrome: CNS with ocular abnormalities; isolated early-onset NS
ITGB4	AR	NEP syndrome-NS, epidermolysis bullosa, and pulmonary disease
ITGA3	AR	Epidermolysis bullosa and pyloric atresia + NS

(Continued)

Table 1 | Continued

Gene	Inheritance	Disease association and onset
LMNA	AD	Familial partial lipodystrophy + NS
COL4A3	AR	Alport's disease
COL4A4	AR	Alport's disease
COL4A5	X-linked	Alport's disease
CD151	AR	NS, pretibial bullous skin lesions, neurosensory deafness, bilateral lacrimal duct stenosis, nail dystrophy, thalassemia minor
OTHER – METABOLIC OR LYSOSOMAL		
PMM2	AR	Congenital disorder of glycosylation
ALG1	AR	Congenital disorder of glycosylation
SCARB2 (lysosome)	AR	Action myoclonus renal failure syndrome ± hearing loss

AR, autosomal recessive; AD, autosomal dominant; CNS, congenital nephrotic syndrome; NS, nephrotic syndrome; ?, inheritance neither clearly autosomal dominant or recessive, possibly complex.

Initial identification of monogenic defects correlating with morphological findings in podocytes in health and disease provided important mechanistic insights. However, this is now too simplistic, a model; podocyte genetic mechanisms are becoming increasingly complex, perhaps not surprising considering their super-specialist role within the GFB. Multiple levels of control involving modifier genes and di-genic and multi-genic inheritance are undoubtedly necessary to deal with epigenetic events and environmental effects (58). It is also likely that some gene variants are not directly causative, but rather modifiers of phenotype that may even arise from primary immune injury or presence of a circulating permeability factor. The advent of NGS and increasingly sophisticated bioinformatics approaches pave the way for exciting exploration of these alternative mechanisms, which undoubtedly play a key role in podocyte pathobiology.

REFERENCES

1. Kriz W, Lemley KV. The role of the podocyte in glomerulosclerosis. *Curr Opin Nephrol Hypertens* (1999) **8**:489–97. doi:10.1097/00041552-199907000-00014
2. Barua M, Shieh E, Schlondorff J, Genovese G, Kaplan BS, Pollak MR. Exome sequencing and in vitro studies identified podocalyxin as a candidate gene for focal and segmental glomerulosclerosis. *Kidney Int* (2014) **85**:124–33. doi:10.1038/ki.2013.354
3. Menon MC, Chuang PY, He CJ. The glomerular filtration barrier: components and crosstalk. *Int J Nephrol* (2012) **2012**:749010. doi:10.1155/2012/749010
4. Grahammer F, Schell C, Huber TB. The podocyte slit diaphragm – from a thin grey line to a complex signalling hub. *Nat Rev Nephrol* (2013) **9**:587–98. doi:10.1038/nrneph.2013.169
5. Mundel P, Shankland SJ. Podocyte biology and response to injury. *J Am Soc Nephrol* (2002) **13**:3005–15. doi:10.1097/01.ASN.0000039661.06947.FD
6. Lasagni L, Lazzeri E, Shankland SJ, Anders HJ, Romagnani P. Podocyte mitosis – a catastrophe. *Curr Mol Med* (2013) **13**:13–23. doi:10.2174/15665240130103
7. Hagemann JH, Haegele H, Muller S, Anders HJ. Danger control programs cause tissue injury and remodeling. *Int J Mol Sci* (2013) **14**(2013):11319–. doi:10.3390/ijms140611319
8. Hayashi K, Sasamura H, Nakamura M, Azegami T, Oguchi H, Sakamaki Y, et al. KLF4-dependent epigenetic remodeling modulates podocyte phenotypes and attenuates proteinuria. *J Clin Invest* (2014) **124**:2523–37. doi:10.1172/JCI69557
9. Ma J, Chadban SJ, Zhao CY, Chen X, Kwan T, Panchapakesan U, et al. TLR4 activation promotes podocyte injury and interstitial fibrosis in diabetic nephropathy. *PLoS One* (2014) **9**:e97985. doi:10.1371/journal.pone.0097985
10. Cai JJ, Petrov DA. Relaxed purifying selection and possibly high rate of adaptation in primate lineage-specific genes. *Genome Biol Evol* (2010) **2**:393–409. doi:10.1093/gbe/evq019
11. Kestila M, Lenkkeri U, Mannikko M, Lamerdin J, McCready P, Putaala H, et al. Positionally cloned gene for a novel glomerular protein – nephrin – is mutated in congenital nephrotic syndrome. *Mol Cell* (1998) **1**:575–82. doi:10.1016/S1097-2765(00)80057-X
12. Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, et al. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* (2000) **24**:349–54. doi:10.1038/74166
13. Pelletier J, Bruening W, Kashtan CE, Mauer SM, Manivel JC, Striegel JE, et al. Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. *Cell* (1991) **67**:437–47. doi:10.1016/0092-8674(91)90194-4
14. Kim JM, Wu H, Green G, Winkler CA, Kopp JB, Miner JH, et al. CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. *Science* (2003) **300**:1298–300. doi:10.1126/science.1081068
15. Donoviel DB, Freed DD, Vogel H, Potter DG, Hawkins E, Barrish JP, et al. Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. *Mol Cell Biol* (2001) **21**:4829–36. doi:10.1128/MCB.21.14.4829-4836.2001
16. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, et al. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* (2000) **24**:251–6. doi:10.1038/73456
17. Brown EJ, Schlondorff JS, Becker DJ, Tsukaguchi H, Tonna SJ, Uscinski AL, et al. Mutations in the formin gene INF2 cause focal segmental glomerulosclerosis. *Nat Genet* (2010) **42**:72–6. doi:10.1038/ng.505
18. Winn MP, Conlon PJ, Lynn KL, Farrington MK, Creazzo T, Hawkins AF, et al. A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science* (2005) **308**:1801–4. doi:10.1126/science.1106215
19. Diomedi-Camassei F, Di GS, Santorelli FM, Caridi G, Piemonte F, Montini G, et al. COQ2 nephropathy: a newly described inherited mitochondriopathy with primary renal involvement. *J Am Soc Nephrol* (2007) **18**:2773–80. doi:10.1681/ASN.2006080833
20. Berkovic SF, Dibbens LM, Oshlack A, Silver JD, Katerelos M, Vears DF, et al. Array-based gene discovery with three unrelated subjects shows SCARB2/LIMP-2 deficiency causes myoclonus epilepsy and glomerulosclerosis. *Am J Hum Genet* (2008) **82**:673–84. doi:10.1016/j.ajhg.2007.12.019
21. Hartleben B, Godel M, Meyer-Schwesinger C, Liu S, Ulrich T, Kobler S, et al. Autophagy influences glomerular disease susceptibility and maintains podocyte homeostasis in aging mice. *J Clin Invest* (2010) **120**:1084–96. doi:10.1172/JCI39492
22. Zeng C, Fan Y, Wu J, Shi S, Chen Z, Zhong Y, et al. Podocyte autophagic activity plays a protective role in renal injury and delays the progression of podocytopathies. *J Pathol* (2014) **234**:203–13. doi:10.1002/path.4382
23. Boerries M, Grahammer F, Eiselein S, Buck M, Meyer C, Goedel M, et al. Molecular fingerprinting of the podocyte reveals novel gene and protein regulatory networks. *Kidney Int* (2013) **83**:1052–64. doi:10.1038/ki.2012.487

24. Kopp JB, Smith MW, Nelson GW, Johnson RC, Freedman BI, Bowden DW, et al. MYH9 is a major-effect risk gene for focal segmental glomerulosclerosis. *Nat Genet* (2008) **40**:1175–84. doi:10.1038/ng.226
25. Kriz W, Shirato I, Nagata M, LeHir M, Lemley KV. The podocyte's response to stress: the enigma of foot process effacement. *Am J Physiol Renal Physiol* (2013) **304**:F333–47. doi:10.1152/ajprenal.00478.2012
26. Cooper DN, Krawczak M, Polychronakos C, Tyler-Smith C, Kehrer-Sawatzki H. Where genotype is not predictive of phenotype: towards an understanding of the molecular basis of reduced penetrance in human inherited disease. *Hum Genet* (2013) **132**:1077–130. doi:10.1007/s00439-013-1331-2
27. Tory K, Menyhard DK, Woerner S, Nevo F, Gribouval O, Kerti A, et al. Mutation-dependent recessive inheritance of NPHS2-associated steroid-resistant nephrotic syndrome. *Nat Genet* (2014) **46**:299–304. doi:10.1038/ng.2898
28. Yamashita M, Millward CA, Inoshita H, Saikia P, Chattopadhyay S, Sen GC, et al. Antiviral innate immunity disturbs podocyte cell function. *J Innate Immun* (2013) **5**:231–41. doi:10.1159/000345255
29. Dering C, Hemmelmann C, Pugh E, Ziegler A. Statistical analysis of rare sequence variants: an overview of collapsing methods. *Genet Epidemiol* (2011) **35**(Suppl 1):S12–7. doi:10.1002/gepi.20643
30. Stitziel NO, Kiezun A, Sunyaev S. Computational and statistical approaches to analyzing variants identified by exome sequencing. *Genome Biol* (2011) **12**(9):227. doi:10.1186/gb-2011-12-9-227
31. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* (2014) **46**:310–5. doi:10.1038/ng.2892
32. Landrum MJ, Lee JM, Riley GR, Jang W, Rubinstein WS, Church DM, et al. ClinVar: public archive of relationships among sequence variation and human phenotype. *Nucleic Acids Res* (2014) **42**:D980–5. doi:10.1093/nar/gkt1113
33. Stenson PD, Mort M, Ball EV, Shaw K, Phillips A, Cooper DN. The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. *Hum Genet* (2014) **133**:1–9. doi:10.1007/s00439-013-1358-4
34. Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. *Genome Res* (2001) **11**:863–74. doi:10.1101/gr.176601
35. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet* (2013) **7**:7. doi:10.1002/0471142905.hg0720s76
36. Gottlieb B, Beitel LK, Trifiro M. Changing genetic paradigms: creating next-generation genetic databases as tools to understand the emerging complexities of genotype/phenotype relationships. *Hum Genomics* (2014) **8**:9. doi:10.1186/1479-7364-8-9
37. Koziell A, Grech V, Hussain S, Lee G, Lenkkeri U, Tryggvason K, et al. Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration. *Hum Mol Genet* (2002) **11**:379–88. doi:10.1093/hmg/11.4.379
38. Lowik M, Levtchenko E, Westra D, Groenen P, Steenbergen E, Weening J, et al. Bigenic heterozygosity and the development of steroid-resistant focal segmental glomerulosclerosis. *Nephrol Dial Transplant* (2008) **23**:3146–51. doi:10.1093/ndt/gfn208
39. Ashraf S, Gee HY, Woerner S, Xie LX, Vega-Warner V, Lovric S, et al. ADCK4 mutations promote steroid-resistant nephrotic syndrome through CoQ10 biosynthesis disruption. *J Clin Invest* (2013) **123**:5179–89. doi:10.1172/JCI69000
40. Gebeshuber CA, Kornauth C, Dong L, Sierig R, Seibler J, Reiss M, et al. Focal segmental glomerulosclerosis is induced by microRNA-193a and its downregulation of WT1. *Nat Med* (2013) **19**:481–7. doi:10.1038/nm.3142
41. He B, Ebarasi L, Zhao Z, Guo J, Ojala JR, Hultenby K, et al. Lmx1b and FoxC combinatorially regulate podocin expression in podocytes. *J Am Soc Nephrol* (2014) **25**(12):2764–77. doi:10.1681/ASN.2012080823
42. Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, et al. Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* (1999) **286**:312–5. doi:10.1126/science.286.5438.312
43. Tang VW, Brieher WM. FSGS3/CD2AP is a barbed-end capping protein that stabilizes actin and strengthens adherens junctions. *J Cell Biol* (2013) **203**:815–33. doi:10.1083/jcb.201304143
44. Hall G, Gbadegesin RA, Lavin P, Wu G, Liu Y, Oh EC. et al. A novel missense mutation of Wilms' tumor 1 causes autosomal dominant FSGS. *J Am Soc Nephrol* (2014). doi:10.1681/ASN.2013101053
45. Boyer O, Woerner S, Yang F, Oakeley EJ, Linghu B, Gribouval O, et al. LMX1B mutations cause hereditary FSGS without extrarenal involvement. *J Am Soc Nephrol* (2013) **24**:1216–22. doi:10.1681/ASN.2013020171
46. Colin E, Huynh Cong E, Mollet G, Guichet A, Gribouval O, Arrondel C, et al. Loss-of-function mutations in WDR73 are responsible for microcephaly and steroid-resistant nephrotic syndrome: Galloway-Mowat syndrome. *Am J Hum Genet* (2014) **95**(6):637–48. doi:10.1016/j.ajhg.2014.10.011
47. Hinkes B, Wiggins RC, Gbadegesin R, Vlangos CN, Seelow D, Nurnberg G, et al. Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be reversible. *Nat Genet* (2006) **38**:1397–405. doi:10.1038/ng1918
48. Wharram BL, Goyal M, Gillespie PJ, Wiggins JE, Kershaw DB, Holzman LB, et al. Altered podocyte structure in GLEPP1 (Ptpro)-deficient mice associated with hypertension and low glomerular filtration rate. *J Clin Invest* (2000) **106**:1281–90. doi:10.1172/JCI7236
49. Ozaltin F, Ibsirlioglu T, Taskiran EZ, Baydar DE, Kaymaz F, Buyukcelik M, et al. Disruption of PTPRO causes childhood-onset nephrotic syndrome. *Am J Hum Genet* (2011) **89**:139–47. doi:10.1016/j.ajhg.2011.05.026
50. Gbadegesin RA, Hall G, Adeyemo A, Hanke N, Tossidou I, Burchette J, et al. Mutations in the gene that encodes the F-actin binding protein anillin cause FSGS. *J Am Soc Nephrol* (2014) **25**:1991–2002. doi:10.1681/ASN.2013090976
51. Gupta IR, Baldwin C, Auguste D, Ha KC, El AJ, Fahiminiya S, et al. ARHGDIA: a novel gene implicated in nephrotic syndrome. *J Med Genet* (2013) **50**:330–8. doi:10.1136/jmedgenet-2012-101442
52. Akilesh S, Suleiman H, Yu H, Stander MC, Lavin P, Gbadegesin R, et al. Arhgap24 inactivates Rac1 in mouse podocytes, and a mutant form is associated with familial focal segmental glomerulosclerosis. *J Clin Invest* (2011) **121**:4127–37. doi:10.1172/JCI46458
53. Mele C, Iatropoulos P, Donadelli R, Calabria A, Maranta R, Cassis P, et al. MYO1E mutations and childhood familial focal segmental glomerulosclerosis. *N Engl J Med* (2011) **365**:295–306. doi:10.1056/NEJMoa1101273
54. Genovese G, Friedman DJ, Ross MD, Lecordier L, Uzureau P, Freedman BI, et al. Association of trypanolytic ApoL1 variants with kidney disease in African Americans. *Science* (2010) **329**:841–5. doi:10.1126/science.1193032
55. Matejas V, Hinkes B, Alkandari F, Al-Gazali L, Annexstad E, Aytac MB, et al. Mutations in the human laminin beta2 (LAMB2) gene and the associated phenotypic spectrum. *Hum Mutat* (2010) **31**:992–1002. doi:10.1002/humu.21304
56. Kambham N, Tanji N, Seigle RL, Markowitz GS, Pulkkinen L, Uitto J, et al. Congenital focal segmental glomerulosclerosis associated with beta4 integrin mutation and epidermolysis bullosa. *Am J Kidney Dis* (2000) **36**:190–6. doi:10.1053/ajkd.2000.8293
57. Malone AF, Phelan PJ, Hall G, Cetincelik U, Homstad A, Alonso AS, et al. Rare hereditary COL4A3/COL4A4 variants may be mistaken for familial focal segmental glomerulosclerosis. *Kidney Int* (2014) **86**(6):1253–9. doi:10.1038/ki.2014.305
58. Stieger N, Worthmann K, Teng B, Engeli S, Das AM, Haller H, et al. Impact of high glucose and transforming growth factor-β on bioenergetic profiles in podocytes. *Metabolism* (2012) **61**(8):1073–86. doi:10.1016/j.metabol.2011.12.003

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Podocyte pathology and nephropathy – sphingolipids in glomerular diseases

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Sphingolipids are components of the lipid rafts in plasma membranes, which are important for proper function of podocytes, a key element of the glomerular filtration barrier. Research revealed an essential role of sphingolipids and sphingolipid metabolites in glomerular disorders of genetic and non-genetic origin. The discovery that glucocerebrosides accumulate in Gaucher disease in glomerular cells and are associated with clinical proteinuria initiated intensive research into the function of other sphingolipids in glomerular disorders. The accumulation of sphingolipids in other genetic diseases including Tay–Sachs, Sandhoff, Fabry, hereditary inclusion body myopathy 2, Niemann–Pick, and nephrotic syndrome of the Finnish type and its implications with respect to glomerular pathology will be discussed. Similarly, sphingolipid accumulation occurs in glomerular diseases of non-genetic origin including diabetic kidney disease (DKD), HIV-associated nephropathy, focal segmental glomerulosclerosis (FSGS), and lupus nephritis. Sphingomyelin metabolites, such as ceramide, sphingosine, and sphingosine-1-phosphate have also gained tremendous interest. We recently described that sphingomyelin phosphodiesterase acid-like 3b (SMPDL3b) is expressed in podocytes where it modulates acid sphingomyelinase activity and acts as a master modulator of danger signaling. Decreased SMPDL3b expression in post-reperfusion kidney biopsies from transplant recipients with idiopathic FSGS correlates with the recurrence of proteinuria in patients and in experimental models of xenotransplantation. Increased SMPDL3b expression is associated with DKD. The consequences of differential SMPDL3b expression in podocytes in these diseases with respect to their pathogenesis will be discussed. Finally, the role of sphingolipids in the formation of lipid rafts in podocytes and their contribution to the maintenance of a functional slit diaphragm in the glomerulus will be discussed.

Keywords: sphingolipid, podocyte, kidney disease, glomerular disease, S1P, ASMase, SMPDL3b, ceramide

Sphingolipids, more precisely sphingomyelin, cerebroside, and cerebrosulfatide were first described in 1884 by Johann L. W. Thudichum who named them for their enigmatic ("Sphinx-like") properties (1). They are important components of the lipid rafts in plasma membranes of mammalian cells and thus contribute to the proper function of cells. Within the kidney, the function and survival of major cell constituents of the glomerular filtration barrier, i.e., podocytes, heavily depends on the integrity of lipid rafts. Podocytes are differentiated cells of the kidney glomerulus consisting of a cell body, major processes, and foot processes (FP). The FP of podocytes are linked to the glomerular basement membrane with their actin cytoskeleton. Processes from neighboring podocytes form a characteristic interdigitating pattern that leaves filtration slits in between them. The latter are bridged by the slit diaphragm (SD) that together with the glomerular basement membrane and the fenestrated endothelium plays an important role in the selective permeability of the filtration barrier of the glomerulus (2–4). Integrity of this filtration barrier is important in order to prevent the loss of protein into the urine (proteinuria) and mutations in genes coding for SD proteins cause proteinuria-associated nephropathies (5–9). Research of the past two decades has revealed an essential role of sphingolipids in glomerular disorders with podocyte involvement.

This review will focus on different types of sphingolipids and sphingolipid metabolites that have been implicated in the pathogenesis of sphingolipidoses of genetic and non-genetic origin with podocyte involvement. We will also discuss sphingolipid signaling in podocytes and its influence on the actin cytoskeleton.

BIOLOGY OF SPHINGOLIPIDS

Sphingolipids are a diverse class of lipids with a varying degree of hydrophobic and hydrophilic properties. The hydrophobic region of sphingolipids consists of a longchain sphingoid base with generally 18 carbons, such as sphingosine, which is linked to a fatty acid via an amide bond. The hydrophilic region consists in the simplest case of a hydroxyl group in the case of ceramide. Fatty acids may vary in their composition but palmitic (C16:0) and stearic (C18:0) are most commonly present. More complex sphingolipids have sugar residues (glycosphingolipids) and phosphates as side chains (phosphosphingolipids) (**Figure 1**).

Glycosphingolipids such as GM1 and phosphosphingolipids such as sphingomyelins are commonly found in eukaryotes,

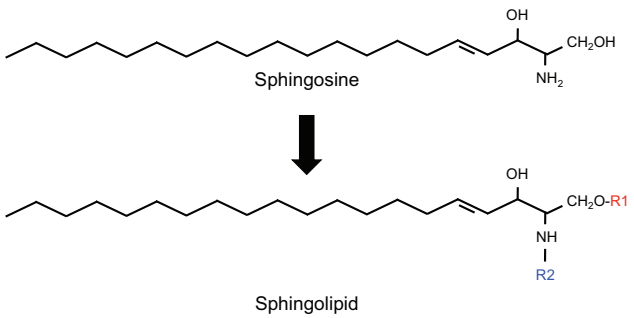

FIGURE 1 | Structure of sphingolipids. In sphingolipids, the hydrophobic region consists of a longchain sphingoid base with generally 18 carbons, such as sphingosine, which is linked to the acyl group of a fatty acid via an amide bond (R2). The hydrophilic region (R1) consists in the simplest case of a hydroxyl group in the case of ceramide.

in some prokaryotes, and in viruses as components of plasma membranes and membranes of organelles such as lysosomes, endosomes, endoplasmatic reticulum (ER), and others. The fluidity of the plasma membrane is tightly regulated by ordered packing of cholesterol between the phospholipid molecules, mainly sphingomyelin (SM) and thus sphingolipids have an important structural function. Sphingolipids are localized in the outer leaflet of the plasma membrane where they are asymmetrically distributed. Lipid rafts or raft-related caveolae are sphingomyelin-rich microdomains of the membrane, which are also enriched with cholesterol and membrane-associated proteins. The formation of lipid rafts is critical for proper cell function, protein–protein interactions, and signal transduction. For example, conversion of SM to ceramide, locally at the plasma membrane, by sphingomyelinases (SMases) will have a direct effect on the biophysical properties of the membrane and cell function as ceramide accumulation will lead to the displacement of cholesterol from the plasma membrane thus altering lipid rafts and signaling properties (10–12). Likewise, an interruption of raft-dependent cell signaling or even cell death can occur as a consequence of a cellular depletion of cholesterol that can be achieved by the use of cholesterol-depleting agents such as beta-cyclodextrin, methyl-beta-cyclodextrin, or 2-hydroxy-propyl-beta-cyclodextrin.

In recent years, it has become clear that, besides being integral part of membranes and having a structural function, sphingolipid metabolites such as ceramides, sphingosine, sphingosine-1-phosphate (S1P), and others play also important roles as second messengers in many biological processes including cell growth (13), differentiation, migration, and apoptosis (14). Complex sphingolipids were shown to interact with growth factor receptors, extracellular matrix, and neighboring cells (15). In addition, studies in yeast mutants revealed that sphingolipids have an important role in cellular stress responses as sphingolipid mutants yeast grew normally under usual culture conditions but were unable to survive if challenged or stressed (16).

Ceramide represents the centerpiece of the sphingolipid metabolic pathway reviewed in (17). Ceramide can be synthesized *de novo* starting with the condensation of L-serine and palmitoyl-CoA by serine palmitoyl transferase (SPT) to generate 3-ketodihydrosphinganine. The latter is then reduced by 3-ketosphinganine reductase to sphinganine, which in turn is N-acylated by ceramide synthetase (CS) to produce dihydroceramide. Finally, dihydroceramide is converted to ceramide by the enzyme dihydroceramide desaturase. Ceramide can also be generated by hydrolysis from sphingomyelin (SM) by SMases producing ceramide and phosphocholine. For sphingolipid biosynthesis, ceramide can be converted to sphingomyelin. This reaction is catalyzed by sphingomyelin synthetase (SMS), an enzyme that transfers the phosphocholine head group from phosphatidylcholine (PC) onto ceramide simultaneously generating diacylglycerol (DAG). Finally, ceramide can be generated by breakdown of glycosphingolipids and galactosylceramide to dihydroceramide and subsequent hydrolyzation (**Figure 2**).

Once ceramide is generated, it can accumulate in the cell or it may be further metabolized. Phosphorylation by ceramide kinase will generate ceramide-1-phosphate whereas deacylation by neutral or acid ceramidases will generate sphingosine, which can be phosphorylated by sphingosine kinase to yield S1P. Endproducts of the ceramide catabolic pathway are ethanolamine-1-phosphate and C16 fatty aldehydes, which are generated from S1P lyase from S1P.

SPHINGOLIPIDS IN GLOMERULAR DISEASES

In the past decade, it has become clear that there is an association between the accumulation of sphingolipids in the kidney and glomerular disease. The accumulation of sphingolipids in form of glycosphingolipids, ceramide, and ceramide metabolites has been described in several models of experimental and clinical nephropathy and is characteristic of some rare genetic glycosphingolipid (GSL) storage disorders. The observation that intracellular accumulation of sphingolipids in glomerular cells such as podocytes is also observed in the absence of genetic mutations and is associated with the development and progression of kidney disease suggests the existence of "acquired" sphingolipid storage disorders.

Most mammalian GSLs are synthesized from glucosylceramide (**Figure 2**) and are primarily present in the outer leaflet of the plasma membrane where they have important functions in mediating cell–cell interactions and modulating activities of proteins in their proximity. They are usually not uniformly distributed within the plasma membrane but cluster in lipid rafts (18, 19). Gangliosides are sialic acid-containing glycosphingolipids in which one or more *N*-acetlyneuraminic acids (NANA) is linked to the sugar chain and are essential components of plasma membranes (20). Gangliosides with one NANA include GM1, GM2, GM3, gangliosides with two NANAs are GD1a, GD1b, GD2, GD3, and the gangliosides GT1b and GQ1 are characterized by three and four NANAs, respectively. Gangliosides were first identified in nervous tissue but are also abundantly present in the kidney (21). GM1, GM2, GM3, GD1a, GD1b, GD2, GD3, GT1a, and GT1b are gangliosides present in normal rat glomeruli (22–24). GM3, GD3, and disialosyllactosylceramide (*O*-acetyl GD3) are the most abundant gangliosides present in kidney and 9-*O*-acetyl GD3 is a podocyte specific ganglioside (25, 26).

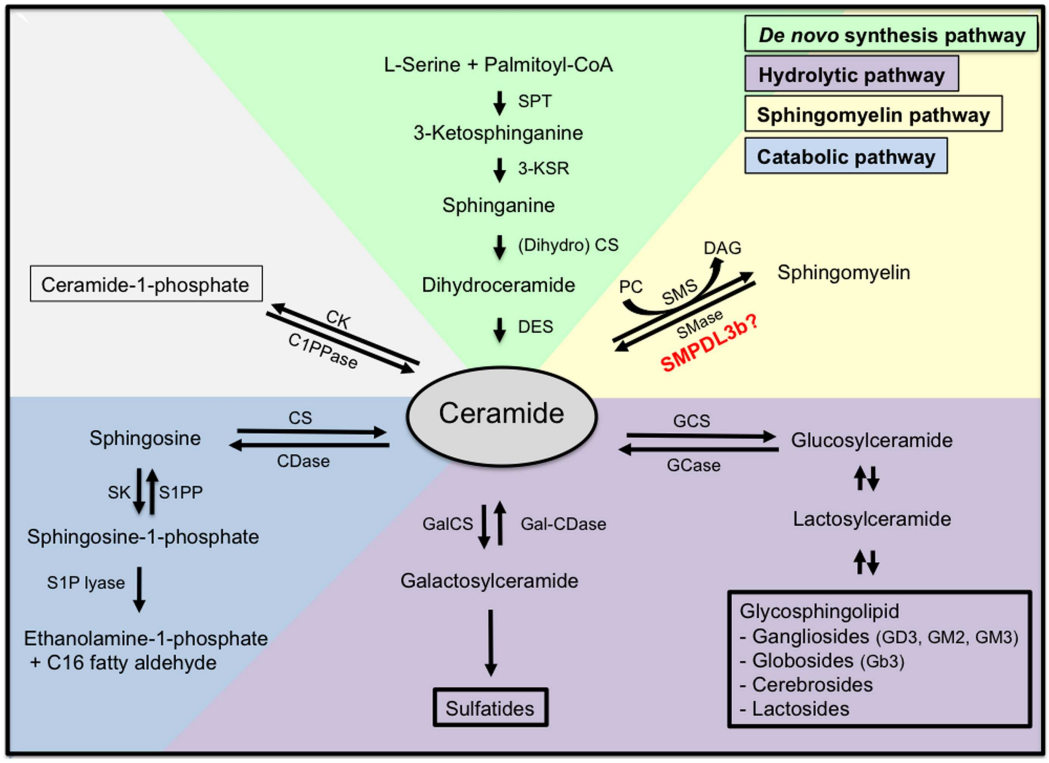

FIGURE 2 | Sphingolipid metabolism. Ceramide is the centerpiece of the sphingolipid metabolic pathway and can be synthesized *de novo* from L-serine and palmitoyl-CoA (green), through hydrolysis of sphingomyelin (yellow), or through hydrolysis of glycosphingolipids and sulfatites (purple). Ceramide can also be synthesized from sphingomyelin through the action of sphingomyelinases, or from ceramide-1-phosphate through the action of ceramide-1-phosphate phosphatase. Finally, ceramide can be further catabolized (blue) to sphingosine and sphingosine-1-phosphate, which are biologically active metabolites and finally to ethanolamine-1-phosphate and C16 fatty aldehydes. SPT, serine palmitoyl transferase; 3-KSR, 3-ketosphinganine reductase; CS, ceramide synthetase; DES, dihydroceramide desaturase; SMase, sphingomyelinase; SMS, sphingomyelin synthetase; PC, phosphatidylcholine; DAG, diacylglycerol; C1PPase, ceramide-1-phosphate phosphatase; CK, ceramide kinase; CDase, ceramidase; CS, ceramide synthase; SK, spingosine kinase; S1PP, spingosine-1-phosphate phosphatase; GCS, glycosylceramide synthase; GCase, glycosylceramidase; GalCS, galactosylceramide synthase; Gal-CDase, galactosylceramidase.

SPHINGOLIPID ACCUMULATION AND GLOMERULAR DISEASE OF GENETIC ORIGIN

Sphingolipidoses are inherited disorders leading to defects in the sphingolipid metabolism resulting in the accumulation of excess glycosphingolipids and phosphosphingolipids. It is interesting to note that different metabolites will tend to accumulate in different cell types, therefore leading to highly variable clinical–pathological findings.

Gaucher disease type 1 (OMIM #230800) is the most prevalent GSL storage disease and is characterized by an accumulation of glucocerebroside (GlcCer) in the affected tissues and cells, mainly in red blood cells, liver, and spleen. Gaucher disease is of autosomal recessive inheritance and it is caused by mutations in the acid beta-glucosidase 1 (*GBA1*) gene on chromosome 1q22 in the vast majority of patients (**Table 1**). This gene encodes for the enzyme that cleaves the beta-glucosidic linkage of glycosylceramide and mutations in this gene lead to accumulation of GlcCer. However, in some patients GlcCer accumulation is due to a lack of saposin C. Enzyme replacement therapy with macrophage-targeted recombinant human glucocerebrosidase is successfully used to treat patients with Gaucher disease (27–29) and drugs that block GlcCer synthesis are currently being tested in clinical trials (30). Although the presence of renal pathology in Gaucher disease is rather rare, it has been described in some patients (31) and it is associated with the accumulation of GlcCer in form of Gaucher bodies in glomerular mesangial and endothelial cells and in interstitial cells of the kidney (31). Sphingolipid activator proteins (saposins A, B, C, and D) are glycoproteins that are encoded in tandem and are derived from a common precursor protein (prosaposin, PSAP). Saposins stimulate the degradation of GSLs by lysosomal enzymes. Defects in saposins are associated with the accumulation of lipids in affected tissues in lysosomal storage disorders. Humans with saposin C deficiency exhibit the clinical presentation of Gaucher-like disease (32). Combined deficiency of Saposin C and D in mice led to accumulation of GSLs and ceramide in brain and kidney due to decreased β-glucosidase activity (33).

Tay–Sachs disease (OMIM #272800) is a genetic disorder with autosomal recessive inheritance that is caused by mutations in the alpha subunit of the hexoseaminidase A (*HEXA*) gene on chromosome 15q23. Sandhoff disease (OMIM #268800) is caused by mutations in the beta subunit of the hexosaminidase gene B (HEXB) on chromosome 5q13 (**Table 1**). Both disorders are

Table 1 | Sphingolipid accumulation in glomerular diseases of genetic and non-genetic origin.

Disease	OMIM	Mutated gene	Chromosomal location	Sphingolipid accumulating
SPHINGOLIPID ACCUMULATION IN GLOMERULAR DISEASE OF GENETIC ORIGIN				
Gaucher	230800	Acid beta-glucosidase 1 (GBA1)	1q22	GlcCer
Tay–Sachs	272800	Hexoseaminidase A (HEXA)	15q23	GM2
Sandhoff	268800	Hexoseaminidase B (HEXB)	5q13	GM2
Fabry	301500	Alpha-galactosidase A (GLA)	Xq22	Gb3, Lyso-Gb3
Hereditary inclusion body myopathy 2	600737	UDP-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE)	9p13	Hyposialylation of glycoproteins such as podocalyxin?
Niemann–Pick	257220 607616 257200	*NPC1 NPC2 SMPD1*	18q11 14q24 11p15	Sphingomyelin
Nephrotic syndrome of the Finnish type	256300	NPHS1	19q13	*O*-actetyl-GD3
SPHINGOLIPID ACCUMULATION IN GLOMERULAR DISEASE OF NON-GENETIC ORIGIN				
Diabetic kidney disease				GlcCer, GM3, S1P, sphingosine?
Puromycin aminonucleoside (PAN)-induced nephropathy				GD3, *O*-actetyl-GD3
HIV-associated nephropathy (HIVAN)				Gb3
Focal segmental glomerulosclerosis (FSGS)				Sphingomyelin
Acute ischemia reperfusion injury				Ceramide

characterized by an accumulation of the ganglioside GM2 in the affected tissues and are clinically indistinguishable from each other. GM2 accumulation occurs mainly in the brain and liver but has also been found in the kidney (34, 35). Unlike in humans, targeted inactivation of the *Hexa* and *Hexb* gene in mice revealed phenotypical differences between the two models. Whereas *Hexa* knockout mice showed GM2 accumulation in the brain and membranous cytoplasmatic bodies in neurons in the absence of neurological manifestations, *Hexb* knockout mice showed profound neurological disturbances. These differences, which are not found in patients with the two diseases, are due to differences in the ganglioside degradation pathway between humans and mice (36).

Fabry disease (OMIM #301500) is caused by mutations in the gene encoding alpha-galactosidase A (*GLA*) on chromosome Xq22, which leads to the systemic accumulation of globotriaoslyceramide (Gb3) (**Table 1**) and related glycosphingolipids in body fluids and affected tissues (37), mainly brain, heart, and kidney. Elevated levels of Gb3 are detected in plasma or urine of patients with Fabry disease (38, 39) and more recently, highly increased levels of globotriaosylsphingosine (lyso-Gb3) were also described (40, 41). In the kidney, Gb3 accumulation occurred mainly within lysosomal, ER, and nuclear markers of renal cells (42). The renal pathology observed in Fabry disease includes hypertrophic podocytes with foamy appearing vacuoles, characteristic inclusion bodies of glycolipids in podocytes (zebrabodies), and mesangial widening (43). Progressive podocyte injury due to accumulation of Gb3 and related glycosphingolipids was shown to be associated with albuminuria and foot process effacement. Thereby, podocyte Gb3 inclusion volume density and foot process effacement increased with age when compared with controls and correlated directly with proteinuria (44, 45). Enzyme replacement therapy using recombinant human α-GalA is the primary treatment for patients with Fabry disease and was shown to attenuate renal complications, halt the progression of renal pathology, and prevent renal failure in patients with Fabry disease (44, 46). Studies in the alpha *GalA* knockout mouse, a mouse model of Fabry disease, revealed reduced levels of glycosylceramide and ceramide in plasma, liver, spleen, kidney, and heart possibly a consequence of Gb3 accumulation. The observation that enzyme replacement therapy in this model normalized glycosylceramide levels possible via increased Gb3 degradation further supported the hypothesis that Gb3 accumulation contributes to the phenotype observed in these mice (46). Thereby, targeting of recombinant α-GalA requires the expression of endocytic receptors, megalin, sortilin, and mannose-6-phosphate receptor (M6PR), which are expressed in human glomerular podocytes (47). Interestingly, lentiviral knockdown of α-GalA in human podocytes led to intracellular Gb3 accumulation, which was associated with a loss of mTOR kinase activity and dysregulated autophagy suggesting a link between autophagy and glomerular injury in Fabry disease (48).

Hereditary inclusion body myopathy 2 (HIBM2) (OMIM #600737) is a genetic disorder with autosomal recessive inheritance that is caused by mutations in the gene encoding UDP-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) on chromosome 9p13 (**Table 1**). GNE is a key enzyme in the sialic acid biosynthetic pathway, which catalyzes the first two steps in NANA biosynthesis, which are main components of gangliosides (49). The disease is a progressive neuromuscular disorder but no renal disorders have been reported in patients with HIBM2. Interestingly, knockin mice carrying a homozygous M712T Gne/Mnk mutation died perinatally in the absence of

myopathic features but were characterized by a renal phenotype. The renal pathology observed included glomerular hematuria, segmental splitting of the glomerular basement membrane, proteinuria, and podocytopathy, including effacement of podocyte FP, and reduced sialylation of the major podocyte sialoprotein, podocalyxin. ManNAc administration to homozygous knockin mice was associated with improved renal histology, increased sialylation of podocalyxin, and increased Gne/Mnk protein expression and Gne-epimerase activities (50). Likewise, mice with a V572L point mutation in the GNE kinase domain show no apparent myopathies or motor dysfunctions but exhibited renal impairment accompanied by massive albuminuria. Histologically, kidneys of the mutant mice showed enlarged glomeruli with mesangial matrix deposition, leading to glomerulosclerosis, and abnormal podocyte foot process morphologies. This phenotype was partially prevented by the administration of *N*-acetylneuraminic acid (Neu5Ac) to the mutant mice (51). These studies indicate that hyposylation of podocyte glycoproteins including podocalyxin may contribute to albuminuria, foot process effacement, and splitting of the glomerular basement membrane and that treatment with sialic acid may represent a new strategy to prevent or treat glomerular phenotypes associated with HIBM2.

Farber disease or Farber lipogranulomatosis (OMIM #228000) is a genetic disorder with autosomal recessive inheritance that is caused by mutations in the gene encoding acid ceramidase (*ASAH1*) on chromosome 8p22, the enzyme, which is responsible for the degradation of ceramide into sphingosine and free fatty acids (**Table 1**). Lipid accumulation is mainly seen in the joints, tissues, and the central nervous system, but also in liver, heart, and kidneys. Ceramide accumulation in the kidney leading to a particular phenotype of lipogranlulomatosis was described (52).

Niemann–Pick is a genetic disorder with autosomal recessive inheritance that is caused by mutations in the *NPC1* gene on chromosome 18q11 (OMIM #257220), by mutations in the *NPC2* gene on chromosome 14q24 (OMIM #607616), and by mutations in the sphingomyelin phosphodiesterase-1 (*SMPD1*) gene on chromosome 11p15 (OMIM #257200). Mutations in these genes lead to the accumulation of lipids in form of cholesterol (NPC1 and NPC2 mutations) and sphingomyelin (SMPD1 mutation) (**Table 1**). Acid sphingomyelinase (ASMase) deficiency in Niemann–Pick disease due to mutations in the *SMPD1* gene leads accumulation of sphingomyelin in the affected tissues including the kidney. The occurrence of lipid-laden macrophages resembling foam cells was described in the bone marrow, liver, and kidney in patients with Niemann–Pick disease and in SMPD1 knockout mice (53, 54). Enzyme replacement therapy using recombinant human ASM in *SMPD1* knockout mice led to significant improvements in the organs of the reticuloendothelial system but neurological deficits remained (55).

Nephrotic syndrome of the Finnish type (OMIM #256300) is a genetic disorder caused by homozygous or compound heterozygous mutations in the gene *NPHS1* encoding for the SD protein nephrin (**Table 1**). Nephrotic syndrome of the Finnish type occurs in association with deposits of disialoganglioside *O*-acetyl GD3 (56). Accumulation of galactosylceramides, mainly sulfatides, was also described in nephrotic syndrome of non-genetic, idiopathic orgin (57). However, what causes the accumulation of *O*-acetyl GD3 in nephrotic syndrome remains unclear. Saposins do not seem to play an important role as mRNA expression in diseased kidneys was found normal and no mutations in the PSAP gene were found in cDNA clones (56). It has been suggested that tumor necrosis factor alpha (TNFα) and CD95 may play an important role in the pathogenesis of nephrotic syndrome. TNFα and CD95 were found significantly increased in patients with nephrotic syndrome (58). It was shown in lymphoid and myeloid tumor cells that accumulation of GD3 induced Fas (APO-1/CD95)-mediated apoptosis in a caspase-independent manner that was the consequence of the disruption of the mitochondrial transmembrane potential. This phenotype was prevented by pharmacological inhibition of GD3 synthesis (59). In other studies, an important role of membrane-associated ASMase was suggested in Fas-mediated apoptosis as activation of ASMase leads to the generation of free ceramide, which then can be converted to GD3 (60, 61). A similar pathway has been recently described in TNFα-mediated apoptosis (62, 63). Significant 9-*O*-acetyl GD3 accumulation, together with increases in GM2 and GM4 gangliosides in glomerular cells was also observed after low level and long term lead exposure and was associated with decreased apoptosis in glomerular cells suggesting that GD3-O acetylation could represent a new strategy to attenuate apoptosis in renal glomerular cells and contribute to cell survival as observed during lead exposure (64).

SPHINGOLIPID ACCUMULATION IN GLOMERULAR DISEASE OF NON-GENETIC ORIGIN

Diabetic kidney disease (DKD) is the most common cause of end-stage renal disease and renal failure in the US and podocyte injury and the consequent loss of podocytes (podocytopenia) is an important feature of DKD in patients with type 1 and type 2 diabetes (65–69). Increased levels of sphingolipids such as glycosphingolipids (70), ceramide (71, 72), sphingosine (73), and sphinganine (72, 73) have been described in the plasma of patients with diabetes. More recently, it has become clear that the intracellular sphingolipid composition in podocytes and other cells of the kidney glomerulus may contribute to the pathogenesis and progression of the disease (**Table 1**).

Several studies investigated the effects of streptozotocin (STZ)-induced diabetes in rats on intracellular sphingolipid accumulation and its association with glomerular cell proliferation and glomerular hypertrophy. Accumulation of S1P was observed in rat glomeruli after 4 days of diabetes induction and was associated with an increase in neutral ceramidase and sphingosine kinase activity, the two enzymes involved in the conversion of ceramide to S1P (74). In another study, accumulation of GlcCer and GM3 occurred in the kidneys of rats 16 days after STZ-induced diabetes (75) whereas reduced GM3 and sialic acid content was detected in the glomeruli of rats 15 days after STZ-induced diabetes (76). Increased ceramide production due to increased expression of SPT, a key enzyme in the ceramide *de novo* synthesis pathway (**Figure 2**), was described in tubular epithelial cells and microvascular endothelial cells and was associated with increased apoptosis. Rapamycin treatment significantly reduced apoptosis and proteinuria indicating an important function of the Akt/mTOR pathway in STZ-induced DKD (77). We recently showed that the expression of sphingomyelin phosphodiesterase acid-like 3b

(SMPDL3b) is increased in glomeruli from patients with DKD, in DKD sera treated human podocytes and in glomeruli of diabetic mice (db/db). Because SMPDL3b is a protein with homology to ASMase, we hypothesized that SMPDL3b may activate SM metabolic pathways leading to the accumulation of sphingolipids other than sphingomyelin. Increased SMPDL3b expression was associated with increased RhoA activity and apoptosis but prevented αVβ3 integrin activation via its interaction with soluble urokinase plasminogen activating receptor (suPAR) in human podocytes cultured in the presence of sera from patients with DKD and in *db/db* mice (78). Because ceramide, sphingosine, and S1P are known sphingolipid metabolites to accumulate in apoptotic cells, we determined the ceramide content in kidney cortexes of db/db mice and found ceramide levels to be decreased in kidney cortexes of these mice. We therefore concluded that increased SMPDL3b levels may lead to increased cellular sphingosine or S1P content in the kidneys of db/db mice as it is the case in glomerular mesangial and tubular cells in *db/db* mice (79, 80), and in adipocytes of *ob/ob* mice (81). Taken together, these studies indicate a possible link between sphingolipid accumulation in form of S1P, GlcCer, and GM3 and glomerular proliferation and hypertrophy in DKD whereas the accumulation of ceramide and other ceramide metabolites such as sphingosine may contribute to podocytopenia observed in DKD. Thus, targeting sphingolipids and sphingolipid metabolites may represent a new strategy to treat patients with DKD.

Puromycin aminonucleoside (PAN) induced nephropathy is a model for human minimal change disease. Following PAN injection in rats, significant decreases in kidney GD3 and *O*-acetyl GD3 occurred in a dose- and time-dependent manner and preceded the development of proteinuria indicating a possible causative effect (82). Because sialoglycoproteins contribute significantly to the negative charge of the glomerular filtration barrier, it seems possible that decreases of GD3 and *O*-acetyl GD3 contribute to decreases in the negative charge of the filtration barrier and to the changes in glomerular permeability observed in PAN-induced nephropathy (83). Likewise, it was shown that PAN treatment of human podocytes led to a loss of sialic acid which was accompanied by increased generation of superoxide anions, a phenotype that was prevented by sialic acid supplementation (84).

HIV-associated nephropathy (HIVAN) is the classic renal disease associated with HIV infection. HIV-1 infection of renal tubular and glomerular podocytes leads to dedifferentiation and increased proliferation of podocytes (85, 86). Because podoyctes do not express HIV-1 receptors, it has been suggested lipid raft mediated endocytosis may facilitate the viral entry (87) underlining an important role for sphingolipids in mediating viral entry into the host cell. Most of our understanding of the pathogenesis of HIVAN has come from the Tg26 transgenic mouse model in which the gag/pol-deleted HIV-1 provirus is expressed. Transgenic mice show glomerular epithelial cell dedifferentiation and proliferation that is associated with proteinuria and renal failure. Renal histology revealed focal segmental glomerulosclerosis (FSGS) and microcystic tubular dilatation, resembling human HIVAN (85, 88). Studies in human podocytes in culture and transgenic mice showed that stable expression of Nef was sufficient to induce increased proliferation and loss of contact inhibition (89–92). In addition, recent studies have shown a strong association between HIVAN and the *APOL1* gene on human chromosome 22 (93) and, although not found in glomeruli, significant accumulation of Gb3 was found in renal tubular epithelial cells of HIV transgenic mice (94) (**Table 1**) indicating a possible role for (sphingo-) lipid metabolism in HIVAN.

Focal segmental glomerulosclerosis is a glomerular disease that is characterized by proteinuria and progression to end-stage renal disease. FSGS is the leading cause of nephrotic syndrome and the most common cause of primary glomerular disease in adults (95). Several mutations in genes coding for proteins that are expressed in podocytes have been shown to cause FSGS. We will focus in this paragraph on non-genetic forms of FSGS, mainly FSGS recurrence after transplantation, which occurs in about one third of patients (96–98) and on primary (idiopathic) forms of FSGS. We recently reported an important role of sphingomyelin-like phosphodiesterase 3b (SMPDL3b) gene in FSGS. Studying 41 patients at high risk for recurrent FSGS, we showed that the number of SMPDL3b-positive podocytes in post-reperfusion biopsies was decreased in patients who developed recurrent FSGS. As mentioned above, SMPDL3b is a protein with homology to ASMase and we hypothesized that decreased expression of SMPDL3b may lead to decreased ASMase activity and accumulation of sphingomyelin contributing to the pathogenesis of FSGS (**Table 1**). Indeed, we were able to show that human podocytes treated with the sera from patients with FSGS had decreased SMPDL3b expression and decreased ASMase activity. In addition, this was associated with actin cytoskeleton remodeling and apoptosis, a phenotype that was prevented by overexpression of SMPDL3b in podocytes or by treatment with rituximab, a monoclonal antibody directed against CD20 that we have found to also bind SMPDL3b in podocytes. The percentage of cells characterized by actin cytoskeleton remodeling in form of a loss of stress fibers correlated with proteinuria suggesting an important role of sphingomyelin in the pathogenesis of FSGS (99). Because decreased SMPDL3b expression in podocytes *per se* does not cause actin cytoskeleton remodeling and apoptosis, it seems possible that accumulation of sphingomyelin renders podocytes more susceptible to apoptosis and may act as a master modulator of danger signaling in podocytes. Supporting an important role of SMPDL3b in actin cytoskeleton remodeling and apoptosis, it was recently demonstrated that administration of rituximab to baboons after xeno-kidney transplantation from pigs delayed, but did not prevent, the post-transplant occurrence of proteinuria. As in the case of our study investigating the role of SMPDL3b in FSGS, this study demonstrated that rituximab was also able to prevent pig podocyte injury and prevented decreases in SMPDL3b expression in podoytes after exposure to naive baboon sera in association with preservation of cell viability (100). Finally, sequestration of plasma membrane lipids by cyclodextrin was shown to prevent suPAR-mediated αVβ3 integrin activation in podocytes (101), a pathway that may be causative of proteinuria in FSGS. Because circulating suPAR levels are elevated in FSGS patients, associate with decreased SMPDL3b expression, and suPAR-dependent αVβ3 integrin activation in podocytes (78, 99, 101, 102), whereas cyclodextrin protects podoctyes from injury in DKD where SMPDL3b expression is increased in podocytes (78, 103), we investigated if SMPDL3b expression modulates the podocyte injury phenotype in these two kidney diseases. We

demonstrated that contrary to what is observed in FSGS, increased SMPDL3b expression in DKD prevented αVβ3 integrin activation via its interaction with suPAR and led to increased RhoA activity rendering podocytes more susceptible to apoptosis (78). These observations suggest that SMPDL3b and thus sphingomyelin or sphingomyelin catabolites are important modulators of podocyte function shifting suPAR-mediated podocyte injury from a migratory to an apoptotic phenotype. Therefore, modulating sphingolipids in podocytes may represent a new strategy to prevent or treat podocyte injury in FSGS and DKD.

SPECIAL CONSIDERATIONS FOCUSING ON S1P AND S1P RECEPTORS IN RENAL DISEASE

Sphingosine-1-phosphate is generated by phosphorylation of sphingosine by sphingosine kinases (SPHK1, SPHK2) in response to various stimuli including growth factors, cytokines, G-protein-coupled receptor agonists, antigens, and others (**Figure 2**). Examples of factors that can transiently increase levels of S1P are TNFα and factors such as angiogenic growth factor, platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) all of which have been implicated in the pathogenesis of glomerular diseases. S1P signaling governs important cellular processes that determine cell fate. Thereby, extracellular S1P signaling is mediated via binding of S1P to G-protein-coupled-receptors (GPCRs). A family of five GPCRs termed $S1P_1$–$S1P_5$ [reviewed in Ref. (104, 105)] has been identified to date. Depending on the receptor subtype being expressed in the target cell, exogenous S1P can bind, and regulate a variety of important cellular functions including cell survival, cytoskeletal rearrangment, mitogenesis, cell differentiation, migration, and apoptosis. In the kidney, the receptors $S1P_1$ (EDG1), $S1P_2$ (EDG5), $S1P_3$ (EDG3), and $S1P_5$ (EDG8) are expressed in glomerular mesangial cells (106, 107) and whereas expression of $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$, but not $S1P_5$, was shown to be expressed in an immortalized mouse podocyte cell line (108). Increases in S1P synthesis mediated by sphingosine kinase, the use of $S1P_1$ receptor agonists, such as FTY720 (an unselective S1P receptor agonist) and SEW2871 (a selective $S1P_1$ receptor agonists) or by SPHK1 gene delivery were shown to protect from renal ischemia reperfusion injury (109–112), which is associated with increased ceramide expression (113–115), from DKD (108), and from various forms of glomerulonephritis (116–118) (**Table 1**). In addition, FTY720 and KRP-203, another $S1P_1$ receptor agonist, have proven highly effective in preventing graft rejection in preclinical models of renal transplantation (119, 120). Whereas the activation of the $S1P/S1P_1$ receptor pathway seems to be beneficial in the context of kidney disease, it was suggested that excessive $S1P/S1P_2$ receptor pathway in renal tubular cells in DKD may play an important role in Rho kinase activation and renal fibrosis (80). Such mechanism could also explain activation of RhoA and increased apoptosis in podocytes in DKD as we previously described (78). In patients with lupus nephritis (LN), an inflammation of the kidney caused by systemic lupus erythematosus (SLE), a disease of the immune system, circulating S1P levels are increased (121). Likewise, S1P and dihydro-S1P levels in serum and kidney tissues from a mouse model of LN were elevated and treatment of these mice with a specific SPHK2 inhibitor, ABC294640 improved renal injury (122). It was suggested that in the case of renal inflammatory disease, extracellular S1P induces COX-2 expression via activation of S1P2, subsequently leading to Gi and p42/p44 MAPK-dependent signaling in renal mesangial cells. Although research of the past two decades has greatly advanced our understanding of the role of S1P and S1P/S1P receptor signaling in the pathogenesis and/or treatment of kidney diseases, more studies are needed to obtain a better and more detailed understanding of their physiological and pathophysiological significance *in vivo*. Certainly, targeting S1P/S1P receptor signaling pathways may represent a novel strategy to treat renal diseases.

SPECIAL CONSIDERATIONS FOCUSING ON THE ACTIN CYTOSKELETON IN RENAL DISEASE

The kidney glomerulus is a highly specialized structure ensuring the selective ultrafiltration of plasma so that essential proteins are retained in the blood (3). Podocytes are glomerular epithelial cells consisting of a cell body, major processes, and FP. FP from neighboring cells are bridged by a 40-μm wide extracellular structure known as the SD (123, 124). Podocyte injury is an important feature of several renal diseases, including FSGS and DKD, in which independent of the underlying disease, a reorganization of the FP structure with fusion of filtration slits and apical displacement of the SD occurs (3, 125, 126). The SD is also required to control actin dynamics, response to injury, endocytosis, and cell viability. These observations make actin the common denominator in podocyte function and dysfunction (127, 128). Regulation of the podocyte actin cytoskeleton is therefore of critical importance for sustained function of the glomerular filter (129, 130). The connection of the actin cytoskeleton to the SD is mediated by several podocyte proteins such as CD2AP, Nephrin, ZO-1, and Podocin (131–134). Lipid rafts in podocytes are critical for the dynamic functional organization of the SD. Nephrin is partially associated with podocyte lipid rafts and co-immunoprecipitates with a podocyte specific 9-O-acetylated ganglioside. Injection of an antibody against the 9-O-acetylated ganglioside causes morphological changes of the filtration slits, resembling FP effacement (135) further underlining the important of intact lipid rafts and sphingolipids in the organization of the SD. Other sphingolipids such as S1P have also been implicated in cytoskeletal remodeling. S1P was shown to induce rapid reorganization of the actin cytoskeleton resulting in stress-fiber formation in 3T3 fibroblast, which was accompanied by transient tyrosine phosphorylation of focal adhesion kinase (FAK) and of the cytoskeleton-associated protein paxillin in association with RhoA activation in 3T3 fibroblasts (136). In renal mesangial cells, the serine/threonine protein kinase LIM kinase-1 (LIMK-1) was identified, which is involved in the regulation of cytoskeletal organization, as a ceramide-induced protein (137). Shiga toxin is a bacterial toxin that induces intracellular signals in a manner that is dependent on glycolipid-enriched membrane domains, or lipid rafts. Shiga-toxin-mediated intracellular signals were shown to induce cytoskeleton remodeling in renal tubular epithelial carcinoma cells (138). VEGF and its receptors, FLK1/KDR and FLT1, are key regulators of angiogenesis. However, recently a new role for FLT1, i.e., the soluble form of FLT, sFLT has been described in podocytes where it binds to the glycosphingolipid GM3 in lipid rafts, promoting adhesion, and rapid

actin reorganization (139). Taken together, these studies underline the important function of sphingolipids in the formation of lipid rafts in podocytes thus contributing to the maintenance of a functional SD under physiological conditions.

CONCLUDING REMARKS

Sphingolipids play an important role in modulating podocyte function in glomerular disorders of genetic and non-genetic origin. Several genetic diseases are characterized by genetic mutations in genes that code for enzymes involved in the sphingolipid metabolism and are characterized by the accumulation of sphingolipids and sphingolipid metabolites in glomerular cells resulting in glomerular pathology. Thus, targeting sphingolipid metabolism in glomerular disease may prove beneficial in the treatment of proteinuric kidney diseases with glomerular involvement. Enzyme replacement therapy has proven to ameliorate disease progression in sphingolipid associated disorders of genetic origin such as Gaucher and Fabry disease. However, less is known about sphingolipid associated disorders of non-genetic origin. While ManNAc and rituximab are promising available therapeutic strategies for sphingolipid associated disorders of non-genetic origin, additional therapeutic strategies specifically targeting proteins such as SMPDL3b remain to be developed. Because sphingolipidoses of non-genetic origin seem to be more complex, additional research needs to be completed in order to elucidate the exact mechanisms by which sphingolipids cause injury to renal cells and thus contribute to the pathology of glomerular diseases.

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REFERENCES

1. McIlwain H. The second thudichum lecture. Cerebral isolates and neurochemical discovery. *Biochem Soc Trans* (1975) **3**:579–90.
2. Pavenstadt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* (2003) **83**:253–307. doi:10.1152/physrev.00020.2002
3. Somlo S, Mundel P. Getting a foothold in nephrotic syndrome. *Nat Genet* (2000) **24**:333–5. doi:10.1038/71724
4. Faul C, Asanuma K, Yanagida-Asanuma E, Kim K, Mundel P. Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. *Trends Cell Biol* (2007) **17**:428–37. doi:10.1016/j.tcb.2007.06.006
5. Kestilä M, Lenkkeri U, Männikkö M, Lamerdin J, McCready P, Putaala H, et al. Positionally cloned gene for a novel glomerular protein – nephrin – is mutated in congenital nephrotic syndrome. *Mol Cell* (1998) **1**:575–82. doi:10.1016/S1097-2765(00)80057-X
6. Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, et al. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* (2000) **24**:349–54. doi:10.1038/74166
7. Li C, Ruotsalainen V, Tryggvason K, Shaw AS, Miner JH. CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere. *Am J Physiol Renal Physiol* (2000) **279**:F785–92.
8. Winn MP, Conlon PJ, Lynn KL, Farrington MK, Creazzo T, Hawkins AF, et al. A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science* (2005) **308**:1801–4. doi:10.1126/science.1106215
9. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, et al. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* (2000) **24**:251–6. doi:10.1038/73456
10. Goni FM, Alonso A. Effects of ceramide and other simple sphingolipids on membrane lateral structure. *Biochim Biophys Acta* (2009) **1788**:169–77. doi:10.1016/j.bbamem.2008.09.002
11. van Blitterswijk WJ, van der Luit AH, Veldman RJ, Verheij M, Borst J. Ceramide: second messenger or modulator of membrane structure and dynamics? *Biochem J* (2003) **369**:199–211. doi:10.1042/BJ20021528
12. Zhang Y, Li X, Becker KA, Gulbins E. Ceramide-enriched membrane domains – structure and function. *Biochim Biophys Acta* (2009) **1788**:178–83. doi:10.1016/j.bbamem.2008.07.030
13. Olivera A, Spiegel S. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* (1993) **365**:557–60. doi:10.1038/365557a0
14. Kaipia A, Chun SY, Eisenhauer K, Hsueh AJ. Tumor necrosis factor-alpha and its second messenger, ceramide, stimulate apoptosis in cultured ovarian follicles. *Endocrinology* (1996) **137**:4864–70. doi:10.1210/endo.137.11.8895358
15. Merrill AH. Ceramide: a new lipid "second messenger"? *Nutr Rev* (1992) **50**:78–80. doi:10.1111/j.1753-4887.1992.tb01273.x
16. Nagiec MM, Wells GB, Lester RL, Dickson RC. A suppressor gene that enables *Saccharomyces cerevisiae* to grow without making sphingolipids encodes a protein that resembles an *Escherichia coli* fatty acyltransferase. *J Biol Chem* (1993) **268**:22156–63.
17. Merrill AH Jr. De novo sphingolipid biosynthesis: a necessary, but dangerous, pathway. *J Biol Chem* (2002) **277**:25843–6. doi:10.1074/jbc.R200009200
18. Mondal S, Mukhopadhyay C. Molecular level investigation of organization in ternary lipid bilayer: a computational approach. *Langmuir* (2008) **24**:10298–305. doi:10.1021/la8015589
19. Hall A, Rog T, Karttunen M, Vattulainen I. Role of glycolipids in lipid rafts: a view through atomistic molecular dynamics simulations with galactosylceramide. *J Phys Chem B* (2010) **114**:7797–807. doi:10.1021/jp912175d
20. Hakomori S. Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. *J Biol Chem* (1990) **265**:18713–6.
21. Shayman JA, Radin NS. Structure and function of renal glycosphingolipids. *Am J Physiol* (1991) **260**:F291–302.
22. Iwamori M, Shimomura J, Tsuyuhara S, Nagai Y. Gangliosides of various rat tissues: distribution of ganglio-N-tetraose-containing gangliosides and tissue-characteristic composition of gangliosides. *J Biochem* (1984) **95**:761–70.
23. Saito M, Sugiyama K. Gangliosides in rat kidney: composition, distribution, and developmental changes. *Arch Biochem Biophys* (2001) **386**:11–6. doi:10.1006/abbi.2000.2206
24. Hoon DS, Okun E, Neuwirth H, Morton DL, Irie RF. Aberrant expression of gangliosides in human renal cell carcinomas. *J Urol* (1993) **150**:2013–8.
25. Reivinen J, Holthofer H, Miettinen A. A cell-type specific ganglioside of glomerular podocytes in rat kidney: an O-acetylated GD3. *Kidney Int* (1992) **42**:624–31. doi:10.1038/ki.1992.327
26. Holthofer H, Reivinen J, Miettinen A. Nephron segment and cell-type specific expression of gangliosides in the developing and adult kidney. *Kidney Int* (1994) **45**:123–30. doi:10.1038/ki.1994.14
27. Barton NW, Brady RO, Dambrosia JM, Di Bisceglie AM, Doppelt SH, Hill SC, et al. Replacement therapy for inherited enzyme deficiency – macrophage-targeted glucocerebrosidase for Gaucher's disease. *N Engl J Med* (1991) **324**:1464–70. doi:10.1056/NEJM199105233242104
28. Barton NW, Furbish FS, Murray GJ, Garfield M, Brady RO. Therapeutic response to intravenous infusions of glucocerebrosidase in a patient with Gaucher disease. *Proc Natl Acad Sci U S A* (1990) **87**:1913–6. doi:10.1073/pnas.87.5.1913
29. Weinreb NJ, Charrow J, Andersson HC, Kaplan P, Kolodny EH, Mistry P, et al. Effectiveness of enzyme replacement therapy in 1028 patients with type 1 Gaucher disease after 2 to 5 years of treatment: a report from the Gaucher registry. *Am J Med* (2002) **113**:112–9. doi:10.1016/S0002-9343(02)01150-6
30. Lukina E, Watman N, Arreguin EA, Banikazemi M, Dragosky M, Iastrebner M, et al. A phase 2 study of eliglustat tartrate (Genz-112638), an oral substrate

reduction therapy for Gaucher disease type 1. *Blood* (2010) **116**:893–9. doi:10.1182/blood-2010-03-273151
31. Chander PN, Nurse HM, Pirani CL. Renal involvement in adult Gaucher's disease after splenectomy. *Arch Pathol Lab Med* (1979) **103**:440–5.
32. Vaccaro AM, Motta M, Tatti M, Scarpa S, Masuelli L, Bhat M, et al. Saposin C mutations in Gaucher disease patients resulting in lysosomal lipid accumulation, saposin C deficiency, but normal prosaposin processing and sorting. *Hum Mol Genet* (2010) **19**:2987–97. doi:10.1093/hmg/ddq204
33. Sun Y, Witte DP, Zamzow M, Ran H, Quinn B, Matsuda J, et al. Combined saposin C and D deficiencies in mice lead to a neuronopathic phenotype, glucosylceramide and alpha-hydroxy ceramide accumulation, and altered prosaposin trafficking. *Hum Mol Genet* (2007) **16**:957–71. doi:10.1093/hmg/ddm040
34. Sandhoff K, Andreae U, Jatzkewitz H. Deficient hexozaminidase activity in an exceptional case of Tay-Sachs disease with additional storage of kidney globoside in visceral organs. *Life Sci* (1968) **7**:283–8. doi:10.1016/0024-3205(68)90024-6
35. Tatematsu M, Imaida K, Ito N, Togari H, Suzuki Y, Ogiu T. Sandhoff disease. *Acta Pathol Jpn* (1981) **31**:503–12.
36. Sango K, Yamanaka S, Hoffmann A, Okuda Y, Grinberg A, Westphal H, et al. Mouse models of Tay-Sachs and Sandhoff diseases differ in neurologic phenotype and ganglioside metabolism. *Nat Genet* (1995) **11**:170–6. doi:10.1038/ng1095-170
37. Nance CS, Klein CJ, Banikazemi M, Dikman SH, Phelps RG, McArthur JC, et al. Later-onset Fabry disease: an adult variant presenting with the cramp-fasciculation syndrome. *Arch Neurol* (2006) **63**:453–7. doi:10.1001/archneur.63.3.453
38. Krüger R, Bruns K, Grünhage S, Rossmann H, Reinke J, Beck M, et al. Determination of globotriaosylceramide in plasma and urine by mass spectrometry. *Clin Chem Lab Med* (2010) **48**:189–98. doi:10.1515/CCLM.2010.048
39. Young E, Mills K, Morris P, Vellodi A, Lee P, Waldek S, et al. Is globotriaosylceramide a useful biomarker in Fabry disease? *Acta Paediatr Suppl* (2005) **94**:51–4; discussion 37–58. doi:10.1111/j.1651-2227.2005.tb02112.x
40. Gold H, Mirzaian M, Dekker N, Joao Ferraz M, Lugtenburg J, Codée JD, et al. Quantification of globotriaosylsphingosine in plasma and urine of fabry patients by stable isotope ultraperformance liquid chromatography-tandem mass spectrometry. *Clin Chem* (2012) **59**:547–56. doi:10.1373/clinchem.2012.192138
41. Auray-Blais C, Ntwari A, Clarke JT, Warnock DG, Oliveira JP, Young SP, et al. How well does urinary lyso-Gb3 function as a biomarker in Fabry disease? *Clin Chim Acta* (2010) **411**:1906–14. doi:10.1016/j.cca.2010.07.038
42. Askari H, Kaneski CR, Semino-Mora C, Desai P, Ang A, Kleiner DE, et al. Cellular and tissue localization of globotriaosylceramide in Fabry disease. *Virchows Arch* (2007) **451**:823–34. doi:10.1007/s00428-007-0468-6
43. Alroy J, Sabnis S, Kopp JB. Renal pathology in Fabry disease. *J Am Soc Nephrol* (2002) **13**(Suppl 2):S134–8. doi:10.1097/01.ASN.0000016684.07368.75
44. Thurberg BL, Rennke H, Colvin RB, Dikman S, Gordon RE, Collins AB, et al. Globotriaosylceramide accumulation in the Fabry kidney is cleared from multiple cell types after enzyme replacement therapy. *Kidney Int* (2002) **62**:1933–46. doi:10.1046/j.1523-1755.2002.00675.x
45. Najafian B, Svarstad E, Bostad L, Gubler MC, Tøndel C, Whitley C, et al. Progressive podocyte injury and globotriaosylceramide (GL-3) accumulation in young patients with Fabry disease. *Kidney Int* (2011) **79**:663–70. doi:10.1038/ki.2010.484
46. Quinta R, Rodrigues D, Assunção M, Macedo MF, Azevedo O, Cunha D, et al. Reduced glucosylceramide in the mouse model of Fabry disease: correction by successful enzyme replacement therapy. *Gene* (2014) **536**:97–104. doi:10.1016/j.gene.2013.11.073
47. Prabakaran T, Nielsen R, Larsen JV, Sørensen SS, Feldt-Rasmussen U, Saleem MA, et al. Receptor-mediated endocytosis of alpha-galactosidase A in human podocytes in Fabry disease. *PLoS One* (2011) **6**:e25065. doi:10.1371/journal.pone.0025065
48. Liebau MC, Braun F, Höpker K, Weitbrecht C, Bartels V, Müller RU, et al. Dysregulated autophagy contributes to podocyte damage in Fabry's disease. *PLoS One* (2013) **8**:e63506. doi:10.1371/journal.pone.0063506
49. Keppler OT, Hinderlich S, Langner J, Schwartz-Albiez R, Reutter W, Pawlita M. UDP-GlcNAc 2-epimerase: a regulator of cell surface sialylation. *Science* (1999) **284**:1372–6. doi:10.1126/science.284.5418.1372
50. Galeano B, Klootwijk R, Manoli I, Sun M, Ciccone C, Darvish D, et al. Mutation in the key enzyme of sialic acid biosynthesis causes severe glomerular proteinuria and is rescued by N-acetylmannosamine. *J Clin Invest* (2007) **117**:1585–94. doi:10.1172/JCI30954
51. Ito M, Sugihara K, Asaka T, Toyama T, Yoshihara T, Furuichi K, et al. Glycoprotein hyposialylation gives rise to a nephrotic-like syndrome that is prevented by sialic acid administration in GNE V572L point-mutant mice. *PLoS One* (2012) **7**:e29873. doi:10.1371/journal.pone.0029873
52. Samuelsson K, Zetterstrom R. Ceramides in a patient with lipogranulomatosis (Farber's disease) with chronic course. *Scand J Clin Lab Invest* (1971) **27**:393–405. doi:10.3109/00365517109080235
53. Brière J, Calman F, Lageron A, Hinglais N, Emerit J, Bernard J. Adult Niemann-Pick disease: a 26 years follow-up. Report of a case with isolated visceral involvement, excess of tissue sphingomyelin, and deficient sphingomyelinase activity (author's transl). *Nouv Rev Fr Hematol Blood Cells* (1976) **16**:185–202.
54. Kuemmel TA, Thiele J, Schroeder R, Stoffel W. Pathology of visceral organs and bone marrow in an acid sphingomyelinase deficient knock-out mouse line, mimicking human Niemann-Pick disease type A. A light and electron microscopic study. *Pathol Res Pract* (1997) **193**:663–71. doi:10.1016/S0344-0338(97)80025-8
55. Miranda SR, He X, Simonaro CM, Gatt S, Dagan A, Desnick RJ, et al. Infusion of recombinant human acid sphingomyelinase into Niemann-pick disease mice leads to visceral, but not neurological, correction of the pathophysiology. *FASEB J* (2000) **14**:1988–95. doi:10.1096/fj.00-0014com
56. Haltia A, Solin ML, Jalanko H, Holmberg C, Miettinen A, Holthöfer H. Sphingolipid activator proteins in a human hereditary renal disease with deposition of disialogangliosides. *Histochem J* (1996) **28**:681–7. doi:10.1007/BF02409005
57. Tamaoki A, Kikkawa Y. The role of sulfatides in autoimmunity in children with various glomerular disease. *Nihon Jinzo Gakkai Shi* (1991) **33**:1045–54.
58. Twfeek DM, Zaki SM. Role of tumour necrosis factor alpha and CD95 as markers of apoptosis in pathogenesis of pediatrics renal diseases. *Egypt J Immunol* (2005) **12**:155–65.
59. De Maria R, Lenti L, Malisan F, d'Agostino F, Tomassini B, Zeuner A, et al. Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. *Science* (1997) **277**:1652–5. doi:10.1126/science.277.5332.1652
60. De Maria R, Rippo MR, Schuchman EH, Testi R. Acidic sphingomyelinase (ASM) is necessary for fas-induced GD3 ganglioside accumulation and efficient apoptosis of lymphoid cells. *J Exp Med* (1998) **187**:897–902. doi:10.1084/jem.187.6.897
61. Cifone MG, De Maria R, Roncaioli P, Rippo MR, Azuma M, Lanier LL, et al. Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. *J Exp Med* (1994) **180**:1547–52. doi:10.1084/jem.180.4.1547
62. Omran OM, Saqr HE, Yates AJ. Molecular mechanisms of GD3-induced apoptosis in U-1242 MG glioma cells. *Neurochem Res* (2006) **31**:1171–80. doi:10.1007/s11064-006-9147-2
63. Wiegmann K, Schwandner R, Krut O, Yeh WC, Mak TW, Krönke M. Requirement of FADD for tumor necrosis factor-induced activation of acid sphingomyelinase. *J Biol Chem* (1999) **274**:5267–70. doi:10.1074/jbc.274.9.5267
64. Aguilar RP, Genta S, Sanchez S. Renal gangliosides are involved in lead intoxication. *J Appl Toxicol* (2008) **28**:122–31. doi:10.1002/jat.1256
65. Meyer TW, Bennett PH, Nelson RG. Podocyte number predicts long-term urinary albumin excretion in Pima Indians with type II diabetes and microalbuminuria. *Diabetologia* (1999) **42**:1341–4. doi:10.1007/s001250051447
66. Steffes MW, Schmidt D, McCrery R, Basgen JM. Glomerular cell number in normal subjects and in type 1 diabetic patients. *Kidney Int* (2001) **59**:2104–13. doi:10.1046/j.1523-1755.2001.0590062104.x
67. Verzola D, Gandolfo MT, Ferrario F, Rastaldi MP, Villaggio B, Gianiorio F, et al. Apoptosis in the kidneys of patients with type II diabetic nephropathy. *Kidney Int* (2007) **72**(10):1262–72. doi:10.1038/sj.ki.5002531
68. White KE, Bilous RW, Marshall SM, El Nahas M, Remuzzi G, Piras G, et al. Podocyte number in normotensive type 1 diabetic patients with albuminuria. *Diabetes* (2002) **51**:3083–9. doi:10.2337/diabetes.51.10.3083
69. Pagtalunan ME, Miller PL, Jumping-Eagle S, Nelson RG, Myers BD, Rennke HG, et al. Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* (1997) **99**:342–8. doi:10.1172/JCI119163
70. Kremer GJ, Atzpodien W, Schnellbacher E. Plasma glycosphingolipids in diabetics and normals. *Klin Wochenschr* (1975) **53**:637–8. doi:10.1007/BF01469685

71. Haus JM, Kashyap SR, Kasumov T, Zhang R, Kelly KR, Defronzo RA, et al. Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance. *Diabetes* (2009) **58**:337–43. doi:10.2337/db08-1228
72. Błachnio-Zabielska AU, Pułka M, Baranowski M, Nikołajuk A, Zabielski P, Górska M, et al. Ceramide metabolism is affected by obesity and diabetes in human adipose tissue. *J Cell Physiol* (2011) **227**:550–7. doi:10.1002/jcp.22745
73. Gorska M, Dobrzyn A, Baranowski M. Concentrations of sphingosine and sphinganine in plasma of patients with type 2 diabetes. *Med Sci Monit* (2005) **11**:CR35–8.
74. Geoffroy K, Troncy L, Wiernsperger N, Lagarde M, El Bawab S. Glomerular proliferation during early stages of diabetic nephropathy is associated with local increase of sphingosine-1-phosphate levels. *FEBS Lett* (2005) **579**:1249–54. doi:10.1016/j.febslet.2004.12.094
75. Zador IZ, Deshmukh GD, Kunkel R, Johnson K, Radin NS, Shayman JA. A role for glycosphingolipid accumulation in the renal hypertrophy of streptozotocin-induced diabetes mellitus. *J Clin Invest* (1993) **91**:797–803. doi:10.1172/JCI116299
76. Kwak DH, Rho YI, Kwon OD, Ahan SH, Song JH, Choo YK, et al. Decreases of ganglioside GM3 in streptozotocin-induced diabetic glomeruli of rats. *Life Sci* (2003) **72**:1997–2006. doi:10.1016/S0024-3205(03)00090-0
77. Liu G, Han F, Yang Y, Xie Y, Jiang H, Mao Y, et al. Evaluation of sphingolipid metabolism in renal cortex of rats with streptozotocin-induced diabetes and the effects of rapamycin. *Nephrol Dial Transplant* (2011) **26**:1493–502. doi:10.1093/ndt/gfq633
78. Yoo TH, Pedigo CE, Guzman J, Correa-Medina M, Wei C, Villarreal R, et al. SMPDL3b expression levels determine podocyte injury phenotypes in glomerular disease. *J Am Soc Nephrol* (2014) **25**(4):737–44. doi:10.1681/ASN.2013111213
79. Brunskill EW, Potter SS. Changes in the gene expression programs of renal mesangial cells during diabetic nephropathy. *BMC Nephrol* (2012) **13**:70. doi:10.1186/1471-2369-13-70
80. Ishizawa S, Takahashi-Fujigasaki J, Kanazawa Y, Matoba K, Kawanami D, Yokota T, et al. Sphingosine-1-phosphate induces differentiation of cultured renal tubular epithelial cells under Rho kinase activation via the S1P2 receptor. *Clin Exp Nephrol* (2014). doi:10.1007/s10157-014-0933-x
81. Samad F, Hester KD, Yang G, Hannun YA, Bielawski J. Altered adipose and plasma sphingolipid metabolism in obesity: a potential mechanism for cardiovascular and metabolic risk. *Diabetes* (2006) **55**:2579–87. doi:10.2337/db06-0330
82. Holthofer H, Reivinen J, Solin ML, Haltia A, Miettinen A. Decrease of glomerular disialogangliosides in puromycin nephrosis of the rat. *Am J Pathol* (1996) **149**:1009–15.
83. Andrews PM. Glomerular epithelial alterations resulting from sialic acid surface coat removal. *Kidney Int* (1979) **15**:376–85. doi:10.1038/ki.1979.49
84. Pawluczyk IZ, Ghaderi Najafabadi M, Patel S, Desai P, Vashi D, Saleem MA, et al. Sialic acid attenuates puromycin aminonucleoside-induced desialylation and oxidative stress in human podocytes. *Exp Cell Res* (2013) **320**:258–68. doi:10.1016/j.yexcr.2013.10.017
85. Barisoni L, Bruggeman LA, Mundel P, D'Agati VD, Klotman PE. HIV-1 induces renal epithelial dedifferentiation in a transgenic model of HIV-associated nephropathy. *Kidney Int* (2000) **58**:173–81. doi:10.1046/j.1523-1755.2000.00152.x
86. Bruggeman LA, Dikman S, Meng C, Quaggin SE, Coffman TM, Klotman PE. Nephropathy in human immunodeficiency virus-1 transgenic mice is due to renal transgene expression. *J Clin Invest* (1997) **100**:84–92. doi:10.1172/JCI119525
87. Mikulak J, Singhal PC. HIV-1 entry into human podocytes is mediated through lipid rafts. *Kidney Int* (2010) **77**:72–3; author reply 73–4. doi:10.1038/ki.2009.366
88. Kopp JB, Klotman ME, Adler SH, Bruggeman LA, Dickie P, Marinos NJ, et al. Progressive glomerulosclerosis and enhanced renal accumulation of basement membrane components in mice transgenic for human immunodeficiency virus type 1 genes. *Proc Natl Acad Sci U S A* (1992) **89**:1577–81. doi:10.1073/pnas.89.5.1577
89. Husain M, Gusella GL, Klotman ME, Gelman IH, Ross MD, Schwartz EJ, et al. HIV-1 Nef induces proliferation and anchorage-independent growth in podocytes. *J Am Soc Nephrol* (2002) **13**:1806–15. doi:10.1097/01.ASN.0000019642.55998.69
90. Kajiyama W, Kopp JB, Marinos NJ, Klotman PE, Dickie P. Glomerulosclerosis and viral gene expression in HIV-transgenic mice: role of nef. *Kidney Int* (2000) **58**:1148–59. doi:10.1046/j.1523-1755.2000.00271.x
91. Sunamoto M, Husain M, He JC, Schwartz EJ, Klotman PE. Critical role for Nef in HIV-1-induced podocyte dedifferentiation. *Kidney Int* (2003) **64**:1695–701. doi:10.1046/j.1523-1755.2003.00283.x
92. Hanna Z, Priceputu E, Hu C, Vincent P, Jolicoeur P. HIV-1 Nef mutations abrogating downregulation of CD4 affect other Nef functions and show reduced pathogenicity in transgenic mice. *Virology* (2006) **346**:40–52. doi:10.1016/j.virol.2005.10.010
93. Kopp JB, Nelson GW, Sampath K, Johnson RC, Genovese G, An P, et al. APOL1 genetic variants in focal segmental glomerulosclerosis and HIV-associated nephropathy. *J Am Soc Nephrol* (2011) **22**:2129–37. doi:10.1681/ASN.2011040388
94. Liu XH, Lingwood CA, Ray PE. Recruitment of renal tubular epithelial cells expressing verotoxin-1 (Stx1) receptors in HIV-1 transgenic mice with renal disease. *Kidney Int* (1999) **55**:554–61. doi:10.1046/j.1523-1755.1999.00278.x
95. Kitiyakara C, Eggers P, Kopp JB. Twenty-one-year trend in ESRD due to focal segmental glomerulosclerosis in the United States. *Am J Kidney Dis* (2004) **44**:815–25. doi:10.1053/j.ajkd.2004.07.008
96. Baum MA. Outcomes after renal transplantation for FSGS in children. *Pediatr Transplant* (2004) **8**:329–33. doi:10.1111/j.1399-3046.2004.00181.x
97. Hubsch H, Montané B, Abitbol C, Chandar J, Shariatmadar S, Ciancio G, et al. Recurrent focal glomerulosclerosis in pediatric renal allografts: the Miami experience. *Pediatr Nephrol* (2005) **20**:210–6. doi:10.1007/s00467-004-1706-7
98. Senggutuvan P, Cameron JS, Hartley RB, Rigden S, Chantler C, Haycock G, et al. Recurrence of focal segmental glomerulosclerosis in transplanted kidneys: analysis of incidence and risk factors in 59 allografts. *Pediatr Nephrol* (1990) **4**:21–8. doi:10.1007/BF00858431
99. Fornoni A, Sageshima J, Wei C, Merscher-Gomez S, Aguillon-Prada R, Jauregui AN, et al. Rituximab targets podocytes in recurrent focal segmental glomerulosclerosis. *Sci Transl Med* (2011) **3**:85ra46. doi:10.1126/scitranslmed.3002231
100. Tasaki M, Shimizu A, Hanekamp I, Torabi R, Villani V, Yamada K. Rituximab treatment prevents the early development of proteinuria following pig-to-baboon xeno-kidney transplantation. *J Am Soc Nephrol* (2014) **25**:737–44. doi:10.1681/ASN.2013040363
101. Wei C, Möller CC, Altintas MM, Li J, Schwarz K, Zacchigna S, et al. Modification of kidney barrier function by the urokinase receptor. *Nat Med* (2008) **14**:55–63. doi:10.1038/nm1696
102. Wei C, Trachtman H, Li J, Dong C, Friedman AL, Gassman JJ, et al. Circulating suPAR in two cohorts of primary FSGS. *J Am Soc Nephrol* (2012) **23**:2051–9. doi:10.1681/ASN.2012030302
103. Merscher-Gomez S, Guzman J, Pedigo CE, Lehto M, Aguillon-Prada R, Mendez A, et al. Cyclodextrin protects podocytes in diabetic kidney disease. *Diabetes* (2013) **62**(11):3817–27. doi:10.2337/db13-0399
104. Pyne NJ, Long JS, Lee SC, Loveridge C, Gillies L, Pyne S. New aspects of sphingosine 1-phosphate signaling in mammalian cells. *Adv Enzyme Regul* (2009) **49**:214–21. doi:10.1016/j.advenzreg.2009.01.011
105. Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat Rev Immunol* (2005) **5**:560–70. doi:10.1038/nri1650
106. Imasawa T, Kitamura H, Ohkawa R, Satoh Y, Miyashita A, Yatomi Y. Unbalanced expression of sphingosine 1-phosphate receptors in diabetic nephropathy. *Exp Toxicol Pathol* (2010) **62**:53–60. doi:10.1016/j.etp.2009.02.068
107. Koch A, Völzke A, Puff B, Blankenbach K, Meyer Zu Heringdorf D, Huwiler A, et al. PPARgamma agonists upregulate sphingosine 1-phosphate (S1P) receptor 1 expression, which in turn reduces S1P-induced [Ca(2+)]i increases in renal mesangial cells. *Biochim Biophys Acta* (2013) **1831**:1634–43. doi:10.1016/j.bbalip.2013.07.011
108. Awad AS, Rouse MD, Khutsishvili K, Huang L, Bolton WK, Lynch KR, et al. Chronic sphingosine 1-phosphate 1 receptor activation attenuates early-stage diabetic nephropathy independent of lymphocytes. *Kidney Int* (2011) **79**:1090–8. doi:10.1038/ki.2010.544
109. Park SW, Kim M, Chen SW, Brown KM, D'Agati VD, Lee HT. Sphinganine-1-phosphate protects kidney and liver after hepatic ischemia and reperfusion in mice through S1P1 receptor activation. *Lab Invest* (2010) **90**:1209–24. doi:10.1038/labinvest.2010.102

110. Kim M, Park SW, Pitson SM, Lee HT. Isoflurane protects human kidney proximal tubule cells against necrosis via sphingosine kinase and sphingosine-1-phosphate generation. *Am J Nephrol* (2010) **31**:353–62. doi:10.1159/000298339
111. Awad AS, Ye H, Huang L, Li L, Foss FW Jr, Macdonald TL, et al. Selective sphingosine 1-phosphate 1 receptor activation reduces ischemia-reperfusion injury in mouse kidney. *Am J Physiol Renal Physiol* (2006) **290**:F1516–24. doi:10.1152/ajprenal.00311.2005
112. Park SW, Kim M, D'Agati VD, Lee HT. Sphingosine kinase 1 protects against renal ischemia-reperfusion injury in mice by sphingosine-1-phosphate1 receptor activation. *Kidney Int* (2011) **80**:1315–27. doi:10.1038/ki.2011.281
113. Zager RA, Conrad S, Lochhead K, Sweeney EA, Igarashi Y, Burkhart KM. Altered sphingomyelinase and ceramide expression in the setting of ischemic and nephrotoxic acute renal failure. *Kidney Int* (1998) **53**:573–82. doi:10.1046/j.1523-1755.1998.00772.x
114. Kalhorn T, Zager RA. Renal cortical ceramide patterns during ischemic and toxic injury: assessments by HPLC-mass spectrometry. *Am J Physiol* (1999) **277**:F723–33.
115. Zager RA, Iwata M, Conrad DS, Burkhart KM, Igarashi Y. Altered ceramide and sphingosine expression during the induction phase of ischemic acute renal failure. *Kidney Int* (1997) **52**:60–70. doi:10.1038/ki.1997.304
116. Peters H, Martini S, Wang Y, Shimizu F, Kawachi H, Krämer S, et al. Selective lymphocyte inhibition by FTY720 slows the progressive course of chronic anti-thy 1 glomerulosclerosis. *Kidney Int* (2004) **66**:1434–43. doi:10.1111/j.1523-1755.2004.00906.x
117. Martini S, Krämer S, Loof T, Wang-Rosenke Y, Daig U, Budde K, et al. S1P modulator FTY720 limits matrix expansion in acute anti-thy1 mesangioproliferative glomerulonephritis. *Am J Physiol Renal Physiol* (2007) **292**:F1761–70. doi:10.1152/ajprenal.00253.2006
118. Schwalm S, Pfeilschifter J, Huwiler A. Targeting the sphingosine kinase/sphingosine 1-phosphate pathway to treat chronic inflammatory kidney diseases. *Basic Clin Pharmacol Toxicol* (2014) **114**:44–9. doi:10.1111/bcpt.12103
119. Ferguson R. FTY720 immunomodulation: optimism for improved transplant regimens. *Transplant Proc* (2004) **36**:549S–53S. doi:10.1016/j.transproceed.2004.01.061
120. Fujishiro J, Kudou S, Iwai S, Takahashi M, Hakamata Y, Kinoshita M, et al. Use of sphingosine-1-phosphate 1 receptor agonist, KRP-203, in combination with a subtherapeutic dose of cyclosporine A for rat renal transplantation. *Transplantation* (2006) **82**:804–12. doi:10.1097/01.tp.0000232687.78242.cd
121. Watson L, Tullus K, Marks SD, Holt RC, Pilkington C, Beresford MW. Increased serum concentration of sphingosine-1-phosphate in juvenile-onset systemic lupus erythematosus. *J Clin Immunol* (2012) **32**:1019–25. doi:10.1007/s10875-012-9710-3
122. Snider AJ, Ruiz P, Obeid LM, Oates JC. Inhibition of sphingosine kinase-2 in a murine model of lupus nephritis. *PLoS One* (2013) **8**:e53521. doi:10.1371/journal.pone.0053521
123. Ruotsalainen V, Ljungberg P, Wartiovaara J, Lenkkeri U, Kestilä M, Jalanko H, et al. Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci U S A* (1999) **96**:7962–7. doi:10.1073/pnas.96.14.7962
124. Tryggvason K. Unraveling the mechanisms of glomerular ultrafiltration: nephrin, a key component of the slit diaphragm. *J Am Soc Nephrol* (1999) **10**:2440–5.
125. Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, et al. A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* (2002) **13**:630–8.
126. Smoyer WE, Mundel P. Regulation of podocyte structure during the development of nephrotic syndrome. *J Mol Med (Berl)* (1998) **76**:172–83. doi:10.1007/s001090050206
127. Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *J Clin Invest* (2001) **108**:1583–7. doi:10.1172/JCI200114629
128. Asanuma K, Mundel P. The role of podocytes in glomerular pathobiology. *Clin Exp Nephrol* (2003) **7**:255–9. doi:10.1007/s10157-003-0259-6
129. Ichimura K, Kurihara H, Sakai T. Actin filament organization of foot processes in rat podocytes. *J Histochem Cytochem* (2003) **51**:1589–600. doi:10.1177/002215540305101203
130. Ichimura K, Kurihara H, Sakai T. Actin filament organization of foot processes in vertebrate glomerular podocytes. *Cell Tissue Res* (2007) **329**:541–57. doi:10.1007/s00441-007-0440-4
131. Yuan H, Takeuchi E, Salant DJ. Podocyte slit-diaphragm protein nephrin is linked to the actin cytoskeleton. *Am J Physiol Renal Physiol* (2002) **282**:F585–91. doi:10.1152/ajprenal.00290.2001
132. Huber TB, Simons M, Hartleben B, Sernetz L, Schmidts M, Gundlach E, et al. Molecular basis of the functional podocin-nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains. *Hum Mol Genet* (2003) **12**:3397–405. doi:10.1093/hmg/ddg360
133. Huber TB, Kottgen M, Schilling B, Walz G, Benzing T. Interaction with podocin facilitates nephrin signaling. *J Biol Chem* (2001) **276**:41543–6. doi:10.1074/jbc.C100452200
134. Fanning AS, Ma TY, Anderson JM. Isolation and functional characterization of the actin binding region in the tight junction protein ZO-1. *FASEB J* (2002) **16**:1835–7. doi:10.1096/fj.02-0121fje
135. Simons M, Schwarz K, Kriz W, Miettinen A, Reiser J, Mundel P, et al. Involvement of lipid rafts in nephrin phosphorylation and organization of the glomerular slit diaphragm. *Am J Pathol* (2001) **159**:1069–77. doi:10.1016/S0002-9440(10)61782-8
136. Wang F, Nobes CD, Hall A, Spiegel S. Sphingosine 1-phosphate stimulates rho-mediated tyrosine phosphorylation of focal adhesion kinase and paxillin in Swiss 3T3 fibroblasts. *Biochem J* (1997) **324**(Pt 2):481–8.
137. Shabahang S, Liu YH, Huwiler A, Pfeilschifter J. Identification of the LIM kinase-1 as a ceramide-regulated gene in renal mesangial cells. *Biochem Biophys Res Commun* (2002) **298**:408–13. doi:10.1016/S0006-291X(02)02483-X
138. Takenouchi H, Kiyokawa N, Taguchi T, Matsui J, Katagiri YU, Okita H, et al. Shiga toxin binding to globotriaosyl ceramide induces intracellular signals that mediate cytoskeleton remodeling in human renal carcinoma-derived cells. *J Cell Sci* (2004) **117**:3911–22. doi:10.1242/jcs.01246
139. Jin J, Sison K, Li C, Tian R, Wnuk M, Sung HK, et al. Soluble FLT1 binds lipid microdomains in podocytes to control cell morphology and glomerular barrier function. *Cell* (2012) **151**:384–99. doi:10.1016/j.cell.2012.08.037

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Role of protein kinase C in podocytes and development of glomerular damage in diabetic nephropathy

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The early glomerular changes in diabetes include a podocyte phenotype with loss of slit diaphragm proteins, changes in the actin cytoskeleton and foot process architecture. This review focuses on the role of the protein kinase C (PKC) family in podocytes and points out the differential roles of classical, novel, and atypical PKCs in podocytes. Some PKC isoforms are indispensable for proper glomerular development and slit diaphragm maintenance, whereas others might be harmful when activated in the diabetic milieu. Therefore, some might be interesting treatment targets in the early phase of diabetes.

Keywords: proteinuria, diabetic podocytopathy, glomerulosclerosis, nephrin endocytosis, effacement

INTRODUCTION

Diabetic nephropathy (DN) is the most common cause of the end-stage renal disease (ESRD) (1). The glomerular changes in DN are characterized by excessive accumulation of extracellular matrix (ECM) with thickening of glomerular and tubular basement membranes and mesangial expansion, which ultimately progress primarily to glomerulosclerosis and secondarily to tubulointerstitial fibrosis. The various cell types involved include glomerular endothelial cells, mesangial cells, podocytes, and tubular epithelia, which are all targets of hyperglycemic injury. However, accumulating evidence suggests that the extent of injury and loss of podocytes is a major prognostic determinant in both, type I and type II DN (2–6). As the terminally differentiated podocytes are believed to play a critical role in maintaining the integrity of the glomerular filtration barrier, effaced podocytes may contribute to the development of albuminuria, a hallmark of DN. Although primarily structure proteins were thought to be the key elements that compose the slit diaphragm initially, it has become clear that the slit diaphragm protein complex is a highly dynamic functional protein complex and is able to initiate cascades of signaling pathways that affect podocyte function (7). More recent data indicate that podocytes express receptors for many circulating hormones and growth factors, which also suggest that a more complex cross-talk between the kidney and other organs affected by diabetes may occur in health and disease (8).

Among various signaling kinases, the protein kinase C (PKC) family seems to play a critical role in the pathogenesis of DN (9). The activation of PKCs in the kidney is a well-known pathway of the diabetic milieu. The PKC family is involved in a variety of signal transduction pathways, cell proliferation, differentiation, cell cycle, and apoptosis. However, the role of PKCs on podocytes in DN has not yet been fully defined. This present review will give an overview of the general role of PKCs and summarize the recent research into the regulatory role of PKCs in podocytes under diabetic conditions.

PKC SUBFAMILIES AND ISOFORMS

All PKC isoforms contain a highly conserved catalytic domain and a regulatory domain. The catalytic domain consists of several motifs and is essential for the ATP/substrate-binding and catalysis, whereas the N-terminal regulatory domain contains an auto inhibitory pseudo-substrate domain and two discrete membrane targeting modules, C1 and C2. The sequence of pseudo-substrate contains an alanine in place of the serine/threonine phospho-acceptor site, but otherwise resembles a PKC substrate (10).

Protein kinase C isoforms are subdivided into three subfamilies based on differences of structure in their N-terminal regulatory domain. The isoforms α, βI, βII, and γ belong to conventional PKCs (cPKCs). The regulatory domains of cPKCs contain a C1 domain that functions as diacylglycerol (DAG)/phorbol 12-myristat 13-acetat (PMA) binding motif and a C2 domain that binds anionic phospholipids in a calcium-dependent manner (10). The novel PKCs (nPKCsδ, ε, η, and θ) also have two C1 domains and a C2 domain. The nPKC C2 domains lack the critical calcium-coordinating acidic residues, which is the distinct difference between cPKC and nPKC. The nPKCs can be maximally activated by agonists that promote DAG accumulation or by PMA, but they are insensitive to calcium. Atypical PKCs (aPKCs, ζ, and λ/ι) lack a calcium-sensitive C2 domain; however, they contain an atypical C1 domain, which binds PIP3 or ceramide, but not DAG or PMA. The activity of aPKCs is primarily regulated by protein–protein interaction and phosphorylation catalyzed by phosphoinositide-dependent kinase-1 (PDK-1). Although a few PKC isoforms are expressed in a tissue-specific manner, most are ubiquitously expressed.

REGULATION OF PKCs

Unless it is post-translationally or co-translationally phosphorylated, PKCs are incapable of being activated by DAG or other cofactors (11, 12). PKC is translated as a catalytically inactive protein, is converted into an active enzyme by an initial phosphate addition and then into a mature form by further phosphorylation (11, 13, 14). In addition to phosphorylation on serine and threonine residues, PKCs also undergo phosphorylation by tyrosine kinases. PKCs are regulated by two sequential and equally critical mechanisms: phosphorylation triggered by PDK-1 and binding to DAG and/or other cofactors such as phosphatidylserine (PS) or phorbol ester (PE). Each mechanism regulates the structure, subcellular localization, and function of PKCs (15).

As a consequence of increased glycolytic flux, chronic hyperglycemia elevates the *de novo* synthesis of DAG and thus leads to an increased activation of DAG dependent classic and novel PKC isoforms in cultured bovine aortic endothelial cells and smooth muscle cells (16). Furthermore, high-glucose induced cellular levels lead to an increased generation of advanced glycation end products (AGEs), which initiate several signaling events by activating PKC, MAP kinase, and transcription factors such as nuclear factor-κB (NF-κB). This would increase the activity of various growth factors, such as TGF-β, and thereby alter expression of ECM proteins (17). In addition, under high-glucose conditions PKC is activated by higher concentrations of reactive oxygen species (ROS) generated following AGE:RAGE (AGE receptor) interactions (15, 18). In turn, the ROS are generated via NADPH-oxidase activated by PKC, ROS/PKC therefore can act in a cyclical manner to activate one another (19).

CONVENTIONAL PKCs IN DIABETIC NEPHROPATHY

Among all the PKC isoforms, the role of PKCα in the pathogenesis of DN has been investigated intensively, and several studies have demonstrated that PKCα-deficient mice show a better outcome after streptozotocin (STZ) induced diabetes with less proteinuria and preserved nephrin expression (20, 21). Studies from our group underline the involvement of PKCα in proteinuria development in DN. The expression of PKCα in podocytes of patients with DN was increased. Mice were treated after STZ-induced diabetes with the synthetic PKCα inhibitor (GÖ6976), which prevented proteinuria development and led to preserved nephrin expression. Furthermore, we could show a central role for PKCα in endocytosis of the slit diaphragm component nephrin (22, 23). Quack et al. further concluded that proteinuria of diabetic mice is as a result of increased endocytosis of nephrin, which is mediated by a complex consisting of PKCα, protein interacting with c kinase-1 (PICK1), and beta-arrestin2. They found rising glucose levels go along with increased binding of beta-arrestin to nephrin *in vitro* as well as in *in vivo*, but only with preceding PKCα phosphorylating activity on nephrin. This fact and the indispensability of PICK1 suggest PKCα and PICK1 as possible drug targets in early stages of DN (23). These studies show that expression of PKCα is regulated by glucose concentration in the external milieu of the podocyte and that PKCα is directly involved in the maintenance of the slit diaphragm. High levels of PKCα in podocytes led to enhanced endocytosis of nephrin and instability of the slit diaphragm.

In addition, Menne and colleagues demonstrated that the high-glucose induced downregulation of nephrin is probably caused by the PKCα mediated reduction of the transcriptional factor Wilms tumor 1 (WT-1), which has previously been described as a directly interacting binding partner of the nephrin promoter (24, 25). These findings are consistent with earlier studies, suggesting one of the PKC isoforms might be pivotal for the regulation of nephrin transcription and expression in podocytes (26), but not for CD2AP and Podocin as they remain at levels similar to those in non-diabetic kidneys (27).

Langham et al. showed that in diabetic patients treated with perindopril, an ACE inhibitor, nephrin levels preserved resembling those of non-diabetic subjects (28) and subsequently could be one factor contributing to the anti-proteinuric effects of ACE inhibitors. Another study suggests that the AGEs, which can be inhibited by aminoguanidine, are also implicated in the downregulation of nephrin in diabetes (29). Interestingly, several groups could demonstrate the reduction of PKC activity under the treatment with ACE inhibitors and aminoguanidine in diabetic subjects that could explain the nephrin protective effects (30, 31).

Most previous studies with specific PKCβ inhibitor ruboxistaurin (LX333531) *in vivo* and *in vitro* indicated that PKCβ isoform is primarily responsible for the high-glucose-induced renal effects in diabetes (32–36). Meier et al. tested this hypothesis by inducing DN in PKCβ deficient mice and did not find a significant preventive effect of PKCβ deficiency on albuminuria. In contrast to non-albuminuric diabetic PKCα$^{-/-}$ mice, the loss of the basement membrane proteoglycan perlecan and the podocyte slit diaphragm protein nephrin were not prevented in the PKCβ$^{-/-}$ mice under diabetic conditions (20, 37). However, the hyperglycemia-induced renal and glomerular hypertrophy as well as increased expression of ECM proteins was reduced in PKCβ deficiency diabetic mice.

In summary, the two important physiological features of DN, renal hypertrophy and albuminuria, are regulated through different PKC isoforms; PKCα is involved in the development of albuminuria and maintenance the glomerular filtration barrier structure, whereas the PKCβ-isoform contributes to hyperglycemia-induced renal fibrosis.

Another study by Menne et al. combined the findings about PKCα and PKCβ and demonstrated that a dual inhibition of both isoforms has a synergistic effect and is capable of preventing the development of experimental DN in streptozocin-induced diabetic mice (38). Blocking both isoforms has a beneficial effect on the development of renal hypertrophy and albuminuria in mice after 8 weeks of diabetes. A pharmacological approach with CGP41252, an inhibitor of PKCα and PKCβ showed that the occurrence of albuminuria could be avoided and preexisting albuminuria could be diminished in both type I and type II diabetic mice (38). But the treatment had little impact on the development of renal hypertrophy. Higher doses treatment also increased mortality.

ESSENTIAL ROLE OF NOVEL AND ATYPICAL PKCs

Only little is known about PKCε in renal function, especially about its role in podocytes, although several previous studies showed increased expression and activation of PKCε isoform in experimental DN (39, 40). Meier et al. investigated the functional role of

PKCε in renal physiology using PKCε-knockout mice and found a renal phenotype with an elevated occurrence of tubulointerstitial fibrosis and glomerulosclerosis, whereas a systemic profibrotic phenotype was not observed (41). Moreover, they demonstrated an increased level of albuminuria in knockout mice whereas the kidney/body ratio remained normal in comparison to non-diabetic wild type mice, indicating that PKCε is probably less implicated in development of renal hypertrophy. Experiments on diabetic mice further showed that knockout of PKCε can exacerbate the renal phenotype with a significantly increased urinary albumin/creatinine ratio and expression of ECM proteins. These data suggest that a rising level of PKCε has a protective function in kidney injury, rather than inducing profibrotic changes.

The atypical isoforms PKCλ and PKCι are both highly expressed in podocytes. Although it is not clear whether both isotypes perform distinct roles in the cell, aPKCs are well-known to be important in the establishment of cell polarity (42, 43). They form an evolutionarily conserved complex consisting of aPKC and PAR3 and PAR6, two PDZ domain containing scaffold proteins (44). Studies showed that the Par3–Par6–aPKC complex interacts with nephrin–podocin through the direct connection of Par3 to nephrin, an essential structural component for the maintenance of integrity of the glomerular filter as well as for the signal transduction (45). Interestingly, our own studies of the glomerular development in regard to the Par polarity complex and slit diaphragm molecules show that the PAR3–PAR6–aPKCλ complex translocates together with other tight junction proteins like ZO-1 from the apical to the basolateral side of the cell preceding the targeting of slit diaphragm components such as nephrin and podocin to the basal membrane, development of foot processes, and the construction of slit diaphragms (46).

The very recent study of Satoh and colleagues shows the necessity of aPKC in the exocytosis of newly produced nephrin and its localization on the surface of podocytes (47). This finding could also partially explain the diminished nephrin expression in proteinuria patients with diabetes (48). Under high-glucose conditions, activation of aPKC presents a protective effect on the nephrin expression (48).

The activation of aPKC is required for the insulin-induced glucose transport and thus defective aPKC activation in muscle and adipocytes has been shown previously in type II diabetic rats, monkeys, and human beings, which leads to a disturbed glucose uptake into muscle and the whole body glucose transfer (49). The knockout of the other isoform PKCζ has no specific renal phenotype but seems to be able compensate for partial functions of aPKCλ loss as the double-knockout leads to glomerular developmental defects with no secondary foot processes (50). This is most likely because of the regulatory cytoskeletal functions of aPKCs with direct influence on small GTPase activation of aPKCs in podocytes (51). Nevertheless, the role of aPKC in DN still remains unclear.

PKC UP-REGULATED GROWTH FACTOR EXPRESSION IN DIABETIC NEPHROPATHY

Podocytes are the major site of vascular endothelial growth factor (VEGF) production in the human kidney (52), and the expression of VEGF is increased in podocytes in diabetic rats and human beings (53, 54). Thus, the up-regulation of VEGF plays a critical role in the progression of DN. Hoshi et al. have shown that under high-glucose conditions, VEGF expression was up-regulated mediated through activation of PKC and extracellular signal-regulated kinase (ERK) in podocytes (55). PKC and ERK are known to regulate activator protein-1 (AP-1) activation (56–58), which promotes the binding of AP-1 to the promoter region of the VEGF gene (59, 60). In addition, AGEs upregulate VEGF mRNA levels through transcription factors such as NF-κB and AP-1. AGEs are also able to activate PKCs, which further increase the expression of VEGF. Although VEGF is required for normal glomerulogenesis and essential for maintenance of glomerular filtration barrier, podocyte-specific overexpression of $VEGF_{164}$ or $VEGF_{165}$ isoform in animals leads to structural and functional renal changes similar to those abnormalities seen in DN, including proteinuria, glomerular hypertrophy, glomerular basement membrane thickening, mesangial expansion, loss of slit diaphragms, and podocyte effacement (61, 62). In DN, the activation of TGF-β1 has been demonstrated to promote podocyte apoptosis and the development of glomerulosclerosis. A reduced expression level of the profibrotic cytokine TGF-β1 was detected in diabetic $PKC\beta^{-/-}$ mice, while the alteration was not observed in diabetic $PKC\alpha^{-/-}$ mice (20). This observation suggested that PKCβ isoform is more important in the up-regulation of TGF-β and for the development of glomerular fibrosis under diabetic conditions, whereas PKCα shows its critical role in the integrity of glomerular filtration barrier (37). However, PKCα plays a key role in the signaling response after stimulation with TGF-β1. An enhanced and prolonged activation of PI3K/AKT and ERK1/2 as well as a reduced proapoptotic signaling via p38MAPK in PKCα-knockout podocytes compared to wild type podocytes was detected after TGF-β1 treatment, which indicated the involvement of PKCα in the TGF-β mediated apoptosis (63).

Of note, deletion of PKCε signaling not only leads to increased expression of TGF-β1 but also induces activation of the TGF-β1 signaling pathway in glomeruli (41). PKCι might also mediate a high-glucose-induced increase in TGF-β receptor II (TGF-βRII) promoter activity, which leads to the up-regulation of TGF-βRII and fibronectin (64).

PKC REGULATED STRUCTURE PROTEINS IN PODOCYTES

P-cadherin as member of the classical cadherins, a superfamily of glycoproteins, is known to be a basic scaffold for the glomerular slit diaphragm (65). Xu et al. have first demonstrated decreased expression of P-cadherin mRNA and protein in experimental diabetic glomeruli and in high-glucose stimulated podocytes, which suggests that a potential role for P-cadherin loss in the development of proteinuria in early DN (66). They also found that PKC inhibitors could ameliorate the decrement of P-cadherin in podocytes under high-glucose conditions. Thus, activation of PKC regulated pathways seems to be involved in the regulation of P-cadherin expression and contributes to the disruption of podocyte integrity (66).

Nevertheless, it seems likely that the molecular changes of the slit diaphragm complex, but not one single slit diaphragm-associated molecule contribute to the pathogenesis of glomerular filtration barrier in DN. P-cadherin, α-, β-, and γ-catenin, and

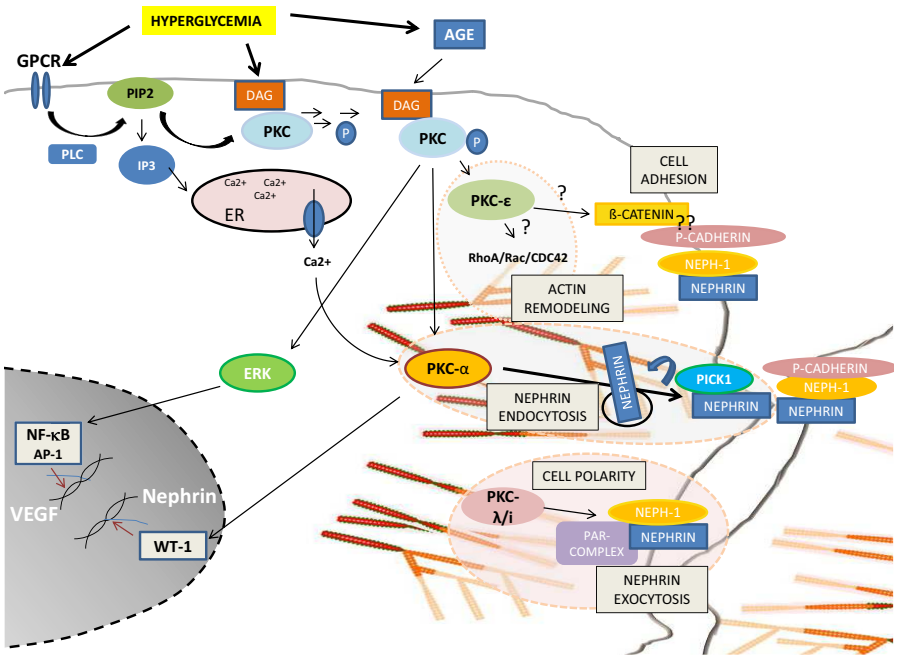

FIGURE 1 | Schematic overview of PKC-isoform functions in podocytes. DAG or calcium activated PKCα induces nephrin endocytosis via the adaptor molecule Pick1. PKCλ/ι is required for foot process formation, cell polarity, and nephrin exocytosis. PKCε might be involved in actin remodeling via small GTPases and might orchestrate cell adhesion and cell–cell contact formation.

ZO-1 are described to compose adherens junctions at the slit diaphragm and establish the link with the actin cytoskeleton (65, 67). However, the role and regulation of β-catenin with P-cadherin and actin cytoskeletal proteins have not been thoroughly explored. In the situation of the podocyte injury induced by high glucose, β-catenin will be released from the destruction complex because of the activation of the Wnt-pathway. Afterwards β-catenin translocates into the nucleus to activate the downstream genes via the aggregation of a transcriptional complex with TCF/LEF, which leads to proteinuria and glomerulosclerosis (68, 69). Several groups demonstrated that PKC activation phosphorylated N-terminal serine residues of β-catenin, which promoted β-catenin degradation (70, 71). In contrast, our ongoing work was focused on several PKC isoforms, whose activation is able to dephosphorylate β-catenin to prevent β-catenin from degradation. It is still unknown, whether this process is protective for the high-glucose-induced cell adherens junction reduction. Nevertheless, PKC activation may be a novel mechanism in regulating β-catenin in glomerular injury, which will be further investigated by our laboratory.

CONCLUSION

Podocyte injury or podocyte loss is a hallmark for the pathogenesis of DN. Based on the above described findings, PKC activation seems to be the most critical pathway involved in the progression of glomerular injury. Obviously, there is a fine balance between activation and inactivation of the different PKC isoforms and their cross-talk involving foot process cytoskeletal architecture, cellular junction formation, and orchestration of turnover and surface expression of slit diaphragm components (**Figure 1**). Therefore, PKCs and their involved pathways are potential therapeutic targets in podocytes to prevent the progression of diabetic glomerulopathy.

REFERENCES

1. Fioretto P, Caramori ML, Mauer M. The kidney in diabetes: dynamic pathways of injury and repair. The Camillo Golgi Lecture 2007. *Diabetologia* (2008) **51**:1347–55. doi:10.1007/s00125-008-1051-7
2. Meyer TW, Bennett PH, Nelson RG. Podocyte number predicts long-term urinary albumin excretion in Pima Indians with type II diabetes and microalbuminuria. *Diabetologia* (1999) **42**:1341–4. doi:10.1007/s001250051447
3. Susztak K, Raff AC, Schiffer M, Bottinger EP. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes* (2006) **55**:225–33. doi:10.2337/diabetes.55.01.06.db05-0894
4. Toyoda M, Najafian B, Kim Y, Caramori ML, Mauer M. Podocyte detachment and reduced glomerular capillary endothelial fenestration in human type 1 diabetic nephropathy. *Diabetes* (2007) **56**:2155–60. doi:10.2337/db07-0019
5. Verzola D, Gandolfo MT, Ferrario F, Rastaldi MP, Villaggio B, Gianiorio F, et al. Apoptosis in the kidneys of patients with type II diabetic nephropathy. *Kidney Int* (2007) **72**:1262–72. doi:10.1038/sj.ki.5002531
6. White KE, Bilous RW, Marshall SM, El NM, Remuzzi G, Piras G, et al. Podocyte number in normotensive type 1 diabetic patients with albuminuria. *Diabetes* (2002) **51**:3083–9. doi:10.2337/diabetes.51.10.3083
7. Benzing T. Signaling at the slit diaphragm. *J Am Soc Nephrol* (2004) **15**:1382–91. doi:10.1097/01.ASN.0000130167.30769.55
8. Diez-Sampedro A, Lenz O, Fornoni A. Podocytopathy in diabetes: a metabolic and endocrine disorder. *Am J Kidney Dis* (2011) **58**:637–46. doi:10.1053/j.ajkd.2011.03.035
9. Li J, Gobe G. Protein kinase C activation and its role in kidney disease. *Nephrology (Carlton)* (2006) **11**:428–34. doi:10.1111/j.1440-1797.2006.00673.x
10. Steinberg SF. Structural basis of protein kinase C isoform function. *Physiol Rev* (2008) **88**:1341–78. doi:10.1152/physrev.00034.2007

11. Pears C, Stabel S, Cazaubon S, Parker PJ. Studies on the phosphorylation of protein kinase C-alpha. *Biochem J* (1992) **283**(Pt 2):515–8.
12. Dietrich A, Rose-John S, Marks F. Expression of the kinase domain of mouse protein kinase C in *E. coli*. *Biochem Int* (1989) **19**:163–72.
13. Cazaubon SM, Parker PJ. Identification of the phosphorylated region responsible for the permissive activation of protein kinase C. *J Biol Chem* (1993) **268**:17559–63.
14. Borner C, Filipuzzi I, Wartmann M, Eppenberger U, Fabbro D. Biosynthesis and posttranslational modifications of protein kinase C in human breast cancer cells. *J Biol Chem* (1989) **264**:13902–9.
15. Liu WS, Heckman CA. The sevenfold way of PKC regulation. *Cell Signal* (1998) **10**:529–42. doi:10.1016/S0898-6568(98)00012-6
16. Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL. Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A* (1992) **89**:11059–63. doi:10.1073/pnas.89.22.11059
17. Jakus V, Rietbrock N. Advanced glycation end-products and the progress of diabetic vascular complications. *Physiol Res* (2004) **53**:131–42.
18. Meinhardt G, Roth J, Totok G. Protein kinase C activation modulates pro- and anti-apoptotic signaling pathways. *Eur J Cell Biol* (2000) **79**:824–33. doi:10.1078/0171-9335-00100
19. Lee HB, Yu MR, Yang Y, Jiang Z, Ha H. Reactive oxygen species-regulated signaling pathways in diabetic nephropathy. *J Am Soc Nephrol* (2003) **14**:S241–5. doi:10.1097/01.ASN.0000077410.66390.0F
20. Menne J, Park JK, Boehne M, Elger M, Lindschau C, Kirsch T, et al. Diminished loss of proteoglycans and lack of albuminuria in protein kinase C-alpha-deficient diabetic mice. *Diabetes* (2004) **53**:2101–9. doi:10.2337/diabetes.53.8.2101
21. Menne J, Meier M, Park JK, Boehne M, Kirsch T, Lindschau C, et al. Nephrin loss in experimental diabetic nephropathy is prevented by deletion of protein kinase C alpha signaling in-vivo. *Kidney Int* (2006) **70**:1456–62. doi:10.1038/sj.ki.5001830
22. Tossidou I, Teng B, Menne J, Shushakova N, Park JK, Becker JU, et al. Podocytic PKC-alpha is regulated in murine and human diabetes and mediates nephrin endocytosis. *PLoS One* (2010) **5**:e10185. doi:10.1371/journal.pone.0010185
23. Quack I, Woznowski M, Potthoff SA, Palmer R, Konigshausen E, Sivritas S, et al. PKC alpha mediates beta-arrestin2-dependent nephrin endocytosis in hyperglycemia. *J Biol Chem* (2011) **286**:12959–70. doi:10.1074/jbc.M110.204024
24. Guo G, Morrison DJ, Licht JD, Quaggin SE. WT1 activates a glomerular-specific enhancer identified from the human nephrin gene. *J Am Soc Nephrol* (2004) **15**:2851–6. doi:10.1097/01.ASN.0000143474.91362.C4
25. Wagner N, Wagner KD, Xing Y, Scholz H, Schedl A. The major podocyte protein nephrin is transcriptionally activated by the Wilms' tumor suppressor WT1. *J Am Soc Nephrol* (2004) **15**:3044–51. doi:10.1097/01.ASN.0000146687.99058.25
26. Wang SX, Mene P, Holthofer H. Nephrin mRNA regulation by protein kinase C. *J Nephrol* (2001) **14**:98–103.
27. Benigni A, Gagliardini E, Tomasoni S, Abbate M, Ruggenenti P, Kalluri R, et al. Selective impairment of gene expression and assembly of nephrin in human diabetic nephropathy. *Kidney Int* (2004) **65**:2193–200. doi:10.1111/j.1523-1755.2004.00636.x
28. Langham RG, Kelly DJ, Cox AJ, Thomson NM, Holthofer H, Zaoui P, et al. Proteinuria and the expression of the podocyte slit diaphragm protein, nephrin, in diabetic nephropathy: effects of angiotensin converting enzyme inhibition. *Diabetologia* (2002) **45**:1572–6. doi:10.1007/s00125-002-0946-y
29. Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, et al. Protein kinase C isozymes and the regulation of diverse cell responses. *Am J Physiol Lung Cell Mol Physiol* (2000) **279**:L429–38.
30. Osicka TM, Yu Y, Panagiotopoulos S, Clavant SP, Kiriazis Z, Pike RN, et al. Prevention of albuminuria by aminoguanidine or ramipril in streptozotocin-induced diabetic rats is associated with the normalization of glomerular protein kinase C. *Diabetes* (2000) **49**:87–93. doi:10.2337/diabetes.49.1.87
31. Thallas-Bonke V, Lindschau C, Rizkalla B, Bach LA, Boner G, Meier M, et al. Attenuation of extracellular matrix accumulation in diabetic nephropathy by the advanced glycation end product cross-link breaker ALT-711 via a protein kinase C-alpha-dependent pathway. *Diabetes* (2004) **53**:2921–30. doi:10.2337/diabetes.53.11.2921
32. Meier M, King GL. Protein kinase C activation and its pharmacological inhibition in vascular disease. *Vasc Med* (2000) **5**:173–85. doi:10.1177/1358836X0000500307
33. Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, et al. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science* (1996) **272**:728–31. doi:10.1126/science.272.5262.728
34. Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL. Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest* (1997) **100**:115–26. doi:10.1172/JCI119503
35. Koya D, Haneda M, Nakagawa H, Isshiki K, Sato H, Maeda S, et al. Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *FASEB J* (2000) **14**:439–47.
36. Kelly DJ, Zhang Y, Hepper C, Gow RM, Jaworski K, Kemp BE, et al. Protein kinase C beta inhibition attenuates the progression of experimental diabetic nephropathy in the presence of continued hypertension. *Diabetes* (2003) **52**:512–8. doi:10.2337/diabetes.52.2.512
37. Meier M, Park JK, Overheu D, Kirsch T, Lindschau C, Gueler F, et al. Deletion of protein kinase C-beta isoform in vivo reduces renal hypertrophy but not albuminuria in the streptozotocin-induced diabetic mouse model. *Diabetes* (2007) **56**:346–54. doi:10.2337/db06-0891
38. Menne J, Shushakova N, Bartels J, Kiyan Y, Laudeley R, Haller H, et al. Dual inhibition of classical protein kinase C-alpha and protein kinase C-beta isoforms protects against experimental murine diabetic nephropathy. *Diabetes* (2013) **62**:1167–74. doi:10.2337/db12-0534
39. Kang N, Alexander G, Park JK, Maasch C, Buchwalow I, Luft FC, et al. Differential expression of protein kinase C isoforms in streptozotocin-induced diabetic rats. *Kidney Int* (1999) **56**:1737–50. doi:10.1046/j.1523-1755.1999.00725.x
40. Whiteside CI, Dlugosz JA. Mesangial cell protein kinase C isozyme activation in the diabetic milieu. *Am J Physiol Renal Physiol* (2002) **282**:F975–80. doi:10.1152/ajprenal.00014.2002
41. Meier M, Menne J, Park JK, Holtz M, Gueler F, Kirsch T, et al. Deletion of protein kinase C-epsilon signaling pathway induces glomerulosclerosis and tubulointerstitial fibrosis in vivo. *J Am Soc Nephrol* (2007) **18**:1190–8. doi:10.1681/ASN.2005070694
42. Henrique D, Schweisguth F. Cell polarity: the ups and downs of the Par6/aPKC complex. *Curr Opin Genet Dev* (2003) **13**:341–50. doi:10.1016/S0959-437X(03)00077-7
43. Macara IG. Par proteins: partners in polarization. *Curr Biol* (2004) **14**:R160–2. doi:10.1016/j.cub.2004.01.048
44. Suzuki A, Yamanaka T, Hirose T, Manabe N, Mizuno K, Shimizu M, et al. Atypical protein kinase C is involved in the evolutionarily conserved par protein complex and plays a critical role in establishing epithelia-specific junctional structures. *J Cell Biol* (2001) **152**:1183–96. doi:10.1083/jcb.152.6.1183
45. Hartleben B, Schweizer H, Lubben P, Bartram MP, Moller CC, Herr R, et al. Neph-Nephrin proteins bind the Par3-Par6-atypical protein kinase C (aPKC) complex to regulate podocyte cell polarity. *J Biol Chem* (2008) **283**:23033–8. doi:10.1074/jbc.M803143200
46. Huber TB, Hartleben B, Winkelmann K, Schneider L, Becker JU, Leitges M, et al. Loss of podocyte aPKClambda/iota causes polarity defects and nephrotic syndrome. *J Am Soc Nephrol* (2009) **20**:798–806. doi:10.1681/ASN.2008080871
47. Satoh D, Hirose T, Harita Y, Daimon C, Harada T, Kurihara H, et al. aPKClambda maintains the integrity of the glomerular slit diaphragm through trafficking of nephrin to the cell surface. *J Biochem* (2014) **156**:115–28. doi:10.1093/jb/mvu022
48. Doublier S, Salvidio G, Lupia E, Ruotsalainen V, Verzola D, Deferrari G, et al. Nephrin expression is reduced in human diabetic nephropathy: evidence for a distinct role for glycated albumin and angiotensin II. *Diabetes* (2003) **52**:1023–30. doi:10.2337/diabetes.52.4.1023
49. Sajan MP, Farese RV. Insulin signalling in hepatocytes of humans with type 2 diabetes: excessive production and activity of protein kinase C-iota (PKC-iota) and dependent processes and reversal by PKC-iota inhibitors. *Diabetologia* (2012) **55**:1446–57. doi:10.1007/s00125-012-2477-5
50. Hartleben B, Widmeier E, Suhm M, Worthmann K, Schell C, Helmstadter M, et al. aPKClambda/iota and aPKCzeta contribute to podocyte differentiation and glomerular maturation. *J Am Soc Nephrol* (2013) **24**:253–67. doi:10.1681/ASN.2012060582
51. Worthmann K, Leitges M, Teng B, Sestu M, Tossidou I, Samson T, et al. Def-6, a novel regulator of small GTPases in podocytes, acts downstream of

atypical protein kinase C (aPKC) lambda/iota. *Am J Pathol* (2013) **183**:1945–59. doi:10.1016/j.ajpath.2013.08.026
52. Brown LF, Berse B, Tognazzi K, Manseau EJ, Van de Water L, Senger DR, et al. Vascular permeability factor mRNA and protein expression in human kidney. *Kidney Int* (1992) **42**:1457–61. doi:10.1038/ki.1992.441
53. Cha DR, Kim NH, Yoon JW, Jo SK, Cho WY, Kim HK, et al. Role of vascular endothelial growth factor in diabetic nephropathy. *Kidney Int Suppl* (2000) **77**:S104–12. doi:10.1046/j.1523-1755.2000.07717.x
54. Lee EY, Shim MS, Kim MJ, Hong SY, Shin YG, Chung CH. Angiotensin II receptor blocker attenuates overexpression of vascular endothelial growth factor in diabetic podocytes. *Exp Mol Med* (2004) **36**:65–70. doi:10.1038/emm.2004.9
55. Hoshi S, Nomoto K, Kuromitsu J, Tomari S, Nagata M. High glucose induced VEGF expression via PKC and ERK in glomerular podocytes. *Biochem Biophys Res Commun* (2002) **290**:177–84. doi:10.1006/bbrc.2001.6138
56. Janknecht R, Ernst WH, Houthaeve T, Nordheim A. C-terminal phosphorylation of the serum-response factor. *Eur J Biochem* (1993) **216**:469–75. doi:10.1111/j.1432-1033.1993.tb18165.x
57. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* (1995) **270**:16483–6. doi:10.1074/jbc.270.28.16483
58. Karin M, Liu Z, Zandi E. AP-1 function and regulation. *Curr Opin Cell Biol* (1997) **9**:240–6. doi:10.1016/S0955-0674(97)80068-3
59. Shima DT, Kuroki M, Deutsch U, Ng YS, Adamis AP, D'Amore PA. The mouse gene for vascular endothelial growth factor. Genomic structure, definition of the transcriptional unit, and characterization of transcriptional and post-transcriptional regulatory sequences. *J Biol Chem* (1996) **271**:3877–83.
60. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* (1991) **266**:11947–54.
61. Veron D, Reidy KJ, Bertuccio C, Teichman J, Villegas G, Jimenez J, et al. Overexpression of VEGF-A in podocytes of adult mice causes glomerular disease. *Kidney Int* (2010) **77**:989–99. doi:10.1038/ki.2010.64
62. Liu E, Morimoto M, Kitajima S, Koike T, Yu Y, Shiiki H, et al. Increased expression of vascular endothelial growth factor in kidney leads to progressive impairment of glomerular functions. *J Am Soc Nephrol* (2007) **18**:2094–104. doi:10.1681/ASN.2006010075
63. Tossidou I, Starker G, Kruger J, Meier M, Leitges M, Haller H, et al. PKC-alpha modulates TGF-beta signaling and impairs podocyte survival. *Cell Physiol Biochem* (2009) **24**:627–34. doi:10.1159/000257518
64. Chuang LY, Guh JY, Liu SF, Hung MY, Liao TN, Chiang TA, et al. Regulation of type II transforming-growth-factor-beta receptors by protein kinase C iota. *Biochem J* (2003) **375**:385–93. doi:10.1042/BJ20030522
65. Reiser J, Kriz W, Kretzler M, Mundel P. The glomerular slit diaphragm is a modified adherens junction. *J Am Soc Nephrol* (2000) **11**:1–8.
66. Xu ZG, Ryu DR, Yoo TH, Jung DS, Kim JJ, Kim HJ, et al. P-Cadherin is decreased in diabetic glomeruli and in glucose-stimulated podocytes in vivo and in vitro studies. *Nephrol Dial Transplant* (2005) **20**:524–31. doi:10.1093/ndt/gfh642
67. Hendriksen J, Jansen M, Brown CM, van der Velde H, van HM, Galjart N, et al. Plasma membrane recruitment of dephosphorylated beta-catenin upon activation of the Wnt pathway. *J Cell Sci* (2008) **121**:1793–802. doi:10.1242/jcs.025536
68. Kato H, Gruenwald A, Suh JH, Miner JH, Barisoni-Thomas L, Taketo MM, et al. Wnt/beta-catenin pathway in podocytes integrates cell adhesion, differentiation, and survival. *J Biol Chem* (2011) **286**:26003–15. doi:10.1074/jbc.M111.223164
69. Dai C, Stolz DB, Kiss LP, Monga SP, Holzman LB, Liu Y. Wnt/beta-catenin signaling promotes podocyte dysfunction and albuminuria. *J Am Soc Nephrol* (2009) **20**:1997–2008. doi:10.1681/ASN.2009010019
70. Gwak J, Cho M, Gong SJ, Won J, Kim DE, Kim EY, et al. Protein-kinase-C-mediated beta-catenin phosphorylation negatively regulates the Wnt/beta-catenin pathway. *J Cell Sci* (2006) **119**:4702–9. doi:10.1242/jcs.03256
71. Raab MS, Breitkreutz I, Tonon G, Zhang J, Hayden PJ, Nguyen T, et al. Targeting PKC: a novel role for beta-catenin in ER stress and apoptotic signaling. *Blood* (2009) **113**:1513–21. doi:10.1182/blood-2008-05-157040

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Diabetes mellitus in the transplanted kidney

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Diabetes mellitus (DM) is the most common cause of chronic kidney disease and end stage renal disease. New onset diabetes mellitus after transplant (NODAT) has been described in approximately 30% of non-diabetic kidney-transplant recipients many years post transplantation. DM in patients with kidney transplantation constitutes a major comorbidity, and has significant impact on the patients and allografts' outcome. In addition to the major comorbidity and mortality that result from cardiovascular and other DM complications, long standing DM after kidney-transplant has significant pathological injury to the allograft, which results in lowering the allografts and the patients' survivals. In spite of the cumulative body of data on diabetic nephropathy (DN) in the native kidney, there has been very limited data on the DN in the transplanted kidney. In this review, we will shed the light on the risk factors that lead to the development of NODAT. We will also describe the impact of DM on the transplanted kidney, and the outcome of kidney-transplant recipients with NODAT. Additionally, we will present the most acceptable data on management of NODAT.

Keywords: diabetes mellitus, diabetic nephropathy, kidney-transplant, podocyte, new onset of diabetes after transplant, suPAR, mTORC1 signaling, B17 receptor

INTRODUCTION

As the epidemic of diabetes mellitus (DM) is growing, diabetic nephropathy (DN) remains the leading cause of end stage renal disease (ESRD) in the American population [US Renal Data System (USRDS) data 2012]. DN and ESRD as its consequence, poses a major healthcare problem especially in developed countries, and as such it represents a great financial burden to the society. In the United States, over 21 million people, or 7% of the general population, are estimated to have diabetes. To date, despite aggressive research conducted in the early diagnosis and treatment of DN, the vast majority of diabetic patients eventually progress to ESRD. For these patients, either dialysis or renal transplantation remain the only options of survival.

Renal transplantation has been considered the therapy of choice in suitable ESRD or pre-ESRD patients since it confers a better survival benefit to these patients compared to dialysis (1–3). Despite renal transplantation addressing the problem of imminent renal failure, DM and DN remain prevalent among kidney-transplant patients leading in some cases to allograft loss and contributing to overall patient's mortality. DM may represent a sequela of the preexisting condition leading to ESRD or can develop as *de novo* after kidney or any other solid organ transplantation in transplant recipients.

Diabetic nephropathy was the etiology of ESRD in approximately 23% of kidney-transplant recipients in the United States in 2008 (4). These numbers unfortunately continue to grow as the number of diabetic patients in the general population increases.

NEW ONSET OF DIABETES MELLITUS AFTER KIDNEY-TRANSPLANT

The incidence of new onset diabetes mellitus after transplant (NODAT) is variable, ranging between 10 and 46% depending on the study design and the definition of NODAT (5–7). More specifically, NODAT has been reported to occur in 4–25% of renal transplant recipients, 2.5–25% of liver transplant recipients, 4–40% of heart transplant recipients, and 30–35% of lung transplant recipients (8–11). In order to establish more precise diagnosis of NODAT, international consensus guidelines defined the criteria for NODAT and these include the following: symptoms of diabetes and random plasma glucose $\geq$200 mg/dL (11.1 mmol/L), fasting plasma glucose $\geq$126 mg/dL (7.0 mmol/L), and 2-h plasma glucose $\geq$200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (8). If at any time point either of these criteria in post transplant patient is met, the diagnosis of NODAT can be established. Levels of glycosylated hemoglobin A1c are unreliable marker of NODAT during the first three to six post transplant months, given the fact that many chronic kidney disease (CKD) stage 5 and ESRD patients are anemic at baseline when receiving a kidney-transplant. Many patients undergo renal and other solid organ transplantation, hence only a subgroup of patients will develop NODAT. It remains poorly understood what predisposes this subgroup of patients to the development of NODAT. The literature describes various modifiable and non-modifiable risk factors for development of NODAT. Non-modifiable risk factors include age, race, genetic background, family history of diabetes, and previous glucose intolerance. Modifiable risk factors are obesity, hepatitis C virus (HCV), cytomegalovirus infections, and immunosuppressive drugs (12, 13). The risk of NODAT development increases with time from transplantation. Therefore, early detection and prompt action are essential in reducing the risk of NODAT and its complications (14).

Among the non-modifiable risk factors age is considered the strongest risk factor for development of NODAT (6, 12, 15). A study by Cosio et al. (7) that included 2078 allograft recipients,

showed that individuals older than 45 were 2.9 times more likely to develop NODAT. Data from the USRDS showed that first kidney-transplant recipients with ages between 45 and 59 years had a relative risk (RR) for NODAT of 1.9 (95% confidence interval (CI) 1.73–2.09; $P < 0.0001$), whereas, individuals ≥60 years had a risk of 2.6 (95% CI 2.32–2.92; $P < 0.0001$) (12, 15). Age increased the risk for development of diabetes 1.5-fold for every 10-year increase in age (12, 16).

RISK FACTORS OF NODAT

Obesity represents a modifiable risk factor and has consistently been shown to be strongly associated with development of NODAT (6, 15, 17). Data from the USRDS revealed that obese patients (BMI ≥30 kg/m^2) have an RR for NODAT of 1.73 (95% CI 1.57–1.90; $P < 0.0001$), this being, along with age, one of the most consistent risk factors (6, 12). The molecular mechanisms of obesity have been studied in detail in the non-transplant population where chronic inflammatory markers synthesis is upregulated. Adipose tissue is known to produce adipocytokines, including leptin, tumor necrosis factor alpha (TNF-α), interleukins, and adiponectin (18, 19). Activation of the TNF-α system has been associated with insulin resistance through the generation of defects in the phosphorylation of the receptor and decreasing the expression of insulin-sensitive glucose transporters. Induction of IL-6 synthesis has been associated with alterations in glucose tolerance and is possibly a predictor of DM2 (19, 20). Adiponectin is a 30-kDa, collagen-like protein synthesized by adipocytes. High adiponectin concentrations have been associated with an independent reduction in the risk of developing type 2 DM in a healthy population (19, 21). In transplant recipients, a low pre-transplant serum concentration of adiponectin was described as an independent risk factor for the development of NODAT (22). A recent study found that for every 1 μg/mL decrease in adiponectin concentration, the risk of developing NODAT is increased by 13% (19).

Race also plays an important role with respect to NODAT development. Even though most of the transplant centers through out the world expose their patients to similar immunosuppressive induction and maintenance protocols, African American and Hispanic patients appear to have a significantly higher risk for development of NODAT compared to Caucasians. Likely, genetic polymorphisms among Black and Hispanic transplant recipients allow for more common disease prevalence compared to their Caucasian counterparts. These polymorphisms remain a subject of research, however precise description is lacking in the transplant literature.

Additionally, infections are also described as culprits of NODAT. Transplanted patients at baseline are more susceptible to infections than the general population. A chronic HCV infection (either acquired peri-operatively from the donor or present in the recipient at the time of transplantation), especially in a setting of uncontrolled viremia, poses a risk factor for NODAT development. The USRDS registry confirmed that the 1-year incidence of NODAT in HCV-positive patients at transplantation was significantly higher compared to the HCV-negative patients (25.6 vs. 15.4%; $P < 0.0001$) (6), clearly demonstrating higher risk for NODAT development in HCV carriers. Recent basic science studies have demonstrated that the HCV elicits an apoptosis-like death of pancreatic beta-cells through endoplasmatic reticulum stress-involved, Caspase 3-Dependent Pathway (23). Interferon has been the drug of choice for treatment of hepatitis C infection in the non-transplant population for several decades. Unfortunately, its use in the HCV infected transplant patients has been widely avoided due to its propensity to elicit acute rejection in the allograft. The novel drugs (protease and nucleotide analog inhibitors) recently released on the market for treatment of HCV infection in non-transplanted patients are still lacking official FDA approval in the transplant cohort. Their use in this cohort has been strictly off label and transplant center dependent. Thus, their prime time for this clinical indication is likely to come in the near future. It would be interesting to see if this will have a positive impact on decreasing the incidence of NODAT in the transplant recipients.

IMMUNOSUPPRESSION DRUGS

In addition to the above mentioned factors influencing NODAT development, several immunosuppressive agents that are commonly used in the transplant arena have been noted to have diabetogenic potential. Herein, commonly used drugs include glucocorticoids, calcineurin inhibitors (CNIs) including tacrolimus (TAC) and cyclosporine (CYC), as well as the mammalian target of rapamycin (mTOR) inhibitors (sirolimus and everolimus). The diabetogenic effect of all of these drugs has been explored in detail and their effect on development of NODAT has been elucidated in several major studies.

Glucocorticoids, as the oldest immunosuppressive agents, have a strongest diabetogenic potential, which is dose dependent. The predominant factor for causing PTDM by corticosteroids seems to be the aggravation of insulin resistance, however, several studies have displayed deleterious effects on insulin secretion and beta-cells apoptosis (24). The precise mechanisms of glucocorticoid-induced insulin resistance are not well understood. *In vivo* and *in vitro* animal studies demonstrate initiation of glucocorticoid related insulin signaling cascade in skeletal muscles, resulting in reduced glucose uptake and glycogen synthesis (25, 26). *In vitro*, the effect of glucocorticoids on β-cell lines has been studied in detail. Glucocorticoids were shown to reduce the expression of GLUT 2 and glucokinase, thereby impairing glucose-stimulated insulin secretion (24, 26). Further, Dexamethasone was shown to stimulate transcription of serum and glucocorticoid-inducible kinase 1, upregulating the activity of voltage-gated K^+ channels and leading to reduced Ca^{2+} entry through voltage-gated Ca^{2+} channels with resultant decreased insulin release (26, 27). In isolated rat islets, dexamethasone decreases the activation of protein kinase C through inhibition of the diacylglycerol–phospholipase C pathway (26, 28).

Additionally, there is some conflicting data regarding the benefit of early corticosteroid withdrawal vs. steroid continuation protocols with respect to NODAT manifestation.

A large randomized controlled study found that early steroid withdrawal does not confer any significant advantage compared to steroid continuation, with the remark that fewer patients with early steroid withdrawal required insulin for NODAT at 5 years [4/107 (3.7%) vs. 10/86 (11.6%), $P = 0.049$] (29). On the other hand, large retrospective study involving more than 25,000 transplant recipients reported significant benefits of early steroid withdrawal

when compared to a steroid-containing regimen with respect to NODAT. The cumulative incidence of NODAT within 3 years post-transplant was 12.3% in steroid-free vs. 17.7% in steroid-containing regimens, $P < 0.001$. Overall, steroid-containing regimens carried a 42% increased risk for NODAT at the time of hospital discharge (30).

Calcineurin inhibitors are also commonly prescribed drugs in the transplant arena. Both CYC and TAC administration correlates strongly with NODAT development, however, TAC appears to have more pronounced diabetogenic effect as demonstrated in prospective and retrospective studies (31, 32). This was observed in kidney, heart, liver, and lung transplants (24). Interestingly, some of the basic science studies involving these agents are not fully supportive of these clinical findings (33).

Calcineurin inhibitors induce NODAT by variety of mechanisms, including decreased insulin secretion and a direct toxic effect on the pancreatic beta-cells. The effect on beta-cells survival implicates the direct effect of CNIs on the nuclear factor of activated T-cell (NFAT) signaling. CNIs regulate the dephosphorylation of (NFAT) protein and CREB (cAMP-responsive element-binding transcription factor) activity-2 (TORC2). The dephosphorylation of these proteins regulates several target genes [insulin, Glut2, (pancreatic and duodenal homeobox 1 (Pdx-1), insulin receptor substrate-2 (Irs2), cyclin D1, cyclin D2, cyclin-dependent kinase 4 (CDK4), etc.], which are critical in β-cell survival, replication, and function. TAC binds intracellularly to FK506-binding protein 1B (FKBP1B) before docking in the calcineurin binding site (Cnb1) of calcineurin, thus inhibiting calcineurin and its downstream pathways and decreasing β-cell replication and survival (26). Moreover, CYC induces inhibition of calcineurin activated leucine zipper-bearing-kinase, leading to beta cell apoptosis (34). Further, mitochondria play a key role in the insulin secretion mainly by providing ATP supply. CYC binds readily to cyclophilin D in the mitochondrial permeability transition pore and blocks the opening of this channel on the mitochondrion and thereby reduces the cytoplasmic free-Ca^{2+} concentration thus interfering with glucose-stimulated insulin secretion (26, 35).

Finally, even though with less frequency, mTOR inhibitors continue to be used among transplant patients despite clinical evidence of their use being associated with greater risks of allograft failure and recipient death compared with a CNI-based regimen (36). Additionally, mTOR inhibitors have been associated with significant risk for NODAT development, especially in combination therapy with TAC, thus with a tendency of sirolimus contributing more to the NODAT development rather than TAC (37).

The effects of sirolimus on the function and survival of β-cells appears paradoxical based on animal studies and vitro studies with cell lines or human islets. *In vitro* sirolimus is noted to increase the insulin content in human islet cells (38) as well the secretion in both basal (50%) and stimulated (40%) states in mini pigs *in vivo* (39). On the other hand, additional *in vitro* studies have shown that sirolimus may facilitate the opening of ATP sensitive potassium channels thereby impairing the insulin secretion (40) in addition to suppressing the glucose-stimulated insulin secretion via direct inhibition of Krebs cycle and decrease of mitochondrial ATP production (41). Further, there is convincing evidence that sirolimus may disrupt regeneration and proliferation of islets, most likely via direct inhibition of the mTOR complex 1 (mTOR C1) signaling and its downstream regulatory effect on cyclin-dependent kinase 4, ultimately leading to reduced cyclin D2 and D3, which are critical regulators of β-cell cycle, proliferation, and mass (38, 42). In summary, the effects of sirolimus on insulin secretion remain the subject of further investigation.

There are no data so far to indicate that mycophenolate and azathioprine are involved in the development of NODAT.

DIABETIC NEPHROPATHY AFTER KIDNEY-TRANSPLANT

Diabetic nephropathy occurs in the transplanted kidney after approximately 5.9 years (43), in patients with pre-transplant DM and those who develop NODAT. In a study of 58 kidney-transplant recipients, 74.1% had history of DM before kidney transplantation and 25.9% had NODAT, of those whom DN histologic findings developed, 69.6% were in patients with history of DM and 30.4% were in NODAT patients. The time from transplantation to the development of DN was slightly longer in NODAT patients (6.68 ± 3.86 vs. 9.93 ± 3.07 years, $P = 0.05$); however, as expected, the duration of diabetes was similar in the two groups at the time of histologic findings of DN (6.68 ± 3.86 vs. 5.90 ± 3.13 years, $P = 0.66$) (43).

The pathological findings of DN post kidney transplantation are in most part similar to those of typical DN in native kidneys. Thickening of GBM and the tubular basement membrane constitutes the first sign of DN. Mesangial matrix expansion develops later on. The extracellular matrix accumulates over time and forms nodular mesangial changes that gradually lead to the compression of the associated glomerular capillaries resulting in glomerular sclerosis and obliteration of capillary lumina. Hyalinosis in the afferent and efferent arteriolar occurs simultaneously with the glomerular lesions leading also to tubulointerstitial chronic changes (44). DN in the transplanted kidney, however, frequently associate with vascular and tubulointerstitial changes caused by allograft rejection, viral infection, or CNI nephrotoxicity, which differentiates it from the DN in the native kidney.

In spite of the extensive data on the pathways that lead to DN in the native kidney, there is still a paucity of such data on DN after kidney transplantation. Although, we believe that the same mechanisms lead to DN in native kidney contribute to the development of post transplant DN, there have been no studies confirming these mechanisms in the transplanted kidney. However, there are few pathways that have been described in the initiating and developing of DN; these may have some significance in the transplanted kidney. Herein, will discuss two of these pathways.

Recently, there have been major changes in our understanding of DN development in the native kidney, with a major focus on the podocyte as the initial site of injury (45), which leads later to the progression of the classic changes of diabetic nodular glomerular sclerosis and interstitial fibrosis.

In a recent study by Fiorina et al., the investigators described the role of podocyte B7-1 in the podocyte injury that results from hyperglycemia (46). The authors found that B7-1 upregulation was induced by hyperglycemia; this upregulation was found to be mediated by activation of the 110-kDa catalytic PI3Kα subunit. Furthermore, the addition of CTLA4-Ig such as abatacept prevented cytoskeleton disruption and adhesion in podocytes that

were exposed to hyperglycemia *in vitro* (46). This data can be of a significant importance in kidney transplantation field. Belatacept, a newer CTLA4-Ig with higher affinity to B7-1 has been approved recently as a maintenance immunosuppressive therapy in kidney-transplant. Hence, it will be of a great interest to evaluate the effect of belatacept in preventing the developing of DN after kidney transplantation.

A second pathway that has been described as a possible contributor to the development and progression of DN in the native kidney is the mTOR. Recent studies suggested that the mTOR pathway of the podocytes plays an important role in the underlying mechanisms of the progression of glomerular diseases (47) and DN (48). In a recent data by Gödel et al., the investigators confirmed that mTOR complex (mTORC) 1 and 2 have crucial roles in the podocyte function. Deletion of mouse podocytes mTORC 1 and 2 induced significant proteinuria and lead to the progression of glomerulosclerosis (48). On the other hand, patients with DN have a significant activation of the podocytes mTOR that associated with early glomerular hypertrophy and hyperfiltration (48). Therefore, there is a potential utilization of mTOR inhibitors such as rapamycin in the prevention of developing DN. Rapamycin has been in use for many years as a maintenance immunosuppressant to prevent kidney-transplant rejection.

A third pathway implies the role of circulating factors that are known to be involved in causing proteinuria. For example, circulating soluble urokinase plasminogen activator receptor uPAR (suPAR) that is known as one of the culprits in native and recurrent Focal and Segmental Glomerulosclerosis (FSGS) has recently been shown to play an active role in patients with DN (49). There, increased suPAR serum levels cause podocyte apoptosis through its association with acid sphingomyelinase-like phosphodiesterase 3b (SMPDL3b) on podocytes. Additional clinical studies further support this concept showing that suPAR is a predictor of proteinuria in patients with DM (50). The neutralization of suPAR may thus be a novel approach to treat DN in the native and possibly also in the transplanted kidney.

MANAGEMENT OF NODAT

The management of NODAT requires a multifaceted approach given that this condition affects multiple organs other than the allograft itself. Other than extensive counseling of pre-transplant patients regarding the higher odds of developing NODAT (especially in high risk groups, as outlined above) and recommendation for implementation of general pre-transplant measures (like weight loss, physical activity, and dietitian referral), most of the transplant centers in the United States have abandoned the screening of transplant recipients with oral glucose tolerance test as this would possibly affect the candidacy for transplantation. Post transplantation screening for NODAT is, however, recommended for all solid organ recipients. Namely, most of the transplant centers would screen the recipients after transplant with weekly fasting glucose testing in the first month after transplantation and continue screening at 5, 6, and 12 months. After this period even though the risk of NODAT is somewhat lower, at least yearly testing with either glycosylated hemoglobin or fasting glucose is recommended.

Early diabetes specialist referral, adequate glycemia control as well as treatment of comorbid conditions remain the backbone of medical approach to this condition. Further, good control of glycemia may even decrease the risk for rejection (51). In patients, who develop DN with overt micro and macroalbuminuria, strict glycemia control in addition to the use of angiotensin inhibitors and statins remains strongly recommended. In transplant patients other than the aforementioned measures extrapolated from the general population, the benefit of decreasing immunosuppression with respect to NODAT prevention and treatment should be carefully weighted against the risk of provoking a rejection in the allograft. Switching from one class of immunosuppressive medication to other should be individualized since the more diabetogenic transplant medications may have other advantages to the longevity of the allograft compared to their competitors.

OUTCOME OF KIDNEY-TRANSPLANT RECIPIENTS WITH NODAT

Even though the two main causes of kidney-transplant loss are chronic allograft nephropathy and death with a functioning allograft, NODAT is strongly associated with impaired patient survival (6, 52) as well as increased cardiovascular mortality (52, 53). Studies demonstrate that kidney-transplant recipients with NODAT are at a two- to three-fold increased risk of fatal and non-fatal cardiovascular disease events as compared with non-diabetic patients (53, 54). In addition, large retrospective registries have concluded that NODAT is a strong, independent predictor of global mortality, graft failure, and death-censored graft failure (6).

The mechanism by which NODAT influences the allograft survival is not well understood, other than predisposing patients to recurrent infections and acute rejection especially when attempts are made to decrease immunosuppression in order to minimize the diabetogenic effect of medications like TAC. In addition to this, NODAT likely contributes to recurrence of DN in the allograft. Similar to the general population, diabetic complications are also commonly encountered in patients with NODAT including ketoacidosis, neurologic, and ophthalmic complications as well as recurrent hypoglycemia and shock (55).

SUMMARY

In summary, NODAT is a common and serious condition that affects the overall health and the survival of a transplant recipient. Inability to control NODAT is associated with significant allograft failure as well as overall transplant recipient morbidity. Strenuous efforts must be undertaken to minimize its detrimental effect on the comorbid conditions that develop as a consequence of NODAT. We are looking forward to newer transplant protocols and medications that do not have the diabetogenic side effects of the ones currently used among the transplant patients and many such are currently underway.

REFERENCES

1. Schnuelle P, Lorenz D, Trede M, Van Der Woude FJ. Impact of renal cadaveric transplantation on survival in end-stage renal failure: evidence for reduced mortality risk compared with hemodialysis during long-term follow-up. *J Am Soc Nephrol* (1998) **9**(11):2135–41.
2. Ojo AO, Port FK, Wolfe RA, Mauger EA, Williams L, Berling DP. Comparative mortality risks of chronic dialysis and cadaveric transplantation in

black end-stage renal disease patients. *Am J Kidney Dis* (1994) **24**(1):59–64. doi:10.1016/S0272-6386(12)80160-0
3. Meier-Kriesche HU, Ojo AO, Port FK, Arndorfer JA, Cibrik DM, Kaplan B. Survival improvement among patients with end-stage renal disease: trends over time for transplant recipients and wait-listed patients. *J Am Soc Nephrol* (2001) **12**(6):1293–6.
4. Gaston RS, Basadonna G, Cosio FG, Davis CL, Kasiske BL, Larsen J, et al. Transplantation in the diabetic patient with advanced chronic kidney disease: a task force report. *Am J Kidney Dis* (2004) **44**(3):529–42. doi:10.1053/j.ajkd.2004.05.030
5. Gunnarsson R, Arner P, Lundgren G, Magnusson G, Ostman J, Groth CG. Diabetes mellitus – a more-common-than-believed complication of renal transplantation. *Transplant Proc* (1979) **11**(2):1280–1.
6. Kasiske BL, Snyder JJ, Gilbertson D, Matas AJ. Diabetes mellitus after kidney transplantation in the United States. *Am J Transplant* (2003) **3**(2):178–85. doi:10.1034/j.1600-6143.2003.00010.x
7. Cosio FG, Pesavento TE, Osei K, Henry ML, Ferguson RM. Post-transplant diabetes mellitus: increasing incidence in renal allograft recipients transplanted in recent years. *Kidney Int* (2001) **59**(2):732–7. doi:10.1046/j.1523-1755.2001.059002732.x
8. Davidson J, Wilkinson A, Dantal J, Dotta F, Haller H, Hernández D, et al. New-onset diabetes after transplantation: 2003 International consensus guidelines. Proceedings of an international expert panel meeting. Barcelona, Spain, 19 February 2003. *Transplantation* (2003) **75**(10 Suppl):SS3–24.
9. Baid S, Cosimi AB, Farrell ML, Schoenfeld DA, Feng S, Chung RT, et al. Post-transplant diabetes mellitus in liver transplant recipients: risk factors, temporal relationship with hepatitis C virus allograft hepatitis, and impact on mortality. *Transplantation* (2001) **72**(6):1066–72. doi:10.1097/00007890-200109270-00015
10. Knobler H, Stagnaro-Green A, Wallenstein S, Schwartz M, Roman SH. Higher incidence of diabetes in liver transplant recipients with hepatitis C. *J Clin Gastroenterol* (1998) **26**(1):30–3. doi:10.1097/00004836-199801000-00009
11. Ye X, Kuo H-T, Sampaio MS, Jiang Y, Bunnapradist S. Risk factors for development of new-onset diabetes mellitus after transplant in adult lung transplant recipients. *Clin Transplant* (2011) **25**(6):885–91. doi:10.1111/j.1399-0012.2010.01383.x
12. Rodrigo E, Fernández-Fresnedo G, Valero R, Ruiz JC, Piñera C, Palomar R, et al. New-onset diabetes after kidney transplantation: risk factors. *J Am Soc Nephrol* (2006) **17**(12 Suppl 3):S291–5. doi:10.1681/ASN.2006080929
13. Kaposztas Z, Gyurus E, Kahan BD. New-onset diabetes after renal transplantation: diagnosis, incidence, risk factors, impact on outcomes, and novel implications. *Transplant Proc* (2011) **43**(5):1375–94. doi:10.1016/j.transproceed.2011.04.008
14. Juan Khong M, Ping Chong C. Prevention and management of new-onset diabetes mellitus in kidney transplantation. *Neth J Med* (2014) **72**(3):127–34.
15. Cosio FG, Kudva Y, van der Velde M, Larson TS, Textor SC, Griffin MD, et al. New onset hyperglycemia and diabetes are associated with increased cardiovascular risk after kidney transplantation. *Kidney Int* (2005) **67**(6):2415–21. doi:10.1111/j.1523-1755.2005.00349.x
16. Gourishankar S, Jhangri GS, Tonelli M, Wales LH, Cockfield SM. Development of diabetes mellitus following kidney transplantation: a Canadian experience. *Am J Transplant* (2004) **4**(11):1876–82. doi:10.1111/j.1600-6143.2004.00591.x
17. Numakura K, Satoh S, Tsuchiya N, Horikawa Y, Inoue T, Kakinuma H, et al. Clinical and genetic risk factors for posttransplant diabetes mellitus in adult renal transplant recipients treated with tacrolimus. *Transplantation* (2005) **80**(10):1419–24. doi:10.1097/01.tp.0000181142.82649.e3
18. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest* (2005) **115**(5):1111–9. doi:10.1172/JCI25102
19. Bayés B, Granada ML, Pastor MC, Lauzurica R, Salinas I, Sanmartí A, et al. Obesity, adiponectin and inflammation as predictors of new-onset diabetes mellitus after kidney transplantation. *Am J Transplant* (2007) **7**(2):416–22.
20. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* (2001) **286**(3):327–34. doi:10.1001/jama.286.3.327
21. Spranger J, Kroke A, Möhlig M, Bergmann MM, Ristow M, Boeing H, et al. Adiponectin and protection against type 2 diabetes mellitus. *Lancet* (2003) **361**(9353):226–8. doi:10.1016/S0140-6736(03)12255-6
22. Bayés B, Lauzurica R, Granada ML, Serra A, Bonet J, Fontseré N, et al. Adiponectin and risk of new-onset diabetes mellitus after kidney transplantation. *Transplantation* (2004) **78**(1):26–30. doi:10.1097/01.TP.0000132561.48217.B1
23. Wang Q, Chen J, Wang Y, Han X, Chen X. Hepatitis C virus induced a novel apoptosis-like death of pancreatic beta cells through a caspase 3-dependent pathway. *PLoS One* (2012) **7**(6):e38522. doi:10.1371/journal.pone.0038522
24. Penfornis A, Kury-Paulin S. Immunosuppressive drug-induced diabetes. *Diabetes Metab* (2006) **32**(5 Pt 2):539–46. doi:10.1016/S1262-3636(06)72809-9
25. Van Raalte DH, Ouwens DM, Diamant M. Novel insights into glucocorticoid-mediated diabetogenic effects: towards expansion of therapeutic options? *Eur J Clin Invest* (2009) **39**(2):81–93. doi:10.1111/j.1365-2362.2008.02067.x
26. Dong M, Parsaik AK, Eberhardt NL, Basu A, Cosio FG, Kudva YC. Cellular and physiological mechanisms of new-onset diabetes mellitus after solid organ transplantation. *Diabet Med* (2012) **29**(7):e1–12. doi:10.1111/j.1464-5491.2012.03617.x
27. Ullrich S, Berchtold S, Ranta F, Seebohm G, Henke G, Lupescu A, et al. Serum- and glucocorticoid-inducible kinase 1 (SGK1) mediates glucocorticoid-induced inhibition of insulin secretion. *Diabetes* (2005) **54**(4):1090–9. doi:10.2337/diabetes.54.4.1090
28. Zawalich WS, Tesz GJ, Yamazaki H, Zawalich KC, Philbrick W. Dexamethasone suppresses phospholipase C activation and insulin secretion from isolated rat islets. *Metabolism* (2006) **55**(1):35–42. doi:10.1016/j.metabol.2005.06.023
29. Woodle ES, First MR, Pirsch J, Shihab F, Gaber AO, Van Veldhuisen P, et al. A prospective, randomized, double-blind, placebo-controlled multicenter trial comparing early (7 day) corticosteroid cessation versus long-term, low-dose corticosteroid therapy. *Ann Surg* (2008) **248**(4):564–77. doi:10.1097/SLA.0b013e318187d1da
30. Luan FL, Steffick DE, Ojo AO. New-onset diabetes mellitus in kidney transplant recipients discharged on steroid-free immunosuppression. *Transplantation* (2011) **91**(3):334–41. doi:10.1097/TP.0b013e318203c25f
31. Vincenti F, Friman S, Scheuermann E, Rostaing L, Jenssen T, Campistol JM, et al. Results of an international, randomized trial comparing glucose metabolism disorders and outcome with cyclosporine versus tacrolimus. *Am J Transplant* (2007) **7**(6):1506–14. doi:10.1111/j.1600-6143.2007.01749.x
32. Heisel O, Heisel R, Balshaw R, Keown P. New onset diabetes mellitus in patients receiving calcineurin inhibitors: a systematic review and meta-analysis. *Am J Transplant* (2004) **4**(4):583–95. doi:10.1046/j.1600-6143.2003.00372.x
33. Øzbay LA, Smidt K, Mortensen DM, Carstens J, Jørgensen KA, Rungby J. Cyclosporin and tacrolimus impair insulin secretion and transcriptional regulation in INS-1E beta-cells. *Br J Pharmacol* (2011) **162**(1):136–46. doi:10.1111/j.1476-5381.2010.01018.x
34. Plaumann S, Blume R, Börchers S, Steinfelder HJ, Knepel W, Oetjen E. Activation of the dual-leucine-zipper-bearing kinase and induction of beta-cell apoptosis by the immunosuppressive drug cyclosporin A. *Mol Pharmacol* (2008) **73**(3):652–9. doi:10.1124/mol.107.040782
35. Düfer M, Krippeit-Drews P, Lembert N, Idahl LA, Drews G. Diabetogenic effect of cyclosporin A is mediated by interference with mitochondrial function of pancreatic B-cells. *Mol Pharmacol* (2001) **60**(4):873–9.
36. Isakova T, Xie H, Messinger S, Cortazar F, Scialla JJ, Guerra G, et al. Inhibitors of mTOR and risks of allograft failure and mortality in kidney transplantation. *Am J Transplant* (2013) **13**(1):100–10. doi:10.1111/j.1600-6143.2012.04281.x
37. Johnston O, Rose CL, Webster AC, Gill JS. Sirolimus is associated with new-onset diabetes in kidney transplant recipients. *J Am Soc Nephrol* (2008) **19**(7):1411–8. doi:10.1681/ASN.2007111202
38. Vodenik B, Rovira J, Campistol JM. Mammalian target of rapamycin and diabetes: what does the current evidence tell us? *Transplant Proc* (2009) **41**(6 Suppl):S31–8. doi:10.1016/j.transproceed.2009.06.159
39. Radu RG, Fujimoto S, Mukai E, Takehiro M, Shimono D, Nabe K, et al. Tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity. *Am J Physiol Endocrinol Metab* (2005) **288**(2):E365–71. doi:10.1152/ajpendo.00390.2004
40. Fuhrer DK, Kobayashi M, Jiang H. Insulin release and suppression by tacrolimus, rapamycin and cyclosporin A are through regulation of the ATP-sensitive potassium channel. *Diabetes Obes Metab* (2001) **3**(6):393–402. doi:10.1046/j.1463-1326.2001.00150.x
41. Shimodahira M, Fujimoto S, Mukai E, Nakamura Y, Nishi Y, Sasaki M, et al. Rapamycin impairs metabolism-secretion coupling in rat pancreatic islets

by suppressing carbohydrate metabolism. *J Endocrinol* (2010) **204**(1):37–46. doi:10.1677/JOE-09-0216
42. Balcazar N, Sathyamurthy A, Elghazi L, Gould A, Weiss A, Shiojima I, et al. mTORC1 activation regulates beta-cell mass and proliferation by modulation of cyclin D2 synthesis and stability. *J Biol Chem* (2009) **284**(12):7832–42. doi:10.1074/jbc.M807458200
43. Bhalla V, Nast CC, Stollenwerk N, Tran S, Barba L, Kamil ES, et al. Recurrent and de novo diabetic nephropathy in renal allografts. *Transplantation* (2003) **75**(1):66–71. doi:10.1097/00007890-200301150-00012
44. Fioretto P, Mauer M. Histopathology of diabetic nephropathy. *Semin Nephrol* (2007) **27**(2):195–207. doi:10.1016/j.semnephrol.2007.01.012
45. Estacio RO, Schrier RW. Diabetic nephropathy: pathogenesis, diagnosis, and prevention of progression. *Adv Intern Med* (2001) **46**:359–408.
46. Fiorina P, Vergani A, Bassi R, Niewczas MA, Altintas MM, Pezzolesi MG, et al. Role of podocyte B7-1 in diabetic nephropathy. *J Am Soc Nephrol* (2014) **25**(7):1415–29. doi:10.1681/ASN.2013050518
47. Mori H, Inoki K, Masutani K, Wakabayashi Y, Komai K, Nakagawa R, et al. The mTOR pathway is highly activated in diabetic nephropathy and rapamycin has a strong therapeutic potential. *Biochem Biophys Res Commun* (2009) **384**(4):471–5. doi:10.1016/j.bbrc.2009.04.136
48. Gödel M, Hartleben B, Herbach N, Liu S, Zschiedrich S, Lu S, et al. Role of mTOR in podocyte function and diabetic nephropathy in humans and mice. *J Clin Invest* (2011) **121**(6):2197–209. doi:10.1172/JCI44774
49. Yoo TH, Pedigo CE, Guzman J, Correa-Medina M, Wei C, Villarreal R, et al. Sphingomyelinase-like phosphodiesterase 3b expression levels determine podocyte injury phenotypes in glomerular disease. *J Am Soc Nephrol* (2014). doi:10.1681/ASN.2013111213
50. Theilade S, Lyngbaek S, Hansen TW, Eugen-Olsen J, Fenger M, Rossing P, et al. Soluble urokinase plasminogen activator receptor levels are elevated and associated with complications in patients with type 1 diabetes. *J Intern Med* (2014). doi:10.1111/joim.12269
51. Schiel R, Heinrich S, Steiner T, Ott U, Stein G. Long-term prognosis of patients after kidney transplantation: a comparison of those with or without diabetes mellitus. *Nephrol Dial Transplant* (2005) **20**(3):611–7. doi:10.1093/ndt/gfh657
52. Fernández-Fresnedo G, Escallada R, de Francisco ALM, Rodrigo E, Zubimendi JA, Ruiz JC, et al. Posttransplant diabetes is a cardiovascular risk factor in renal transplant patients. *Transplant Proc* (2003) **35**(2):700. doi:10.1016/S0041-1345(03)00605-5
53. Hjelmesaeth J, Hartmann A, Leivestad T, Holdaas H, Sagedal S, Olstad M, et al. The impact of early-diagnosed new-onset post-transplantation diabetes mellitus on survival and major cardiac events. *Kidney Int* (2006) **69**(3):588–95. doi:10.1038/sj.ki.5000116
54. Ojo AO. Cardiovascular complications after renal transplantation and their prevention. *Transplantation* (2006) **82**(5):603–11. doi:10.1097/01.tp.0000235527.81917.fe
55. Burroughs TE, Swindle J, Takemoto S, Lentine KL, Machnicki G, Irish WD, et al. Diabetic complications associated with new-onset diabetes mellitus in renal transplant recipients. *Transplantation* (2007) **83**(8):1027–34. doi:10.1097/01.tp.0000259617.21741.95

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Non-immunologic actions of calcineurin inhibitors in proteinuric kidney diseases

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Diseases affecting the glomerulus are the most common cause of end-stage kidney disease in developed countries (1). These disorders are characterized by significant proteinuria, and the level of proteinuria is an independent risk factor for disease progression (2). Podocytes are thought to play a key role in the pathogenesis of glomerular diseases (3, 4). The importance of podocytes in glomerular diseases is highlighted by genetic studies, which have identified mutant podocyte proteins that cause familial forms of nephrosis (5). Because podocytes are terminally differentiated cells with little capacity for replication, their ability to compensate for podocyte loss is limited (3). A component of current therapy is, therefore, focused on reducing podocyte injury by decreasing systemic blood pressure (BP) and inhibition of the renin–angiotensin system (2, 6).

Historically, the immune system was thought to play a significant role in non-genetic forms of nephrosis including acquired diseases such as minimal-change disease (MCD) and focal segmental glomerulosclerosis (FSGS) (7). As a result, corticosteroids and CNIs are often used to treat these disorders (6). Indeed, the response to steroid therapy is an important prognostic indicator for both MCD and FSGS (8, 9). Recent studies, however, suggest that these agents may have actions that are independent of their immunosuppressive properties. For example, while not a universal finding (10, 11), steroids and/or CNIs are reported to induce partial or complete remissions of proteinuria in a subset of patients with genetic forms of nephrosis (7, 11, 12). Although we acknowledge that these reports have significant limitations, the data support the concept that that steroids and/or CNIs may have beneficial effects unrelated to their immunosuppressive actions. Similarly, CNIs inhibit death of cultured podocytes after apoptotic stimuli despite the absence of immune effector mechanisms in the tissue culture model (13, 14). Moreover, genetic activation of the CN effector NFAT (nuclear factor of activated T cells) in podocytes promotes proteinuria, glomerulosclerosis, and a decrease in podocyte numbers in mice despite restricting the experimental manipulation to glomerular podocytes (15).

As shown in **Figure 1**, non-immunological actions of CNIs can be broadly divided into effects on the podocyte cytoskeleton and effects on podocyte survival. A seminal observation was that the actin-associated protein synaptopodin (SYN) was phosphorylated by either protein kinase A (PKA) or calcium/calmodulin-dependent protein kinase II (CaMKII). Phosphorylation of SYN provided a docking site for 14–3–3 proteins and prevented degradation of SYN by the cysteine proteinase cathepsin L (16). Dephosphorylation of the 14–3–3 docking site by calcium sensitive phosphatase CN promoted SYN degradation. This group further demonstrated that SYN competitively antagonized ubiquitination of Rho A by Smurf1 (SMAD specific E3 ubiquitin protein ligase 1), and promoted Rho A activation and stress fiber formation (17). While Rho A activity is also stimulated by calcium-dependent mechanisms (18, 19), the SYN dependence of these effects appeared relevant to glomerular diseases because expression of a degradation resistant SYN in podocytes protected mice from proteinuric stimuli (16). In this scenario, CNIs promote a podocyte phenotype that is resistant to the development of proteinuria by stabilizing the actin cytoskeleton.

CNIs also protect podocytes from apoptotic stimuli (13, 14). At least one mechanism is dependent on gene transcription induced by NFAT (13, 14). NFAT transcription factors were originally discovered in cells of the lymphoid lineage, but abundant evidence indicates that NFAT isoforms are expressed in non-immune cells with some family members expressed ubiquitously (20). In quiescent cells, NFAT isoforms are phosphorylated and located in the cytoplasm (20). CN dephosphorylates NFAT, which permits translocation to the nucleus and stimulation of gene transcription. In cultured podocytes, expression of a constitutively active CN construct causes apoptosis, and this apoptotic effect is blocked by the pharmacologic CNI FK506 as well as by a peptide inhibitor of CN termed VIVIT (13). Similarly, hyperglycemia induces nuclear localization of NFAT isoforms as well as promotes apoptosis of cultured podocytes, and this apoptotic effect is also attenuated by VIVIT (14). Moreover, CN activity is enhanced in kidneys of diabetic rodents (13, 21), and treatment with FK506 attenuates hyperglycemia-induced podocyte apoptosis in diabetic mice (13). Because VIVIT specifically inhibits CN-dependent NFAT activation (22), these data suggest that CN causes podocyte apoptosis by mechanisms that require NFAT mediated gene transcription. In this regard, TRPC6 (transient receptor potential channel C6) is an important gene target of NFAT transcription factors (23). Indeed, gain-of-function mutations

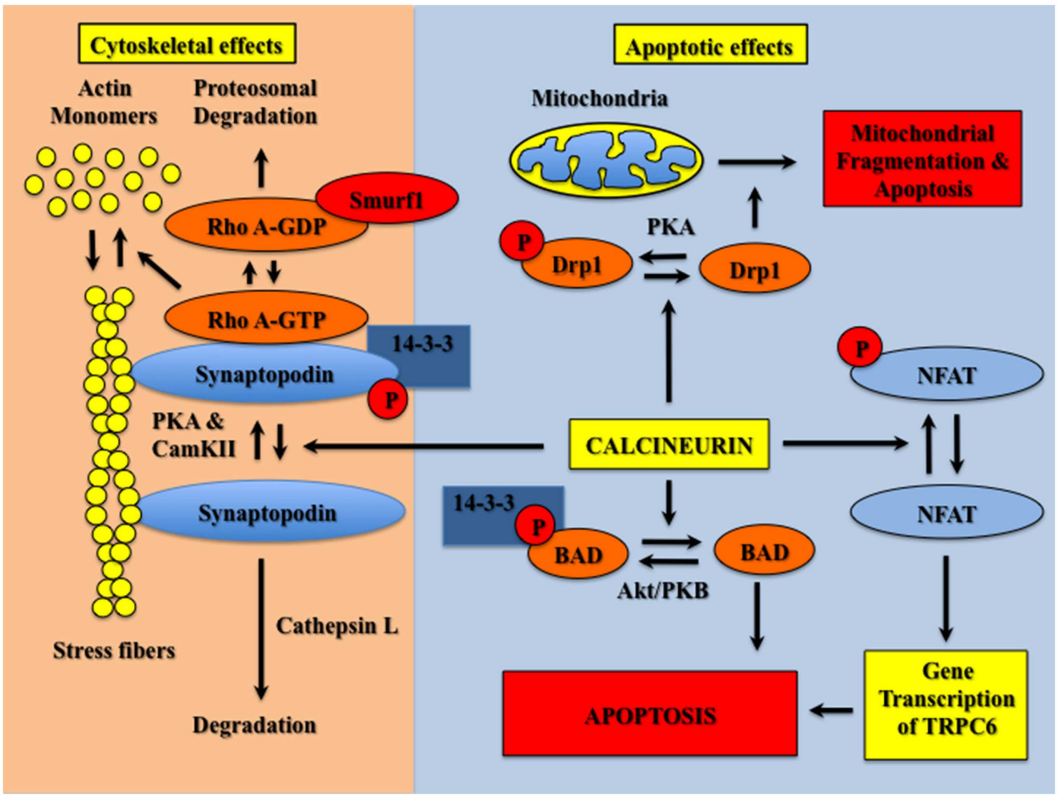

FIGURE 1 | CN activation destabilizes the actin cytoskeleton and causes podocyte apoptosis. Phosphorylation of SYN is mediated by PKA and CamKII. Phosphorylated SYN promotes 14–3–3 binding, which protects SYN from degradation by cathepsin L. SYN also binds Rho A, and competitively inhibits binding of Rho A to the ubiquitin ligase Smurf1, which prevents targeting of Rho A for proteasomal degradation. Binding of Rho A to SYN activates Rho A (GTP bound Rho A) and, in turn, induces stress fiber formation and stabilizes the podocyte cytoskeleton. CN dephosphorylates the 14–3–3 docking site in SYN and promotes SYN degradation by cathepsin L. In the absence of SYN, Rho A is targeted for proteosomal degradation, which reduces stress fiber formation and destabilizes the actin cytoskeleton. CN also promotes podocyte apoptosis by dephosphorylation of either NFAT isoforms, Drp1 or BAD. This apoptotic effect is mediated both directly by Drp1- or BAD-dependent activation of mitochondrial apoptotic pathways, as well as indirectly by stimulation of NFAT-dependent gene transcription.

in TRPC6 cause FSGS (24, 25). TRPC6 is also up-regulated in primary glomerular diseases (26) and over-expression of TRPC6 in podocytes causes proteinuric kidney disease (27). Thus, TRPC6 may be an important downstream gene target of CN signaling in glomerular disorders. In contrast, one study reported that CNIs induced podocyte apoptosis (28). This report, however, is controversial, and we and others (7) have not been able to reproduce this observation.

As shown in **Figure 1**, other mechanisms of CN-mediated podocyte apoptosis include induction of mitochondrial fragmentation by Drp1 (dynamin related protein 1) as well as activation of the apoptosis inducing Bcl-2 family member BAD (Bcl-2 associated death promoter). Drp1 is phosphorylated and inhibited by PKA (29); BAD is phosphorylated by Akt, which causes sequestration of BAD by 14–3–3 proteins and inhibits apoptosis (30). CN dephosphorylates both proteins and induces apoptotic cell death through the mitochondrial pathway (29, 30). Both Drp1 and BAD have been implicated in the pathogenesis of glomerular diseases by promoting podocyte apoptosis (31–33), with the extent of apoptosis presumably dependent on the relative activities of CN and the relevant kinases. Based on these observations as well as the NFAT-dependent apoptotic effects described above, we speculate that CNIs might be useful therapies for attenuating podocyte apoptosis in diseases with either enhanced CN activity or in diseases associated with reduced activity of the relevant kinase.

In summary, CNIs may have important beneficial effects for both the podocyte cytoskeleton and podocyte viability. These agents attenuate podocyte apoptosis as well as promote a podocyte phenotype that is resistant to the development of proteinuria. The beneficial effects of CNIs may be mediated by mechanisms that are independent of the immune system. Given the potential role of CN in diverse glomerular diseases, the use of CNIs might be useful for a broader range of kidney disorders. We acknowledge that CNI nephrotoxicity is a concern (34), but the development of more specific agents with fewer off-target effects (35, 36) may be an effective strategy for expanding the use of CN inhibition to a broader range of glomerular disease processes.

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REFERENCES

1. Collins AJ, Foley RN, Chavers B, Gilbertson D, Herzog C, Johansen K, et al. United States renal data system 2011 annual data report: atlas of chronic kidney disease & end-stage renal disease in the United States. *Am J Kidney Dis* (2012) **59**(1 Suppl 1):e1–420. doi:10.1053/j.ajkd.2011.11.015
2. Jafar TH, Stark PC, Schmid CH, Landa M, Maschio G, Marcantoni C, et al. Proteinuria as a modifiable risk factor for the progression of non-diabetic renal disease. *Kidney Int* (2001) **60**(3):1131–40. doi:10.1046/j.1523-1755.2001.0600031131.x
3. Kriz W, Gretz N, Lemley KV. Progression of glomerular diseases: is the podocyte the culprit? *Kidney Int* (1998) **54**(3):687–97. doi:10.1046/j.1523-1755.1998.00044.x
4. Shankland SJ. The podocyte's response to injury: role in proteinuria and glomerulosclerosis. *Kidney Int* (2006) **69**(12):2131–47. doi:10.1038/sj.ki.5000410
5. Winn MP, Daskalakis N, Spurney RF, Middleton JP. Unexpected role of TRPC6 channel in familial nephrotic syndrome: does it have clinical implications? *J Am Soc Nephrol* (2006) **17**(2):378–87. doi:10.1681/ASN.2005090962
6. Hogan J, Mohan P, Appel GB. Diagnostic tests and treatment options in glomerular disease: 2014 update. *Am J Kidney Dis* (2014) **63**(4):656–66. doi:10.1053/j.ajkd.2013.09.019
7. Schonenberger E, Ehrich JH, Haller H, Schiffer M. The podocyte as a direct target of immunosuppressive agents. *Nephrol Dial Transplant* (2011) **26**(1):18–24. doi:10.1093/ndt/gfq617
8. Waldman M, Crew RJ, Valeri A, Busch J, Stokes B, Markowitz G, et al. Adult minimal-change disease: clinical characteristics, treatment, and outcomes. *Clin J Am Soc Nephrol* (2007) **2**(3):445–53. doi:10.2215/CJN.03531006
9. Schwartz MM, Evans J, Bain R, Korbet SM. Focal segmental glomerulosclerosis: prognostic implications of the cellular lesion. *J Am Soc Nephrol* (1999) **10**(9):1900–7.
10. Ruf RG, Lichtenberger A, Karle SM, Haas JP, Anacleto FE, Schultheiss M, et al. Patients with mutations in NPHS2 (podocin) do not respond to standard steroid treatment of nephrotic syndrome. *J Am Soc Nephrol* (2004) **15**(3):722–32. doi:10.1097/01.ASN.0000113552.59155.72
11. Buscher AK, Kranz B, Buscher R, Hildebrandt F, Dworniczak B, Pennekamp P, et al. Immunosuppression and renal outcome in congenital and pediatric steroid-resistant nephrotic syndrome. *Clin J Am Soc Nephrol* (2010) **5**(11):2075–84. doi:10.2215/CJN.01190210
12. Bensman A, Niaudet P. Non-immunologic mechanisms of calcineurin inhibitors explain its antiproteinuric effects in genetic glomerulopathies. *Pediatr Nephrol* (2010) **25**(7):1197–9. doi:10.1007/s00467-010-1469-2
13. Wang L, Chang JH, Paik SY, Tang Y, Eisner W, Spurney RF. Calcineurin (CN) activation promotes apoptosis of glomerular podocytes both in vitro and in vivo. *Mol Endocrinol* (2011) **25**(8):1376–86. doi:10.1210/me.2011-0029
14. Li R, Zhang L, Shi W, Zhang B, Liang X, Liu S, et al. NFAT2 mediates high glucose-induced glomerular podocyte apoptosis through increased Bax expression. *Exp Cell Res* (2013) **319**(7):992–1000. doi:10.1016/j.yexcr.2013.01.007
15. Wang Y, Jarad G, Tripathi P, Pan M, Cunningham J, Martin DR, et al. Activation of NFAT signaling in podocytes causes glomerulosclerosis. *J Am Soc Nephrol* (2010) **21**(10):1657–66. doi:10.1681/ASN.2009121253
16. Faul C, Donnelly M, Merscher-Gomez S, Chang YH, Franz S, Delfgaauw J, et al. The actin cytoskeleton of kidney podocytes is a direct target of the antiproteinuric effect of cyclosporine A. *Nat Med* (2008) **14**(9):931–8. doi:10.1038/nm.1857
17. Asanuma K, Yanagida-Asanuma E, Faul C, Tomino Y, Kim K, Mundel P. Synaptopodin orchestrates actin organization and cell motility via regulation of RhoA signalling. *Nat Cell Biol* (2006) **8**(5):485–91. doi:10.1038/ncb1400
18. Vassiliadis J, Bracken C, Matthews D, O'Brien S, Schiavi S, Wawersik S. Calcium mediates glomerular filtration through calcineurin and mTORC2/Akt signaling. *J Am Soc Nephrol* (2011) **22**(8):1453–61. doi:10.1681/ASN.2010080878
19. Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, et al. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* (2004) **6**(11):1122–8. doi:10.1038/ncb1183
20. Horsley V, Pavlath GK. NFAT: ubiquitous regulator of cell differentiation and adaptation. *J Cell Biol* (2002) **156**(5):771–4. doi:10.1083/jcb.200111073
21. Gooch JL, Barnes JL, Garcia S, Abboud HE. Calcineurin is activated in diabetes and is required for glomerular hypertrophy and ECM accumulation. *Am J Physiol Renal Physiol* (2003) **284**(1):F144–54. doi:10.1152/ajprenal.00158.2002
22. Aramburu J, Yaffe MB, Lopez-Rodriguez C, Cantley LC, Hogan PG, Rao A. Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science* (1999) **285**(5436):2129–33. doi:10.1126/science.285.5436.2129
23. Kuwahara K, Wang Y, McAnally J, Richardson JA, Bassel-Duby R, Hill JA, et al. TRPC6 fulfills a calcineurin signaling circuit during pathologic cardiac remodeling. *J Clin Invest* (2006) **116**(12):3114–26. doi:10.1172/JCI27702
24. Winn MP, Conlon PJ, Lynn KL, Farrington MK, Creazzo T, Hawkins AF, et al. A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science* (2005) **308**(5729):1801–4. doi:10.1126/science.1106215
25. Reiser J, Polu KR, Moller CC, Kenlan P, Altintas MM, Wei C, et al. TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. *Nat Genet* (2005) **37**(7):739–44. doi:10.1038/ng1592
26. Moller CC, Wei C, Altintas MM, Li J, Greka A, Ohse T, et al. Induction of TRPC6 channel in acquired forms of proteinuric kidney disease. *J Am Soc Nephrol* (2007) **18**(1):29–36. doi:10.1681/ASN.2006091010
27. Krall P, Canales CP, Kairath P, Carmona-Mora P, Molina J, Carpio JD, et al. Podocyte-specific overexpression of wild type or mutant trpc6 in mice is sufficient to cause glomerular disease. *PLoS One* (2010) **5**(9):e12859. doi:10.1371/journal.pone.0012859
28. Fornoni A, Li H, Foschi A, Striker GE, Striker LJ. Hepatocyte growth factor, but not insulin-like growth factor I, protects podocytes against cyclosporin A-induced apoptosis. *Am J Pathol* (2001) **158**(1):275–80. doi:10.1016/S0002-9440(10)63966-1
29. Cribbs JT, Strack S. Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. *EMBO Rep* (2007) **8**(10):939–44. doi:10.1038/sj.embor.7401062
30. Shibasaki F, Hallin U, Uchino H. Calcineurin as a multifunctional regulator. *J Biochem* (2002) **131**(1):1–15. doi:10.1093/oxfordjournals.jbchem.a003063
31. Wang W, Wang Y, Long J, Wang J, Haudek SB, Overbeek P, et al. Mitochondrial fission triggered by hyperglycemia is mediated by ROCK1 activation in podocytes and endothelial cells. *Cell Metab* (2012) **15**(2):186–200. doi:10.1016/j.cmet.2012.01.009
32. Huber TB, Hartleben B, Kim J, Schmidts M, Schermer B, Keil A, et al. Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Mol Cell Biol* (2003) **23**(14):4917–28. doi:10.1128/MCB.23.14.4917-4928.2003
33. Yu SY, Qi R. Role of bad in podocyte apoptosis induced by puromycin aminonucleoside. *Transplant Proc* (2013) **45**(2):569–73. doi:10.1016/j.transproceed.2012.07.160
34. Naesens M, Kuypers DR, Sarwal M. Calcineurin inhibitor nephrotoxicity. *Clin J Am Soc Nephrol* (2009) **4**(2):481–508. doi:10.2215/CJN.04800908
35. Yu H, van Berkel TJ, Biessen EA. Therapeutic potential of VIVIT, a selective peptide inhibitor of nuclear factor of activated T cells, in cardiovascular disorders. *Cardiovasc Drug Rev* (2007) **25**(2):175–87. doi:10.1111/j.1527-3466.2007.00011.x
36. Noguchi H, Matsushita M, Okitsu T, Moriwaki A, Tomizawa K, Kang S, et al. A new cell-permeable peptide allows successful allogeneic islet transplantation in mice. *Nat Med* (2004) **10**(3):305–9. doi:10.1038/nm994

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Use and isolation of urinary exosomes as biomarkers for diabetic nephropathy

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Diabetes represents a major threat to public health and the number of patients is increasing alarmingly in the global scale. Particularly, the diabetic kidney disease (nephropathy, DN) together with its cardiovascular complications cause immense human suffering, highly increased risk of premature deaths, and lead to huge societal costs. DN is first detected when protein appears in urine (microalbuminuria). As in other persisting proteinuric diseases (like vasculitis) it heralds irreversible damage of kidney functions up to non-functional (end-stage) kidney and ultimately calls for kidney replacement therapy (dialysis or kidney transplantation). While remarkable progress has been made in understanding the genetic and molecular factors associating with chronic kidney diseases, breakthroughs are still missing to provide comprehensive understanding of events and mechanisms associated. Non-invasive diagnostic tools for early diagnostics of kidney damage are badly needed. Exosomes – small vesicular structures present in urine are released by all cell types along kidney structures to present with distinct surface assembly. Furthermore, exosomes carry a load of special proteins and nucleic acids. This "cargo" faithfully reflects the physiological state of their respective cells of origin and appears to serve as a new pathway for downstream signaling to target cells. Accordingly, exosome vesicles are emerging as a valuable source for disease stage-specific information and as fingerprints of disease progression. Unfortunately, technical issues of exosome isolation are challenging and, thus, their full potential remains untapped. Here, we review the molecular basis of exosome secretion as well as their use to reveal events along the nephron. In addition to novel molecular information, the new methods provide the needed accurate, personalized, non-invasive, and inexpensive future diagnostics.

Keywords: exosomes, extracellular vesicles, urine, diabetic nephropathy, podocyte

BACKGROUND

Diabetes, chronic kidney disease (CKD), and cardiovascular disease (CVD), [DCC] are significant causes of morbidity and excess mortality and with an alarmingly increasing global trend. Together, they constitute the DCC-disease complex that accounts for major human suffering and already more than 15% of the economic burden on all Healthcare systems (1, 2) Consequently, there are ever increasing academic, industrial, and societal investment needs in combating this worsening epidemic.

The DCC complex shares key symptoms (e.g., microalbuminuria, high cholesterol, and elevated blood pressure) and risk factors (e.g., genetic traits, exercise, and dietary deficiencies) (3, 4). Many lines of evidence show that an early common denominator for DCC diseases is malfunction of the kidney (glomerular) filtration barrier and, particularly, its epithelial component, the podocyte. Recent milestone discoveries have revealed molecules, mechanisms, and pathways of the podocyte, which associate directly with in the onset and progression of the disease (5, 6).

The alarming statistics of DCC highlights the importance of understanding the molecular and cellular pathways thereof. Important advances in understanding the mechanisms leading to the loss of kidney function have been made. Accordingly, abnormal glucose level is known to distort several molecular pathways like the renin–angiotensin system (RAS), inflammatory cytokines (transforming growth factor β (TGF-β) and tumor necrosis factor-α (TNF-α)), protein kinase C (PKC), and the Janus kinase pathway and, oxidative stress mediators, such as nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase). All of these

Abbreviations: Alix, ALG-2 interacting protein; AMBP, alpha-1-microglobulin/bikunin precursor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio] -1-propanesulfonic; CHMP4C, charged multivesicular body protein 4C; CKD, chronic kidney disease; CR1, complement receptor 1; CVD, cardiovascular diseases; DCC, diabetes chronic complications; DN, diabetic nephropathy; DNA, deoxyribonucleic acid; DPPIV, dipeptidyl peptidase IV; DTT, dithiothreitol; ESCRT, endosomal sorting complex required for transport; EV, extracellular vesicles; GPI, glycosylphosphatidylinositol; HD, hydrostatic dialysis; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; HSA, human serum albumin; ILV, intraluminal vesicles; LBPA – lysobisphosphatidic acid; miRNA, micro ribonucleic acids; MLL3, histone-lysine N-methyltransferase; MVB, multivesicular body; MWCO, molecular weight cutoff; NEP, nephrilysin; Oli-neu cells, oligodendroglial cell line; PLP, proteolipid protein; Pmel17, melanosomal protein; RCF, g – relative centrifugal force; SEC, size exclusion chromatography; siRNA, silencing ribonucleic acids; STAM1, signal transducing adapter molecule 1; THP, Tamm–Horsfall protein; TSG101, tumor susceptibility gene 101; UMOD, uromodulin; VDAC1, voltage-dependent anion-selective channel protein 1; VPS4B, vacuolar protein sorting 4 homolog B; VTA1, vacuolar protein sorting-associated protein; WT-1, Wilm's tumor-1 protein.

have been associated with pathogenesis of diabetic nephropathy (DN) [for dedicate review see Ref. (7–9)] while, the clinical management of patients to protect the kidney function in diabetes are not satisfactory (10, 11). Therefore, more accurate early diagnostics and better disease management is needed.

A key consideration relates to the availability of clinically relevant biomarkers for personalized disease management. Notably, the value of albumin excretion rate to urine, presently an important indication of CKD, is decreased by the following observations. First, diabetic patients presenting with microalbuminuria already show advanced damage in kidney filtration barrier associated with disease progression (10, 11). Second, one-third of diabetic patients developed diabetic kidney damage (nephropathy; DN) soon after the onset of microalbuminuria, and this was not dependent on the presence of relevant proteinuria (12). Finally, the albumin assay methodology in use has shown serious flaws for the presence of fragmented and biochemically modified albumin (12). Furthermore, up to 10% of general public shows occasional albumin leakage into urine.

EXTRACELLULAR VESICLES: BIOGENESIS, CLASSIFICATION, AND FUNCTIONS

Extracellular vesicles (see **Figure 1**) are spherical structures composed with a lipid bilayer carrying a cargo of proteins and nucleic acids, released by cells into extracellular space. This term comprises different classes of particles, which had been assigned by van der Pol and colleagues into three major groups: exosomes, microvesicles (MV), and apoptotic bodies (13). This classification subjects all membrane derived vesicles (shed vesicles, exosome-like vesicles, membrane particles, ectosomes, and retrovirus vesicles) into one group and endosomal pathway derived exosomes into other group. The distinction is not perfect as viruses can hijack Multivesicular Body (MVB) pathway and occur also inside of exosomes (14). Apoptotic blebs constitute a separate class as their presence is not associated with a continuously living cell, but with programed cell death process. Although more comprehensive classification based e.g., on size, density, sedimentation force, or vesicle-group specific biomarkers exists, these properties also overlap between EVs populations (15).

Biogenesis of exosomes includes inward budding of vesicles into endosomal lumen to become MVBs fusing with the cell membrane and simultaneously releasing exosomes into extracellular space (**Figure 1**) (16). Unlike the vesicles shedding directly from cell membrane, exosomes contain distinct cargo, closely mirroring the inner compartments of their cells of origin.

Frequently presented exosome diameter ranges from 40 to 100 nm and up to 150 nm (13, 15, 17). Unfortunately, this wide range includes a variety of other EV classes and cannot be considered as a solid identifier. The well-characterized pathway of exosome biogenesis includes proteins of the ESCRT complex (the

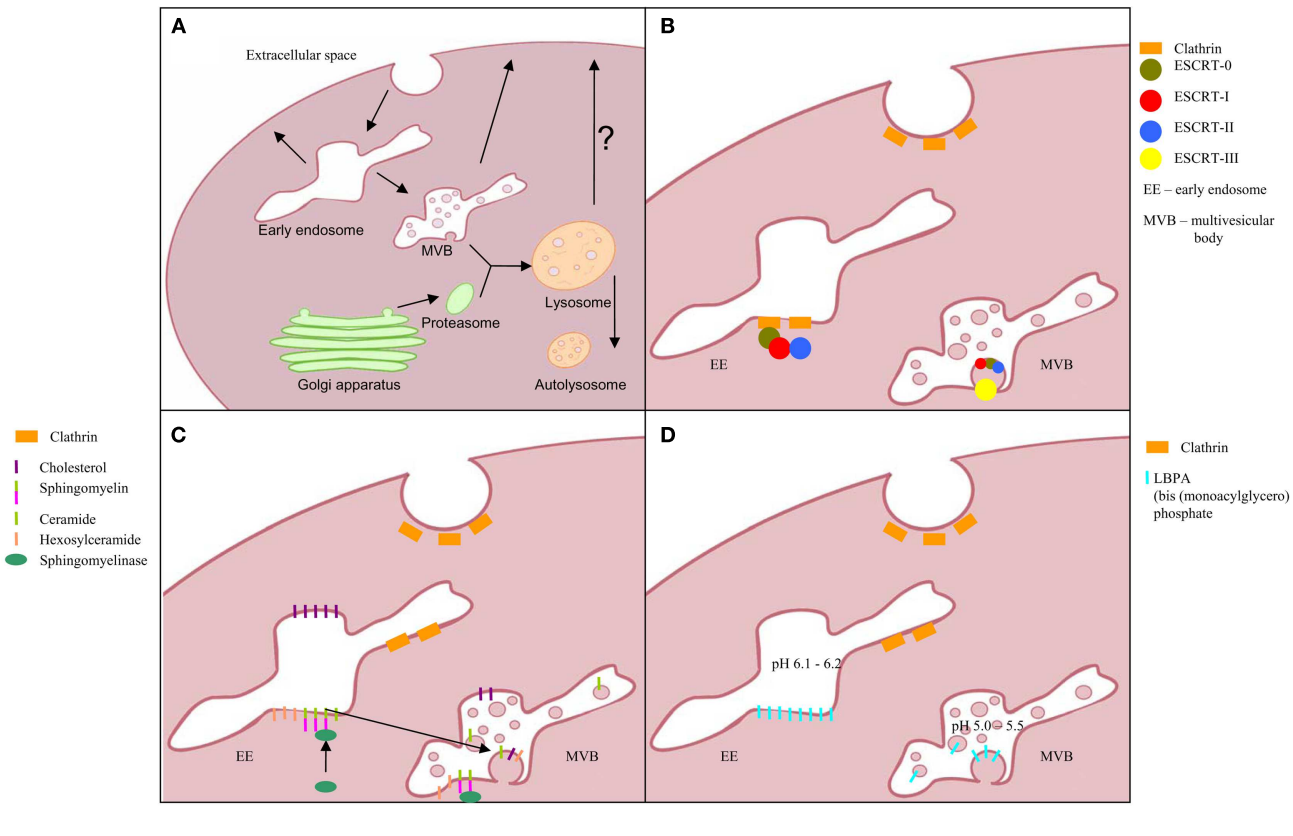

FIGURE 1 | Exosomes biogenesis; (A) multivesicular body formation; intraluminal vesicles formation pathway based on: (B) ESCRT complex involvement, (C) transformation of sphingomyelin into ceramide, and (D) triggered by phospholipid lysobisphosphatidic acid (LBPA) in acidic pH.

endosomal sorting complex required for transport), which is also shared by lysosomal pathway of degradation. Ubiquitinated proteins are recruited by proteins of this complex and clustered in intraluminal vesicles (ILV, future exosomes) within an endosome to yield MVB (16).

Proteins associated with this pathway are grouped into four main complexes (ESCRT-0, -I, -II, and -III) and a class of accessory components (ALIX). ESCRT-0 is built by STAM1 and Hrs, which recognize the clathrin presence on the surface of early endosome as well as the ubiquitin on degradation-directed proteins. Hrs interact with Tumor susceptibility gene 101 (TSG101), an ESCRT-I protein and thus hires this complex. Together with ESCRT-II, ESCRT-I is responsible for membrane deformation into buds, whereas, ESCRT-III recruited by ALIX is required for scission of the bud neck. For more detailed description, the recent comprehensive reviews of ESCRT machinery and role of each protein (16, 18, 19) should be consulted.

This pathway clearly describes the mechanism of directing and anchoring protein cargo into ILV as well as the vesicle formation. Interestingly, considering importance of ESCRT machinery in exosome biogenesis, only 7 from 23 of ESCRT genes after silencing in HeLa-CIITA-OVA cells presented significant influence on this pathway. Furthermore, knock-down of four of these (ESCRT-III complex proteins CHMP4C, VPS4B, VTA1, and ALIX) induced increase in exosome release (20). Thus, alternative pathways of ILV formation have been proposed. Trajkovic and colleagues described a ceramide-dependent process of ILV inward budding in mouse oligodendroglial cell line (Oli-neu cells) (21). In these cells, ceramide is taken from microdomains of early endosomal membranes by the action of sphingomyelinases and lead to fusion of microdomains into larger domains favoring a domain-induced budding. However, the study was focused on membrane trafficking of the proteolipid protein (PLP) and thus do not describe the mechanism of cargo entrapping in ILVs. This pathway seems to yield the vesicles for extracellular release as the PLP is found in exosomes. Alternative pathway suggests a role for lysobisphosphatidic acid (LBPA) in ILV formation. However, lack of LBPA in released vesicles may indicate the determination of lysosomal degradation pathway or different mechanism of ILV formation in which LBPA is involved indirectly (18, 22). Another ESCRT-independent pathway is suggested by Theos et al. (23). In their publication, a ubiquitin-independent mechanism of directing cargo into ILV was postulated using the melanosomal protein Pmel17 as a thus contrasting the proposed ceramide or LBPA pathway.

In the literature, there is disagreement over the next steps after ILV formation within the MVBs. Accordingly, fusion with lysosome/proteasome before directing the MVB to the plasma membrane has been proposed. This could explain the presence of a variety of lysosome specific proteins (proteasome subunits, cathepsin E and B, kallikrein 1, DPPIV, lipoprotein lipase, prolyl 4-hydroxylase, transmembrane protease, serine carboxypeptidase I, and lysosomal-associated membrane protein 1 and 2) within EVs. On the other hand, urinary extracellular vesicle (UEV) protein databases contain a number of molecules of apoptotic blebs (e.g., histones) and MV in general (e.g., cholesterol and diacylglycerol) (24, 25). This suggests that any EV populations may in fact be highly heterogeneous. This raises a real need to better define exosomes as those in diameter <100 nm recovered in pellet 200,000 g.

Term "*microvesicles*" includes all structures budding directly from the cell membrane by rearrangement of the lipid bilayer, especially in phospholipid distribution and with help of cytoskeletal proteins (26). Full mechanism of vesicles budding from plasma membrane is not well known. Nonetheless, direct involvement of some ESCRT machinery proteins like TSG101 into process of vesicle shedding not related with hijack performed by viruses has been presented. Those results are coming from mammalian cell cultures (HeLa, HEK293T, and A549) and from *C. elegans* embryos, suggesting more wide-spread mechanism (22, 27). This once again raises the question whether TSG101 is suitable as exosome-specific marker.

Apoptotic bodies are released into extracellular space as the consequence of apoptosis – the programed cell death. Their diameter range varies from 20–500 nm to 1,000–5,000 nm (15, 28). In this biological process, activated by p53 and caspases, the apoptotic cell is splitting into multiple apoptotic blebs, which are autolyzed by release of lysosomal content or phagocytozed without inflammatory reaction (29). DNA degradation in the nucleus is characteristic for this process and, thus, histones associated with nucleic acid can be used as biomarkers for apoptotic blebs. However, a great number of histone fragments have also been found in exosomes (24, 25).

International Society for extracellular vesicles (EVs) emphasized in 2012 that most EV preparations are heterogeneous and also contain membrane budding vesicles and apoptotic bodies. Thus, an urge for standardized protocol has been expressed (30).

Importance of exosomes increased after discover of their role in cell-to-cell communication. Although it is a function reserved for exosomes, there are also publications describing membrane derived vesicles as a special type of messengers (15) Thus, vesicles released by one cell, can be taken up by other distal cell (31) and the respective contents recognized as useful and important message (32). Of special note is that microRNAs of vesicle cargo can directly affect processes in the target cell by silencing specific genes. Moreover, vesicles can transport not only to neighboring cells but also on relatively long distance (33). It is intriguing to speculate that in the special case of kidney nephrons, substantial distant communication between the epithelial cells can take place (see **Figure 2**). This distant signaling is used by healthy donor-cells as well as cancer cells, making it an efficient benefit for cancer progression and tumor expansion (32). On the other hand, promising data are presented on using vesicles as treatment of human cells *in vitro*. Silencing RNAs (siRNAs) were thus introduced into recovered exosomes, and exposed to HeLa and HT1080 cells *in vitro*, efficiently silencing targeted genes (34).

Thus, being EVs carrier of information a relative long list of functions has been attributed to them depending on the cell (tissue) type specificity. Characterization of exosomes secreted from B-lymphocytes and dendritic cells showed that these vesicles may carry the full set of proteins to induce an immunoresponse (35–37). Moreover, EVs isolated from various body fluids and stored blood can induce the production *in vitro* of inflammatory cytokines like interleukin (IL)-1, tumor necrosis factor α (TNF-α), IL-23a for example (38, 39). In addition to exosomes, also MV have

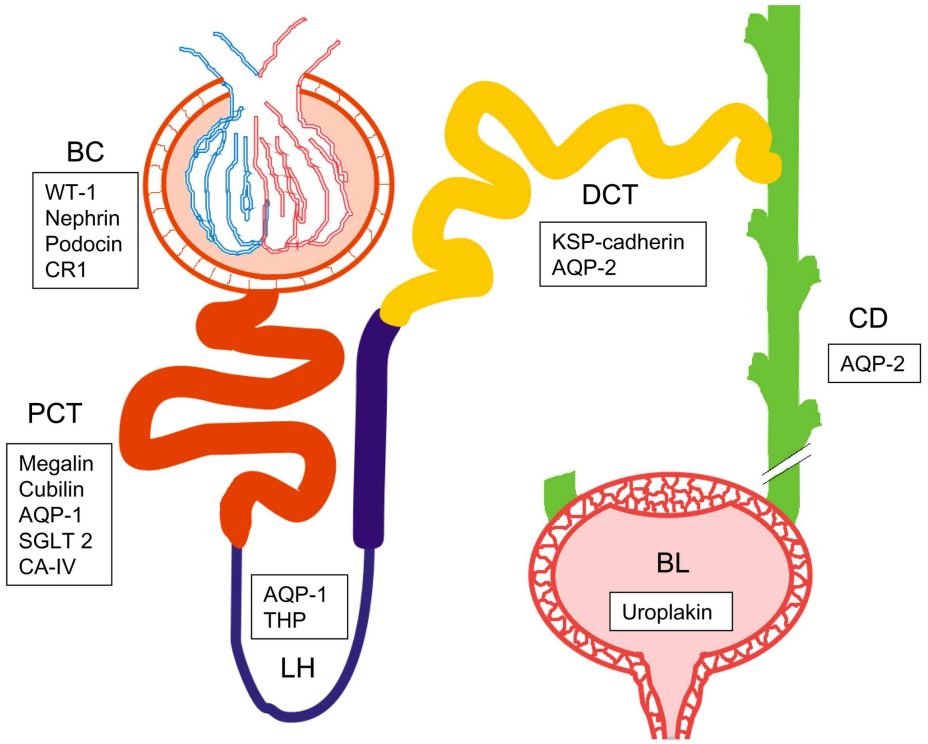

FIGURE 2 | Schematic picture of the urogenital route with one nephron and bladder. Bowman's capsule (BC), proximal convoluted tubule (PCT), loop of Henle (LH), distal convoluted tubule (DCT), collecting duct (CD), and bladder (BL). List of specific biomarkers for each nephron segment are presented in frames. WT-1, Wilm's tumor 1; CR1, complement receptor 1; AQP1, aquaporin 1; SGLT2, sodium-glucose linked transporter 2; CA-IV, carbonic anhydrase IV; THP, Tamm-Horsfall protein; KSP-cadherin, kidney specific cadherin; AQP2, aquaporin 2.

been proposed to serve as mediators of inflammation (40). Interestingly, MV concentration increases in diseases involving vascular and microvascular organ injury like diabetes (41–43). In addition to inflammation, MVs may regulate e.g., the endothelial production of reactive oxygen species as mediated by NADPH oxidase (44, 45). Conversely, exosomes released by cells under oxidative stress can induce neighboring cells to have a higher resistance to ROS, therefore becoming more tolerant to the damage of oxidative stress (46). Interestingly, very recently Gildea and colleagues (47) showed that in their *in vitro* model of exosomes, cell communication between proximal and distal tubules, proximal exosomes could induce distal and proximal duct cells to limit ROS production after stimulation with a dopamine receptor agonist. Thus, not only this report reinforces the concept of cell-to-cell communication and consequently modulator of signal pathway but also extends it to a new level: nephron segment-to-segment communication.

URINARY VESICLES AS POTENTIAL SOURCE FOR BIOMARKERS

One of the first publications describing vesicles in the urine dates back to 1987 when Wiggins et al. described the presence of lipid MV with procoagulant activity (48) in urine of rabbits in nephrotoxic nephritis. This was followed by series of reports by Scherberich (49) to describe shedding of membrane – bound enzymes in a vacuolar membrane morphology (50–500 nm in diameter) from the proximal tubule in patients with kidney injury after administration of potentially nephrotoxic drugs. Later on Pascual et al. characterized vesicles carrying complement receptor 1 (CR1) in urine unequivocally shown to originate from the podocyte (50). Consequently, these were proposed as markers of podocyte injury. However, the interest and follow-up remained low until the first comprehensive description of exosomes in healthy urine by Pisitkun et al. (51). This publication can be considered the milestone in the UEV field, opening possibilities for accurate cell type-specific molecular changes in nephrology and urology.

The unprecedented characterization of the vesicle associated proteins and protein fractions (apparently due to enzymatic degradation within urine) using especially advanced mass spectrometry certainly laid a solid basis for their potential applications in clinical diagnostics and disease follow-up and, consequently, brought UEVs to a wider public awareness. Since then the number of publications exploring urinary exosomes and/or other MV have rapidly and exponentially increased. Furthermore, in addition to focus strictly on exosomes, other types of vesicles have been described in urine including, among others, exosome-like vesicles (52) and shedding vesicles (53). Notably, all the UEV subsets have their distinct intracellular secretory pathways. These are also reflected in the more or less carefully defined isolation protocols, respectively, while their distinct potential for diagnostics-disease management largely overlaps. This emphasizes the need to establish robust protocols to enable UEVs isolation and analysis *in toto* for such purposes.

Proteomic profiling of UEVs has identified a surprising number, abundance, and distinct character of proteins originated from cells all along the nephron epithelium: from the visceral glomerular epithelial cell (podocyte) to the proximal tubule, thick ascending limb of Henle, distal convoluted tubule, and the collecting duct (54–58). Additionally, mass spectrometry analysis has described amino acid sequences originating of proteins of the lower part of the genitourinary path including the urinary bladder and prostate gland (51, 52, 54–58).

Consequently, analysis of urinary exosomes has proven to be an attractive, non-invasive approach to the physiological state of epithelial cells of distinct origin. This clearly offers a true opportunity for future "fluid biopsy" to complement – or up to replacing – information from the invasive kidney biopsies or cystoscopy.

Accordingly, Prunotto et al. (59) were recently able to pinpoint biomarkers and follow the physio-pathological state of specific cell types by enriching and characterizing vesicles released specifically from the podocyte. Interestingly, their almost 1,200 protein identifications discovered 14 previously unidentified urinary proteins. Many of these were subsequently classified (by gene ontology analysis) as brain-specific proteins whose expression in the kidney was then confirmed. Unless these specific proteins are from vesicles gaining direct passage through the kidney glomerular filter, this evidence highlights, once again, similarities between podocyte and set of neuron specific proteins (60). Thus, it seems obvious that exosomes (together with the other vesicular structures found in the urine) will genuinely offer an interesting new approach to dig into the distinct nephron segment-specific molecules and detect changes induced by disease processes (61).

In addition to proteins, UEVs carry a wide range of genetic information molecules like mRNA – encoding proteins native to all nephron regions and, especially small RNA (62, 63) species. While the few targeted methods may show promise of better RNA yields (64–66), in general methods for RNA isolation also need serious optimization. Interestingly, the lipid-bilayered UEVs appear to be protected of the ubiquitous RNases, suggesting that UEVs do have a true physiological role in downstream signaling.

While the finding of the variety of viable RNA species in UEVs is intriguing, the full meaning of this needs to be better understood. It is interesting to speculate again a role for special downstream modulation as a form for distant cell-to-cell communication and, hence, for biomarkers and targets for treatment.

Recently, CD2AP (of the podocyte) specific mRNA has been proposed as biomarker of kidney disease (67). Among the 194 microRNA species thus far reported from exosomes, 45 had appeared to present with significant association to blood pressure modulation (68). Similarly, microRNA-29c in UEVs correlates with renal function and degree of general fibrosis thus emerging as a potential urinary marker of renal fibrosis. Barutta and colleagues recently showed that the expression of miR-130, miR-145, miR-155, and miR-424 were significantly alterated in type 1 diabetic patients with incipient DN (69). This result deserves full validation and additional studies. Finally, in addition to RNA and miRNa, UEVs may serve as carriers of mitochondrial DNA, which has shown to be significantly decreased in DN patients (70) to suggest that an altered bioenergy supply of kidney cells may promote ROS production and localized inflammation both contributing to progress of renal damage [reviewed in Ref. (71)].

For DN, considerable advances have been achieved with biomarkers derived from UEVs. Accordingly, Wilm's tumor-1 (WT-1) protein expression appears to increase with the decline of the renal function in DN (72). Interestingly, urinary WT-1, most likely derived from the podocyte, may thus qualify as a simple marker of podocyte injury rather then a specific marker of DN (73–75).

The amount and activity of dipeptidyl peptidase IV (DPPIV/CD26) in urinary MV coming from proximal tubule cells was shown to positively correlate with progression of DN in type 2 diabetic patients (76) suggesting an early tubular impairment, which may be considered an early marker of renal damage even before the onset of albuminuria.

Zubiri and colleagues (77) carried out a proteomic quantitative analysis on urine samples from DN patients in advanced disease stages (CKD stages III–V). A panel of three potential proteins were included: protein fragment of alpha-1-microglobulin/bikunin precursor (AMBP), isoform 1 of histone-lysine N-methyltransferase (MLL3), and voltage-dependent anion-selective channel protein 1 (VDAC1). These showed differential expression in the samples studied. If verified in larger sample sets, this represents still another promising finding of UEVs as biomarkers.

Finally, very recently in the animal models of DN an increase of MVs secreted from podocytes before the onset of albuminuria (78) was reported. This finding highlights the potential of MV and other UEVs as early markers of glomerular injury.

Furthermore, changes in WT-1 were seen in DN (72), focal segmental glomerulosclerosis, and in minimal change nephropathy (73, 74). Studies performed in other kidney diseases like IgA nephropathy and thin basement membrane nephropathy (TBMN) (79) have identified four candidate proteins (aminopeptidase N, vasorin, ceruloplasmin, and α1 anti-trypsin) associated to UEVs, which appear differently expressed in disease. Among the four protein candidates ceruloplasmin showed highest sensitivity and specificity to distinguish IgA from TBMN. However, for full verification more samples are needed.

Several candidate biomarkers proposed have not followed yet. For example, Fetuin-A has been proposed as predictive biomarkers for acute kidney injury (80) while no further evidence to validate the original finding has been presented. Similarly, aquaporin-1 (AQP-1) was presented as a novel specific urinary biomarker to follow-up ischemia reperfusion injury (81) while further studies are needed for validation.

METHODS FOR EXTRACELLULAR VESICLE AND URINARY EXTRACELLULAR VESICLE ISOLATION

Although the number of publications describing a variety of protein and RNA based potential disease biomarkers is still increasing, there is no consensus of standard protocols for EV isolation.

In most studies of UEVs, the method of differential centrifugation is used for their isolation with the aim to obtain enriched exosomal fraction. However, minor technical variability is often described, which may lead to significant impact on final results (82). Notably, there is no agreement in cell and cell debris removal from either cell culture media or from biofluids. Some studies use

centrifugations with low different relative centrifugal forces (RCF) (lower than 10,000 *g*) whereas, others prefer direct approach with force over 16,000 *g* for cell debris and apoptotic bleb removal (29, 37). Unfortunately, little systematic analytics of the discarded fractions have been published.

Still another point of inconsistency is the ultracentrifugation step in which forces achieved may vary from 100,000 *g* up to 200,000 *g*. In this respect, it is important to note that Thery and colleagues could show that sedimentation point for most types of vesicles may range between 100,000 and 200,000 *g* and highlighted that obtained fractions are heterogeneous (37).

Relative centrifugal force (*g*) given in most publications is describing only force applied on the bottom of vial. Lack of technical information like type of rotor (fixed angle or swing bucket), diameter, volume, and viscosity of the sample do not allow estimated the force affecting upper part of media/biofluid. This leads to variable yield obtained from similar type of samples, which may cause challenges in downstream applications (83) of the material isolated.

In some studies, ultrafiltration is applied *between* differential centrifugation steps. Here also lack of standardization leads to use special filter device with varying porous filter from 0.1 or 0.2 μm up to 0.8 μm (26, 29, 30) and the appropriate moment for use (after low force centrifugation step or directly before ultracentrifugation). Additionally, little systematics has been devoted to lost yield caused by clogs of the filter membrane or variability of exosome size (82). In terms of membrane-derived vesicles, removal at this step is questionable as size of these structures vary from 20 up to 1,000 nm (30, 37), and we do not know if particular disease can have impact on their size distribution.

Lately, commercial products for exosome enrichment have been released (ExoQuick®System Biosciences and Total exosome isolation reagent ®Invitrogen), which allow to omit the ultracentrifugation step (84). It is worth to emphasize that regardless the product names, they are enriching heterogeneous populations of vesicles but their use carry also disadvantages. They are cost-efficient only for very concentrated samples like plasma and, furthermore, these methods are poorly applicable for diluted biofluids (e.g., urine). Additionally, they may cause co-precipitation of most abundant soluble proteins in media/biofluid and thus are not suitable for protein profiling using Mass Spectrometry.

Methods for isolation of UEV are mainly based on differential centrifugation with some modification due to the intrinsic and peculiar characteristics of urine as sample. The first subfraction was reported by Pisitkun et al. (51) while detailed analysis of their efficiency, specificity for the targeted fractions has not been systematically studied. Moreover, efficiency of these methods so as not to lose valuable UEV fractions remains to be defined. Furthermore, as stated above, the true practical importance of obtaining highly pure subpopulations still remains elusive. A major reason for this is that while the intracellular pathways in the formation of various vesicle classes have been well established (20), their contents indiscriminately reflect events interior of the cell. Thus, from the applications point of view to reflect cell type-specific changes, the whole variety of UEVs may be more important to harvest for analysis. As an example, CR1, among the full array of complement pathway-specific proteins has been recognized among the 1,195 protein identifications with the method used (pullout by podocyte-specific antibody) (59). Presently available methods, furthermore, do not distinguish abundance of each of the protein species among the full UEV repertoire leaving opportunity for method improvement. Likewise, the same problem of cross-contamination with UEV subspecies, loss of substantial fractions of specific target vesicles usually occurs when the separation is made on physical characteristics of the vesicles e.g., by their density buoyancy (52, 79, 85–87).

The most abundant proteins in the UEVs include e.g., human serum albumin (or, more precisely, its fragments) and Tamm–Horsfall Protein (THP) (produced in cells of ascending limb of the loop of Henle), Aquaporin-1 (of the proximal tubulus), Aquaporin-2 (distal tubulus and collecting duct), uroplakin (urinary bladder) in addition to apolipoproteins, immunoglobulins, and other soluble proteins (54, 88, 89). Their presence in the whole UEV proteome has been well established and thus, already provide source for nephron segment-specific detailing.

Tamm–Horsfall glycoprotein represents a special case among UEV proteins as it actively forms urinary protein meshworks to entrap a variety of vesicles (89). This may reflect a physiological role for THP to modulate UEV abundance. Consequently, adherence to THP may result in huge differences in the UEV subtype yields if the THP "contaminant" is not removed from urine at the early steps of UEV isolation. It is interesting to note that our recent observations clearly show high fluctuations of daily, intraindividual THP levels in the urine (unpublished data). As THP is secreted mainly from the distal parts of nephron, this could reflect the proposed physiological role of THP to modulate UEV access to more downstream targets. Interestingly, UEVs are known to display a distinct surface proteome (as opposed to their respective "cargo") consisting of specific proteins also capable for receptor binding. Thus, modulating the UEV availability may be constantly in control by other secreted urinary proteins like THP.

The conventional isolation protocol of UEVs starts with a pre-concentration step, followed by series of differential centrifugations or direct ultrafiltration (mostly to reduce the final volume). For density gradients to distinguish UEV subclasses, the same density layer proteins "characteristic" of exosomes (i.e., with marker protein TSG101), MV (marker prominin-1), apoptotic markers (marker histone proteins) along with matrix proteins (collagens, fibronectin fractions), soluble proteins (THP/HSA), or apolipoproteins (E, D, A1, AIV) can be found indiscriminately. Up to now no study has evaluated the possibility of vesicle–matrix, vesicle–soluble protein, vesicle–vesicle, and vesicle–lipoprotein interaction or whether this is a technical artifact caused by the pre-enrichment step. For comprehensive evaluation of UEV usefulness for physiological analytics and not to cross-contaminate them in different density gradient layers, systematics to yield optimized protocols should be achieved. Moreover, no studies have carefully addressed the localization of UEV protein contents themselves: are they present on outer surface or inside the vesicles is important to understand their possible role in distant signaling along the nephron. Our recent results indicate considerable differences in these two UEV domains to suggest of distinct "address tag" and/or effector functions, respectively. Notably, one report

has shown that in urine of patients with light chain amyloidosis, multiple myeloma, and monoclonal gammopathy of undetermined significance (MGUS), light chains were found on the exosome surfaces (90).

BOTTLENECKS OF UEV ISOLATION AND FUTURE ASPECTS

As reported previously, one of the factors limiting wide implementation of UEVs includes the ambiguity of the isolation methods in use. The current golden standard method initially established by Pisitkun et al. (51) includes differential centrifugations followed by treatment of the yield either with a reducing agent (like dithiothreitol) followed by a second ultracentrifugation step (9, 51, 54, 55, 61, 63, 77, 91) or/and by centrifugation in density cushion/gradient (52, 88, 92, 93) or/and by size exclusion chromatography (94) or/and by ultrafiltration devices (9, 62, 64) or/and ExoQuick precipitation reagent (7, 54). All these protocols have their advantages and limitations (**Table 1**) while aim to obtain pure population of vesicles, especially exosomes. At the same time these protocols attempt to eliminate the undesirable interference from THP also known as uromodulin (UMOD). THP forms polymers in urine that sediment during centrifugation and easily entraps vesicles (95). Our results fully recapitulate this problem and call for better solutions to avoid it (manuscript in submission). The most commonly used and fast approach uses a reduction step by dithiothreitol (DTT) to reduce the 24 disulfide bonds of THP in order to increase the yield of vesicles after ultracentrifugation. Unfortunately, this extra step further complicates the UEV isolation process and, despite the de-folding of THP, this still co-sediments with UEVs. In fact such a denaturation step can cause aggregation due to the exposure of hydrophobic domains in an aqueous environment. Furthermore, this leads to alterations in the three-dimensional structure of THP to minimize its free energy by optimized hiding of hydropobic groups and, conversely, exposing as many polar residues as possible in the unnatural, non-urine environment. The hydrophobic groups can also interact with each other to form intermolecular hydrophobic bonds further catalyzing aggregation and co-precipitation with abundant UEVs attached. This mechanism is plausible and was recently exploited to deplete plasma from high abundant disulfide-rich proteins for mass spectrometry analysis (96).

Factors in UEV isolation conditions such as variable pH, temperature, and ionic strength can further influence the ratio of the interaction between hydrophobic residues themselves and/or interaction with the solvent (97). These evidences may directly explain the differences in results obtained and call for better standardized isolation protocols. Our hydrostatic dialysis (HD) method (manuscript in submission) utilizes simple dialysis with defined cut-off membrane directly for the whole void urine and achieves a superior yield of UEVs while simultaneously eliminating THP contamination and, furthermore, standardizes the electrolyte content of the sample. By additionally eliminating the need for ultracentrifugation steps and yielding reasonable volumes e.g., for biobanking purposes at low cost, a very high throughput of samples can be obtained easily.

We have also replaced DTT treatment with a more gentle zwitterionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic (CHAPS) to better preserve the tertiary structure stabilized by disulfide bonds (98). CHAPS is a non-denaturating detergent designed to disrupt non-specific protein interactions and preserving protein conformation (99). Moreover, the average size of CHAPS derived micelles is ~6 kDa and, therefore, it can easily be removed by a simple dialysis step. With the introduction of CHAPS we also manage to preserve the activity of two indicator enzymes associated to UEVs-dipeptidyl dipeptidase IV (DPPIV) and nephrilysin (NEP) (99). Especially, NEP activity benefits of the replacement of DTT to CHAPS. As an obvious explanation for these results, the extracellular domain of NEP contains 12 cysteine residues, forming 6 disulfide bridges. Four of these are located within the catalytic domain while the other two participate in maintaining the structure consisting of two multiply connected folding domains embracing a central cavity containing the active site (100). Evaluation the proteolytic activity of NEP after reduction and re-oxidation of disulfide bridges during dialysis highlights that an incorrect re-folding of the enzyme could have happened leading to an impairment of NEP activity. Therefore, for the aforementioned reasons, CHAPS can be considered an optimized substitute of DTT if remaining physiological activity in the sample is of importance.

However, both UEV treatments, with DTT and CHAPS, did not completely remove the THP interference from the pellet recovered. It is, however, to remember that THP is a glycosylphosphatidylinositol (GPI) anchor protein synthetized exclusively by the epithelial cells at the ascending limb of Henle's loop. It is transported to the apical side of the plasma membrane and released in the tubular lumen by a still undefined protease action [see Rampoldi et al. for more details, (101)].

As a GPI-anchored protein and in such abundance in the urine, THP may be involved in the formation of membrane cargo vesicles and, perhaps, also in the signal transduction by providing a receptor structure for ligands like cytokines (102–105). In support of its involvement as receptor and vesicle trafficking, THP was recently found in basolateral translocation process followed by secretion in the circulation. This was, interestingly, associated with recovery of kidney function in a murine model of ischemic acute kidney injury (106). Thus it should not come as a surprise if THP, as a GPI-anchored protein, is a key constituent of MV shed in the tubular lumen by direct budding of plasma membrane or as secreted through the endosomal pathway. More detailed studies to elucidate these proposed functions are warranted.

Although differential centrifugation is widely used to recover UEVs it has several shortcomings. One major drawback is the less-optimal efficiency of recovery in the distinct pellet fraction (107) but also in the final supernatant. Rotor type, g-forces applied, and centrifugation time significantly affect the final yield (108). Moreover, such a methodology does not allow high throughput of samples.

Alternative methods proposed to avoid these limitations are the use of customized nano-membrane concentrators and microfiltration disk membranes (57, 59, 62, 76, 77, 89, 91, 109, 110). Exosomes and abundant soluble protein fractions bind to the membrane surfaces of these device non-selectively and irrespective of the chemical composition of the filters. This leads to

Table 1 | Overview of the main methods to isolate urinary vesicles.

Reference	Short method description	Advantages	Disadvantages	
Barutta et al. (69) Gildea et al. (47) Kalani et al. (72) Lv et al. (65)	Differential centrifugation Differential centrifugation Differential centrifugation Differential centrifugation	Vesicles enrichment as a pellet	No fixed conditions for: 1. number of centrifugations; 2. relative centrifugation force; 3. time; 4. rotor type; 5. sample volume; 6. temperature during centrifugation; and 7. presence/absence of protease inhibitors	Not applicable for large volume of samples and not suitable for large cohort of patients. Expensive equipment like ultracentrifuge and HPLC are necessary to implement UEVs enrichment
Musante et al. (98)	Differential centrifugation + CHAPS treatment	Protein activity prevention	Labor intensive	
Gonzales et al. (54) Fernandez-Llama et al. (95) Wang et al. (55) Cheng et al. (63)	Differential centrifugation + DTT treatment Differential centrifugation + DTT treatment Differential centrifugation + DTT treatment Differential centrifugation + DTT treatment	Increase of the exosomal yield in the ultracentrifugation pellet from THP interference	Not suitable for protein activity assessment designated samples. Incomplete removal of THP from the ultracentrifugation pellet	
Rood et al. (91)	Differential centrifugation + SEC	Vesicle separation according to size	Labor intensive	
Rood et al. (91) Merchant et al. (89) Miranda et al. (62) Principe et al. (57) Prunotto et al. (59)	Nano-membrane filtration Differential centrifugation + microfiltration Differential centrifugation + nanofiltration Differential centrifugation + ultrafiltration Differential centrifugation + ultrafiltration	Concentration and removal of soluble proteins	Differences in removal of bigger (>0.22 μm or more) vesicles without assessment of their importance for biomarkers screening. Adsorption of vesicles on the surface of ultrafiltration membrane	
Hogan et al. (52) Raimondo et al. (116)	Differential centrifugation + sucrose gradient Differential centrifugation + sucrose gradient	Vesicle separation according to density	Extremely labor intensive	
Ramirez-Alvarado et al. (90)	Ultrafiltration + differential centrifugation	Vesicle separation according to density	Extremely labor intensive	
Zubiri et al. (77) Alvarez (64)	Exoquick Exoquick	No need of ultracentrifugation step. Suitable for extraction of RNA and DNA	Precipitation solution is expensive especially when applied for large volumes of urine and number of samples. For proteomic analysis, extra steps are necessary to remove the interference from precipitating agent	
Musante et al. (unpublished)	Hydrostatic dialysis	Inexpensive, large volume of urine		

excessive obstruction of the nano-membrane. Thus, only smallish volumes of urine can be processed and, therefore, centrifugation still remains the preferential method to enrich and isolate UEVs. However, independently from the method used some common problems remain. First, the volume of urine to be processed is limited while the time required is excessive.

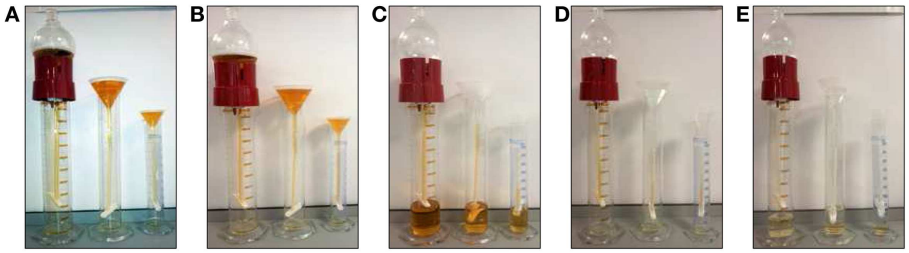

FIGURE 3 | Hydrostatic dialysis systems set up for large (0.5–1 l) medium (200–600 ml) and small volume (10–100 ml) of urine. The dialysis membrane tube (MWCO 1,000 kDa) is connected to the separating funnel and/or a normal funnel. The bottom end is sealed with a universal dialysis tube closure. The separating funnel is filled in with supernatant from 2,000 *g* centrifugation **(A)**. The hydrostatic pressure of urine in funnel pushes the solvent (water) trough the meshwork of dialysis membrane and liquid below the MWCO, which fells to the bottom of the cylinder **(B)**. When urine is concentrated up to 7–8 ml **(C)** the funnel is re-filled with milliQ water (~200 ml equivalent to 25 volumes of concentrated urine), which flushes away all analytes left inside the tube **(D)**. This dialysis step is kept going until the solution is completely clear from yellow pigments (as internal natural control) and concentrated to a desired final volume **(E)**. The whole process has been named hydrostatic dialysis. Finally, it is possible to observe the diffusion of urine from the tube and precipitation at the bottom of the cylinder **(E)**.

We propose that most direct benefit emerging from understanding of EV biology will come from individualized measurements: personalized datasets of efficiency, side-effects, and general performance of medications. Furthermore, it is easy to speculate that simple measurements based on non-invasive sampling of biofluids like urine will prove to be very important in disease progression, prognostics, and disease management in general.

Among the main limitation of working on UEV we would like to emphasize the dilution of the sample, limited amount of starting material (biobanking) and the extreme range of reference interval for analytes (urine is a waste product) responsible of a massive intra- and inter-individual analytical variability (111) In order to overcome such limitations, we recently designed and characterized an alternative approach for vesicle enrichment using basic, cost-efficient laboratory equipment we named our system HD from its operating principle (**Figure 3**).

In HD, urine samples are initially centrifuged at a RCF of 2,000 *g* to remove cells, bacteria, cellular casts, and the bulk of the prevalent THP macropolymers. Thereafter, the resulting supernatant is poured in the dialysis system consisting of a funnel connected with simple dialysis membrane with molecular weight cut-off (MWCO) of 1,000 kDa. The cut-off mol weight is easy to modify if needed for special purposes.

The hydrostatic pressure created by the sheer column of urinary solution is strong enough to push the solvent through the mesh of dialysis membrane together with all constituents, often containing unnecessary contaminants below the selected MWCO. This step simultaneously concentrates the sample, while the funnel is re-filled with deionized water and/or buffer of choice to rinse away all the analytes below the selected MWCO. This step optimizes and standardizes both sample concentration, electrolyte content, and sample volume to be suitable directly e.g., to biobanking of urine even from large volumes of starting material.

Full characterization of this methods revealed that it can enrich UEVs with far superior efficiency and minimal loss of vesicles on the dialysis membrane and in a short time compared to serial centrifugations.

We established that the filtration rate in HD is proportional to the volume of urine contained in the funnel with an average of 75 ml per hour of urine filtrate which takes also into consideration the fact that for large volume of urine (>200 ml) the system may loose some of its efficiency due the adsorption of soluble protein and/or vesicles in the dialysis. We found, however, that for the volume of 200 ml of urine, up to 18% of TSG101 and 45% of THP signal, respectively is adsorbed in the dialysis membrane. However, this method is a gentle way to concentrate first and then dialyze samples. In fact, urinary concentration already takes place at 1 g gravity at atmospheric pressure of sea level with respect to the 3,000 *g* RCF needed for the nano-membrane concentrator with 60 psi of special atmosphere of nitrogen pressure (64, 89, 91).

As urine is by far the simplest biofluid to collect in large quantities for biobanking purposes, our method vastly simplifies the process and practical volumes can easily be achieved for storaging.

However, it is worth to notice that urine in normal condition is a diluted solution and membrane protein most likely associated to UEVs represents an estimated 3% of the urinary proteome (112). Thus, by using HD the void urine can be collected and the whole volume processes soon after the first centrifugation. We have thus far handled up to 500 ml of urine within 24 h without changing in the protein pattern obtained. Moreover, several HD device can be run simultaneously and with minimal costs.

Notably, this new approach also allows normalizing the physical and chemical parameters of the retained solutions by eliminating all the urinary constituents' analytes below the chosen MWCO. As recently reported, different sample compositions have an effect on the viscosity of the samples, which distorts the recovery of vesicles as obtained by differential centrifugation (113). Although urine is a diluted solution with a density and viscosity close to water we noticed that when the tip of the dialysis bag is immersed in water the stream of urine flowing from the tube sank on the bottom of the cylinder. Factors affecting the viscosity of urine include temperature, protein content (114), and hydration status (115). These are of pivotal importance when a comparative analysis between different subjects and patients' samples with different grade of hydration and proteinuria are compared and a differential centrifugation protocol is employed.

In conclusion, although more than a decade has passed, interest and number of publications have been soaring, the simple UEV isolation still meets several challenges to overcome. Urine collection and methodology to enrich vesicles need to be optimized

especially for clinical settings of sample collection and storaging. One particular challenge to overcome includes definition of suitable housekeeping genes and proteins to yield comparable results. Large scale validation of biomarkers derived from UEVs similarly remains to be accomplished. However, it is apparent that comprehensive understanding of the role of UEVs, both their surface proteome and internal cargo molecules and respective roles in distant addressing and cell-to-cell communication even to distant downstream location has started while careful future optimization of protocols and methods as well as standardization are needed.

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REFERENCES

1. Solomon SD, Uno H, Lewis EF, Eckardt KU, Lin J, Burdmann EA, et al. Erythropoietic response and outcomes in kidney disease and type 2 diabetes. *N Engl J Med* (2010) **363**:1146–55. doi:10.1056/NEJMoa1005109
2. Clarke PM, Glasziou P, Patel A, Chalmers J, Woodward M, Harrap SB, et al. Event rates, hospital utilization, and costs associated with major complications of diabetes: a multicountry comparative analysis. *PLoS Med* (2010) **7**:236. doi:10.1376/journal.pmed.1000236
3. Albertson DT, Liu J, Xue JL, Louis TA, Solid CA, Ebben JP, et al. Projecting the number of patients with end-stage renal disease in the United States to the year 2015. *J Am Soc Nephrol* (2005) **16**:3736–41. doi:10.1681/ASN.2005010112
4. de Jong PE, Gansevoort RT. Prevention of chronic kidney disease: the next step forward. *Nephrology* (2006) **11**:240–4. doi:10.1111/j.1440-1797.2006.00575.x
5. Liu S, Hartleben B, Kretz O, Wiech T, Igarashi P, Mizushima N, et al. Autophagy plays a critical role in kidney tubule maintenance, aging and ischemia-reperfusion injury. *Autophagy* (2012) **8**:826–37. doi:10.4161/auto.19419
6. Hartleben B, Widmeier E, Wanner N, Schmidts M, Kim ST, Schneider L, et al. Role of the polarity protein scribble for podocyte differentiation and maintenance. *PLoS One* (2012) **7**:36705. doi:10.1376/journal.pone.0036705
7. Reidy K, Kang HM, Hostetter T, Susztak K. Molecular mechanisms of diabetic kidney disease. *J Clin Invest* (2014) **124**:2333–40. doi:10.1172/JCI72271
8. Badal SS, Danesh FR. New insights into molecular mechanisms of diabetic kidney disease. *Am J Kidney Dis* (2014) **63**:S63–83. doi:10.1053/j.ajkd.2013.10.047
9. Arora MK, Singh UK. Molecular mechanisms in the pathogenesis of diabetic nephropathy: an update. *Vascul Pharmacol* (2013) **58**:259–71. doi:10.1016/j.vph.2013.01.001
10. Perkins BA, Ficociello LH, Roshan B, Warram JH, Krolewski AS. In patients with type 1 diabetes and new-onset microalbuminuria the development of advanced chronic kidney disease may not require progression to proteinuria. *Kidney Int* (2010) **77**:57–64. doi:10.1038/ki.2009.399
11. Viswanathan G. Upadhyay A. Assessment of proteinuria. *Adv Chronic Kidney Dis* (2011) **18**:243–8. doi:10.1053/j.ackd.2011.03.002
12. Drummond K, Mauer M. The early natural history of nephropathy in type 1 diabetes: II. Early renal structural changes in type 1 diabetes. *Diabetes* (2002) **51**:1580–7. doi:10.2337/diabetes.51.5.1580
13. van der Pol E, Boing AN, Harrison P, Struck A, Nieuwland R. Classification, functions and clinical relevance of extracellular vesicles. *Pharmacol Rev* (2012) **64**:676–705. doi:10.1124/pr.112.005983
14. Blot V, Perugi F, Gay B, Prévost MC, Briant L, Tangy F, et al. Nedd4.1-mediated ubiquitination and subsequent recruitment of Tsg101 ensure HTLV-1 Gag trafficking towards the multivesicular in C. Lipid organization in erythrocyte membrane microvesicles. *Biochem J* (1984) **224**:285–90.
15. Regev-Rudzki N, Wilson DW, Carvalho TG, Sisquella X, Coleman BM, Rug M, et al. Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. *Cell* (2013) **153**:1120–33. doi:10.1016/j.cell.2013.04.029
16. Schuh AL, Audhya A. The ESCRT machinery: from the plasma membrane to endosomes and back again. *Crit Rev Biochem Mol Biol* (2014) **49**:242–61. doi:10.3109/10409238.2014.881777
17. Hurley JH, Emr SD. The ESCRT complexes: structure and mechanism of membrane-trafficking network. *Annu Rev Biophys Biomol Struct* (2006) **35**:277–98. doi:10.1146/annurev.biophys.35.040405.102126
18. Gruenberg J, Stenmark H. The biogenesis of multivesicular endosomes. *Nat Rev Mol Cell Biol* (2004) **5**:317–23. doi:10.1038/nrm1360
19. Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci* (2013) **126**:5553–65. doi:10.1242/jcs.128868
20. Akers JC, Gonda D, Kim R, Carter BS, Chen CC. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles and apoptotic bodies. *J Neurooncol* (2013) **113**:1–11. doi:10.1007/s11060-013-1084-8
21. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* (2008) **319**:1244–7. doi:10.1126/science.1153124
22. Nabhan JF, Hu R, Cohen SN, Lu Q. Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. *Proc Natl Acad Sci U S A* (2012) **109**:4146–51. doi:10.1073/pnas.1200448109
23. Theos AC, Truschel ST, Tenza D, Hurbain I, Harper DC, Berson JF, et al. A lumenal domain-dependent pathway for sorting to intralumenal vesicles of multivesicular endosomes involved in organelle morphogenesis. *Dev Cell* (2006) **10**:343–54. doi:10.1016/j.devcel.2006.01.012
24. Mathivanan S, Simpson RJ. ExoCarta: a compendium of exosomal proteins and RNA. *Proteomics* (2009) **9**:4997–5000. doi:10.1002/pmic.200900351
25. Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, et al. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol* (2012) **10**:e1001450. doi:10.1371/journal.pbio.1001450
26. Borges FT, Reis LA, Schor N. Extracellular vesicles: structure, function and potential clinical uses in renal diseases. *Braz J Med Biol Res* (2013) **46**:824–30. doi:10.1590/1414-431X20132964
27. Wehman AM, Poggioli C, Schweinsberg P, Grant BD, Nance J. The P4-ATPase TAT-5 inhibits the budding of extracellular vesicles in *C. elegans* embryos. *Curr Biol* (2011) **21**:1951–9. doi:10.1016/j.cub.2011.10.040
28. Johansson AC, Appelqvist H, Nilsson C, Kågedal K, Roberg K, Ollinger K. Regulation of apoptosis-associated lysosomal membrane permeabilization. *Apoptosis* (2010) **15**:527–40. doi:10.1007/s10495-009-0452-5
29. Crescitelli R, Lässer C, Szabó TG, Kittel A, Eldh M, Dianzani I, et al. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *J Extracell Vesicles* (2013) **2**. doi:10.3402/jev.v2i0.20677
30. Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, et al. Standarization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* (2013) **2**. doi:10.3402/jev.v2i0.20360
31. Ludwig AK, Giebel B. Exosomes: small vesicles participating in intercellular communication. *Int J Biochem Cell Biol* (2012) **44**:11–5. doi:10.1016/j.biocel.2011.10.005
32. Corrado C, Raimondo S, Saieva L, Flugy AM, De Leo G, Alessandro R. Exosome-mediated crosstalk between chronic myelogenous leukemia cells and human bone marrow stromal cells triggers and interleukin 8-dependent survival of leukemia cells. *Cancer Lett* (2014) **348**:71–6. doi:10.1016/j.canlet.2014.03.009
33. Villarroya-Beltri C, Gutiérrez-Vázquez C, Sánchez-Madrid F, Mittelbrunn M. Analysis of microRNA and protein transfer by exosomes during an immune synapse. *Methods Mol Biol* (2013) **1024**:41–51. doi:10.1016/j.canlet.2014.03.009
34. Shtam TA, Kovalev RA, Varfolomeeva EY, Makarov EM, Kil YV, Filatov MV. Exosomes are natural carriers of exogenous siRNA to human cells in vitro. *Cell Commun Signal* (2013) **11**:88. doi:10.1186/1478-811X-11-88
35. Wubbolts R, Leckie RS, Veenhuizen PT, Schwarzmann G, Mobius W, Hoernschemeyer J, et al. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J Biol Chem* (2003) **278**:10963–72. doi:10.1074/jbc.M207550200
36. Clayton A, Court J, Navabi H, Adams M, Mason MD, Hobot JA, et al. Analysis of antigen presenting cell-derived exosomes, through immuno-magnetic isolation and flow cytometry. *J Immunol Methods* (2001) **247**:163–74. doi:10.1016/S0022-1759(00)00321-5
37. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* (2009) **9**:581–93. doi:10.1038/nri2567
38. Bretz NP, Ridinger J, Rupp AK, Rimbach K, Keller S, Rupp C, et al. Body fluid exosomes promote secretion of inflammatory cytokines in monocytic

cell via toll-like receptor signalling. *J Biol Chem* (2013) **288**:36691–702. doi:10.1074/jbc.M113.512806
39. Danesh A, Inglis HC, Jackman RP, Wu S, Deng X, Muench MO, et al. Exosomes from RBC units bind to monocytes and induce pro-inflammatory cytokines, boosting T cell responses in vitro. *Blood* (2014) **123**:687–96. doi:10.1182/blood-2013-10-530469
40. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol* (2010) **10**:826–37. doi:10.1038/nri2873
41. Feng B, Chen Y, Luo Y, Chen M, Li X, Ni Y. Circulating level of microparticles and their correlation with arterial elasticity and endothelium-dependent dilation in patients with type 2 diabetes mellitus. *Atherosclerosis* (2010) **208**:264–9. doi:10.1016/j.atherosclerosis.2009.06.037
42. Tramontano AF, Lyubarova R, Tsiakos J, Palaia T, Deleon JR, Ragolia L. Circulating endothelial microparticles in diabetes mellitus. *Mediators Inflamm* (2010) **2010**:250476. doi:10.1155/2010/250476
43. Omoto S, Nomura S, Shouzu A, Hayakawa T, Shimizu H, Miyake Y, et al. Significance of platelet-derived microparticles and activated platelets in diabetic nephropathy. *Nephron* (1999) **81**:271–7. doi:10.1159/000045292
44. Brodsky SV, Zhang F, Nasjletti A, Goligorsky MS. Endothelium-derived microparticles impair endothelial function *in vitro*. *Am J Physiol Heart Circ Physiol* (2004) **286**:H1910–5. doi:10.1152/ajpheart.01172.2003
45. Yang C, Mwaikambo BR, Zhu T, Gagnon C, Lafleur J, Seshadri S, et al. Lymphocytic microparticles inhibit angiogenesis by stimulating oxidative stress and negatively regulating VEGF-induced pathways. *Am J Physiol Regul Integr Comp Physiol* (2008) **294**:R467–76. doi:10.1152/ajpregu.00432.2007
46. Eldh M, Ekstrom K, Valadi H, Sjostrand M, Olsson B, Jernas M, et al. Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. *PLoS One* (2010) **5**:e15353. doi:10.1371/journal.pone.0015353
47. Gildea JJ, Seaton JE, Victor KG, Reyes CM, Bigler Wang D, Pettigrew AC, et al. Exosomal transfer from human renal proximal tubule cells to distal tubule and collecting duct. *Clin Biochem* (2014). doi:10.1016/j.clinbiochem.2014.06.018
48. Wiggins R, Glatfelter A, Kshirsagar B, Beals T. Lipid microvesicles and their association with procoagulant activity in urine and glomeruli of rabbits with nephrotoxic nephritis. *Lab Invest* (1987) **56**:264–72.
49. Scherberich JE. Immunological and ultrastructural analysis of loss of tubular membrane-bound enzymes in patients with renal damage. *Clin Chim Acta* (1989) **185**:276–82. doi:10.1016/0009-8981(89)90217-9
50. Pascual M, Steiger G, Sadallah S, Paccaud JP, Carpentier JL, James R, et al. Identification of membrane-bound CR1 (CD35) in human urine: evidence for its release by glomerular podocytes. *J Exp Med* (1994) **179**:889–99. doi:10.1084/jem.179.3.889
51. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* (2004) **101**:13368–73. doi:10.1073/pnas.0403453101
52. Hogan MC, Manganelli L, Woollard JR, Masyuk AI, Masyuk TV, Tammachote R, et al. Characterization of PKD protein-positive exosome-like vesicles. *J Am Soc Nephrol* (2009) **20**:278–88. doi:10.1681/ASN.2008060564
53. Hara M, Yanagihara T, Hirayama Y, Ogasawara S, Kurosawa H, Sekine S, et al. Podocyte membrane vesicles in urine originate from tip vesiculation of podocyte microvilli. *Hum Pathol* (2010) **41**:1265–75. doi:10.1016/j.humpath.2010.02.004
54. Gonzales PA, Pisitkun T, Hoffert JD, Tchapyjnikov D, Star RA, Kleta R, et al. Large-scale proteomics and phosphoproteomics of urinary exosomes. *J Am Soc Nephrol* (2009) **20**:363–79. doi:10.1681/ASN.2008040406
55. Wang Z, Hill S, Luther JM, Hachey DL, Schey KL. Proteomic analysis of urine exosomes by multidimensional protein identification technology (MudPIT). *Proteomics* (2012) **12**:329–38. doi:10.1002/pmic.201100477
56. Chen CL, Lai YF, Tang P, Chien KY, Yu JS, Tsai CH, et al. Comparative and targeted proteomic analyses of urinary microparticles from bladder cancer and hernia patients. *J Proteome Res* (2012) **11**:5611–29. doi:10.1021/pr3008732
57. Principe S, Kim Y, Fontana S, Ignatchenko V, Nyalwidhe JO, Lance RS, et al. Identification of prostate-enriched proteins by in-depth proteomic analyses of expressed prostatic secretions in urine. *J Proteome Res* (2012) **11**:2386–96. doi:10.1021/pr2011236
58. Principe S, Jones EE, Kim Y, Sinha A, Nyalwidhe JO, Brooks J, et al. In-depth proteomic analyses of exosomes isolated from expressed prostatic secretions in urine. *Proteomics* (2013) **13**:1667–76. doi:10.1002/pmic.201200561
59. Prunotto M, Farina A, Lane L, Pernin A, Schifferli J, Hochstrasser DF, et al. Proteomic analysis of podocyte exosome-enriched fraction from normal human urine. *J Proteomics* (2013) **82**:193–229. doi:10.1016/j.jprot2013.01.012
60. Rastaldi MP, Armelloni S, Berra S, Li M, Pesaresi M, Poczewski H, et al. Glomerular podocytes possess the synaptic vesicle molecule Rab3A and its specific effector rabphilin-3a. *Am J Pathol* (2003) **163**:889–99. doi:10.1016/S0002-9440(10)63449-9
61. Salih M, Zietse R, Hoorn EJ. Urinary extracellular vesicles and the kidney: biomarkers and beyond. *Am J Physiol Renal Physiol* (2014) **306**:F1256–9. doi:10.1152/ajprenal.00128.2014
62. Miranda KC, Bond DT, McKee M, Skog J, Păunescu TG, Da Silva N, et al. Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. *Kidney Int* (2010) **78**:191–9. doi:10.1038/ki.2010.106
63. Cheng L, Sun X, Scicluna BJ, Coleman BM, Hill AF. Characterization and deep sequencing analysis of exosomal and non exosomal miRNA in human urine. *Kidney Int* (2013) **86**(2):433–44. doi:10.1038/ki.2013.502
64. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. *Kidney Int* (2012) **82**:1024–32. doi:10.1038/ki.2012.256
65. Lv LL, Cao Y, Liu D, Xu M, Liu H, Tang RN, et al. Isolation and quantification of microRNAs from urinary exosomes/microvesicles for biomarker discovery. *Int J Biol Sci* (2013) **9**:1021–31. doi:10.7650/ijbs.6100
66. Channavajjhala SK, Rossato M, Morandini F, Castagna A, Pizzolo F, Bazzoni F, et al. Optimizing the purification and analysis of miRNAs from urinary exosomes. *Clin Chem Lab Med* (2014) **52**:345–54. doi:10.1515/cclm-2013-0562
67. Lv LL, Cao YH, Pan MM, Liu H, Tang RN, Ma KL, et al. CD2AP mRNA in urinary exosome as biomarker of kidney disease. *Clin Chim Acta* (2014) **428**:26–31. doi:10.1016/j.cca.2013.10.003
68. Glidea JJ, Carlson JM, Schoeffel CD, Carey RM, Felder RA. Urinary exosomes miRNome analysis and its applications to salt sensitivity of blood pressure. *Clin Biochem* (2013) **46**:113–4. doi:10.1516/cllinbiochem.2013.05.052
69. Barutta F, Tricarico M, Corbelli A, Annaratone L, Pinach S, Grimaldi S, et al. Urinary exosomal microRNAs in incipient diabetic nephropathy. *PLoS One* (2013) **8**(11):e73798. doi:10.1376/journal.pone.0073798
70. Sharma K, Karl B, Mathew AV, Gangoiti JA, Wassel CL, Saito R, et al. Metabolomics reveals signature of mitochondrial dysfunction in diabetic kidney disease. *J Am Soc Nephrol* (2013) **24**:1901–12. doi:10.1681/ASN.2013020126
71. Higgins GC, Coughlan MT. Mitochondrial dysfunction and mitophagy: the beginning and end to diabetic nephropathy? *Br J Pharmacol* (2014) **171**:1917–42. doi:10.111/bph.12503
72. Kalani A, Mohan A, Godbole MM, Bhatia E, Gupta A, Sharma RK, et al. Wilm's tumor-1 protein levels in urinary exosomes from diabetic patients with or without proteinuria. *PLoS One* (2013) **8**:e60177. doi:10.1376/journal.pone.0060177
73. Zhou H, Cheruvanky A, Hu X, Matsumoto T, Hiramatsu N, Cho ME, et al. Urinary exosomal transcription factors, a new class of biomarkers for renal disease. *Kidney Int* (2008) **74**:613–21. doi:10.38/ki.2008.206
74. Lee H, Han KH, Lee SE, Kim SH, Kang HG, Cheong HI. Urinary exosomal WT1 in childhood nephrotic syndrome. *Pediatr Nephrol* (2012) **27**:317–20. doi:10.1007/s00467-011-2035-2
75. Zhou H, Kajiyama H, Tsuji T, Hu X, Leelahavanichkul A, Vento S. Urinary exosomal Wilms' tumor-1 as a potential biomarker for podocyte injury. *Am J Physiol Renal Physiol* (2013) **305**:F553–9. doi:10.1152/ajprenal.00056.2013
76. Sun AL, Deng JT, Guan GJ, Chen SH, Liu YT, Cheng J, et al. Dipeptidyl peptidase-IV is a potential molecular biomarker in diabetic kidney disease. *Diab Vasc Dis Res* (2012) **9**:301–8. doi:10.1177/1479164111434318
77. Zubiri I, Posada-Ayala M, Sanz-Maroto A, Calvo E, Martin-Lorenzo M, Gonzalez-Calero L, et al. Diabetic nephropathy induces changes in the proteome of human urinary exosomes as revealed by label-free comparative analysis. *J Proteomics* (2014) **96**:92–102. doi:10.1016/j.jprot.2013.10.03
78. Burger D, Thibodeau JF, Holterman CE, Burns KD, Touyz RM, Kennedy CR. Urinary podocyte microparticles identify prealbuminuric diabetic glomerular injury. *J Am Soc Nephrol* (2014) **25**:1401–7. doi:10.1681/ASN.2013070763
79. Moon PG, Lee JE, You S, Kim TK, Cho JH, Kim IS, et al. Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy. *Proteomics* (2011) **11**:2459–75. doi:10.1002/pmic.201000443
80. Zhou H, Pisitkun T, Aponte A, Yuen PS, Hoffert JD, Yasuda H, et al. Exosomal fetuin-A identified by proteomics: a novel urinary biomarker for

detecting acute kidney injury. *Kidney Int* (2006) **70**:1847–57. doi:10.1038/sj.ki.5001874
81. Sonoda H, Yokota-Ikeda N, Oshikawa S, Kanno Y, Yoshinaga K, Uchida K, et al. Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol* (2009) **297**:F1006–16. doi:10.1152/ajprenal.00200.2009
82. Cvjetkovic A, Lötvall J, Lässer C. The influence of rotor type and centrifugation time on the yield and purity of extracellularvesicles. *J Extracell Vesicles* (2014) **25**. doi:10.3402/jev.v3.23111
83. Mullier F, Bailly N, Chatelain C, Chatelain B, Dogné JM. Pre-analytical issues in the measurement of circulating microparticles: current recommendations and pending questions. *J Thromb Haemost* (2013) **11**:693–6. doi:10.1111/jth.12171
84. Schageman J, Zeringer E, Li M, Barta T, Lea K, Gu J, et al. The complete exosome workflow solution: from isolation to characterization of RNA cargo. *Biomed Res Int* (2013) **2013**:253957. doi:10.1155/2013/253957
85. Raj DA, Fiume I, Capasso G, Pocsfalvi G. A multiplex quantitative proteomics strategy for protein biomarker studies in urinary exosomes. *Kidney Int* (2012) **81**:1263–72. doi:10.1038/ki.2012.25
86. Hogan MC, Johnson KL, Zenka RM, Cristine Charlesworth M, Madden BJ, Mahoney DW, et al. Subfractionation, characterization, and in-depth proteomic analysis of glomerular membrane vesicles in human urine. *Kidney Int* (2014) **85**:1225–37. doi:10.1038/ki.2013.422
87. Fraser KB, Moehle MS, Daher JP, Webber PJ, Williams JY, Stewart CA, et al. LRRK2 secretion in exosomes is regulated by 14-3-3. *Hum Mol Genet* (2013) **22**:4988–5000. doi:10.1093/hmg/ddt346
88. Welton JL, Khanna S, Giles PJ, Brennan P, Brewis IA, Staffurth J, et al. Proteomics analysis of bladder cancer exosomes. *Mol Cell Proteomics* (2010) **9**:1324–38. doi:10.1074/mcp.M000063-MCP201
89. Merchant ML, Powell DW, Wilkey DW, Cummins TD, Deegens JK, Rood IM, et al. Microfiltration isolation of human urinary exosomes for characterization by MS. *Proteomics Clin Appl* (2010) **4**:84–96. doi:10.1001/prca.200800093
90. Ramirez-Alvarado M, Ward CJ, Huang BQ, Gong X, Hogan MC, Madden BJ, et al. Differences in immunoglobulin light chain species found in urinary exosomes in light chain amyloidosis (Al). *PLoS One* (2012) **7**(6):e38061. doi:10.1376/journal.pone.0038061
91. Rood IM, Deegens JK, Merchant ML, Tamboer WP, Wilkey DW, Wetzels JF, et al. Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome. *Kidney Int* (2010) **78**:810–6. doi:10.1038/ki.2010.26
92. Mitchell PJ, Welton J, Staffurth J, Court J, Mason MD, Tabi Z, et al. Can urinary exosomes act as treatment response markers in prostate cancer? *J Transl Med* (2009) **7**:4. doi:10.1186/1479-5876-7-4
93. Keller S, Ridinger J, Rupp AK, Janssen JW, Altevogt P. Body fluid derived exosomes as a novel template for clinical diagnostics. *J Transl Med* (2011) **9**:86. doi:10.1186/1479-5876-9-86
94. Benito-Martin A, Ucero AC, Zubiri I, Posada-Ayala M, Fernandez-Fernandez B, Cannata-Ortiz P, et al. Osteoprotegerin in exosome-like vesicles from human cultured tubular cells and urine. *PLoS One* (2013) **8**:e72387. doi:10.1371/journal.pone.0072387
95. Fernandez-Llama P, Khositseth S, Gonzalez PA, Star RA, Pisitkun T, Knepper MA. Tamm-Horsfall protein and urinary exosome isolation. *Kidney Int* (2010) **77**:736–42. doi:10.1038/ki.2009.550
96. Warder SE, Tucker LA, Strelitzer TJ, McKeegan EM, Meuth JL, Jung PM, et al. Reducing agent-mediated precipitation of high-abundance plasma proteins. *Anal Biochem* (2009) **387**:184–93. doi:10.1016/j.ab.2009.01.013
97. Ho NF, Hiquchi WI. Kinetics of aggregation of denatured proteins. I. Methodology based on the multichannel particle size analyzer. *J Pharm Sci* (1967) **56**:248–54. doi:10.1002/jps.2600560221
98. Musante L, Saraswat M, Duriez E, Byrne B, Ravidà A, Domon B, et al. Biochemical and physical characterisation of urinary nanovesicles following CHAPS treatment. *PLoS One* (2012) **7**(7):e37279. doi:10.1376/journal.pone.0037279
99. Hjelmeland LM. A nondenaturing zwitterionic detergent for membrane biochemistry: design and synthesis. *Proc Natl Acad Sci U S A* (1980) **77**:6368–70. doi:10.1073/pnas.77.11.6368
100. Emoto N, Yanagisawa M. Endothelin-converting enzyme-2 is a membrane bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J Biol Chem* (1995) **270**:15262–8. doi:10.1074/jbc.270.25.15262
101. Rampoldi L, Scolari F, Amoroso A, Ghiggeri G, Devuyst O. The rediscovery of uromodulin (Tamm-Horsfall protein): from tubulointerstitial nephropathy to chronic kidney disease. *Kidney Int* (2011) **80**:338–47. doi:10.1038/ki.2011.134
102. Paladino S, Pocard T, Catino MA, Zurzolo C. GPI-anchored proteins are directly targeted to the apical surface in fully polarized MDCK cells. *J Cell Biol* (2006) **172**:1023–34. doi:10.1083/jcb.200507116
103. Rajendran L, Simons K. Lipid rafts and membrane dynamics. *J Cell Sci* (2005) **118**:1099–102. doi:10.1242/jcs.01681
104. Sabharanjak S, Sharma P, Parton RG, Mayor S. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell* (2002) **2**:411–23. doi:10.1016/S1534-5807(02)00145-4
105. Liu Y, El-Achkar TM, Wu XR. Tamm-Horsfall protein regulates circulating and renal cytokines by affecting glomerular filtration rate and acting as a urinary cytokine trap. *J Biol Chem* (2010) **287**:16365–78. doi:10.1074/jbc.M112.348243
106. El-Achkar TM, McCracken R, Liu Y, Heitmeier MR, Bourgeois S, Ryerse J, et al. Tamm-Horsfall protein translocates to the basolateral domain of thick ascending limbs, interstitium amd circulation during recovery from acute kidney injury. *Am J Physiol Renal Physiol* (2013) **204**:F1066–75. doi:10.1152/ajprenal.00543.2012
107. Musante L, Saraswat M, Ravidà A, Byrne B, Holthofer H. Recovery of urinary nanovesicles from ultracentrifugation supernatants. *Nephrol Dial Transplant* (2013) **28**:1425–33. doi:10.1093/ndt/gfs564
108. Cvjetkovic A, Lotvall J, Lasser C. The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. *J Extracell Vesicles* (2014) **25**:3. doi:10.3402/jev.v3.23111
109. Cheruvanky A, Zhou H, Pisitkun T, Kopp JB, Knepper MA, Yuen PS, et al. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. *Am J Physiol Renal Physiol* (2007) **292**:F1657–61. doi:10.1152/ajprenal.00434.2006
110. Ben-Dov IZ, Tan YC, Morozov P, Wilson PD, Rennert H, Blumenfeld JD, et al. Urine microRNA as potential biomarkers of autosomal dominant polycystic kidney disease progression: description of miRNA profiles at baseline. *PLoS One* (2014) **9**:e86856. doi:10.1376/journal.pone.0086856
111. Nagaraj N, Mann M. Quantitative analysis of the intra- and inter-individual variability of the normal urinary proteome. *J Proteome Res* (2011) **10**:637–45. doi:10.1021/pr100835s
112. Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. *Genome Biol* (2006) **7**:R80. doi:10.1186/gb-2006-7-9-r80
113. Momen-Heravi F, Balaj L, Alian S, Trachtenberg AJ, Hochberg FH, Skog J, et al. Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles. *Front Physiol* (2012) **3**:162–7. doi:10.3389/fphys.2012.00162
114. Inman BA, Etienne W, Rubin R, Owusu RA, Oliveira TR, Rodriques DB, et al. The impact of temperature and urinary constituents on urine viscosity and its relevance to bladder hyperthermia treatment. *Int J Hyperthermia* (2013) **29**:206–10. doi:10.3109/02656736.2013.775355
115. Burton-Opitz R, Dinegar R. The viscosity of urine. *Am J Physiol* (1918) **47**:220–30.
116. Raimondo F, Morosi L, Corbetta S, Chinello C, Brambilla P, Della Mina P, et al. Differential protein profiling of renal cell carcinoma urinary exosomes. *Mol Biosyst* (2013) **9**:1220–33. doi:10.1039/c3mb25582d

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