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Podocyte Biology and Pathogenesis of Kidney Disease

Jochen Reiser¹ and Sanja Sever²

Jochen Reiser: jochen_reiser@rush.edu

¹Department of Medicine, Rush University Medical Center, Chicago, Illinois 60612

²Nephrology Division, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129

Abstract

Proteinuric chronic kidney disease (CKD), once a rare affliction believed to be mainly caused by genetic mutations, has become a global pandemic that severely diminishes the quality of life for millions. Despite the changing face of CKD, treatment options and resources remain woefully antiquated and have failed to arrest or reverse the effects of kidney-related diseases. Histological and genetic data strongly implicate one promising target: the podocyte. Podocytes are terminally differentiated cells of the kidney glomerulus that are essential for the integrity of the kidney filter. Their function is primarily based on their intricate structure, which includes foot processes. Loss of these actin-driven membrane extensions is tightly connected to the presence of protein in the urine, podocyte loss, development of CKD, and ultimately renal failure.

Keywords

CKD; actin cytoskeleton; dynamin; suPAR; novel therapeutics

CURRENT TREATMENTS FOR CHRONIC KIDNEY DISEASE

The global pandemic of chronic kidney disease (CKD) is progressing at an alarming rate. Up to 11% of the general population is affected in the United States, Australia, Japan, and Europe (1). India, China, and South-East Asia have also seen a steady increase in the prevalence of type II diabetes mellitus and its associated kidney complications (2, 3). Despite the dramatic increase in the number of patients suffering from CKD, current treatment options remain limited and unchanged from the last century.

The first line of treatment uses old but important approaches to reduce kidney tissue damage by trying to control the underlying conditions of CKD, such as hypertension, diabetes, and obesity. For example, the most commonly used drugs for patients suffering from diabetic nephropathy are angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs). Both drugs are primarily used for the treatment of hypertension. One of the best-known ACEIs, Captopril, was approved by the United States Food and Drug Administration (FDA) in 1981. However, the inception of Captopril's development can be traced to 1956, when angiotensin-converting enzyme was first discovered. For patients suffering from the kidney-scarring disease termed focal segmental glomerulosclerosis (FSGS), corticosteroids are frequently prescribed. Among them is prednisone, a

glucocorticoid that was first commercially produced in 1955. In addition to blood pressure drugs and corticosteroids, CKD patients are prescribed a bevy of other drugs and compounds such as non-steroid inhibitors, anti-inflammatory drugs, anticancer drugs and antibiotics in what could easily be depicted as a last-ditch effort to stem the progression of CKD. If these treatments fail and the patient begins to experience kidney failure, the final available option is dialysis, yet another mid-century development, or kidney transplantation. Thus, while the face of CKD has changed drastically in the past 60 years, treatment options have remained frozen in a period when diabetes was rare and the burden of CKD was unknown or underestimated.

There are two ways to interpret the dearth of recent innovations in CKD treatment. Either the existing treatments offer completely effective relief for CKD patients or the nephrology community has failed to find new treatment options. Those who believe that it is impossible to improve on the past have continued to adjust compounds targeting already known pathways. Indeed, this is the present trend for CKD treatments. Current human trials for CKD drugs seek approval for existing FDA-approved drugs, such as anti-inflammatories, ACEIs, and ARBs, to be used outside their approved indications. In line with this trend is Reata's bardoxolone methyl, an investigational anti-inflammatory, anti-oxidative-stress compound, which has completed phase II trials for CKD yet was recently halted owing to safety concerns, and Retrophin's RE-021, an angiotensin/endothelin receptor antagonist for treatment of FSGS. Although targeting these pathways is justified and does provide relief for some CKD patients, epidemiologic data show a doubling of the number of patients on dialysis within the past 20 years. These alarming numbers are unlikely to change significantly with these new candidate drugs. Clearly, current pharmaceutical treatments are woefully inadequate in preventing kidney failure. Although dialysis has long been considered the ultimate life-saving procedure for patients with kidney failure awaiting transplantation, it is far from perfect. In 2009 alone, Medicare spent \$20.9 billion on CKD patients as reported by the United States Renal Data System. It is not clear whether the highly profitable dialysis industry is comfortable with the efficacy of dialysis as the ultimate treatment option for end-stage renal disease or whether the pharmaceutical industry is getting frustrated in the absence of alternatives. CKD patients desperately need a treatment that targets new pathways that will stop or even reverse disease progression. It is increasingly evident that the current CKD pandemic demands a true innovation in CKD care.

The globalization of CKD is a modern disaster that will require an innovative solution. Researchers studying CKDs such as FSGS and diabetic nephropathy recognize that basic and translational scientific research is needed to clarify the pathogenesis of CKD and identify novel druggable pathways. The authors of this article believe that the time is ripe for a new discovery in the field of CKD treatments—one that will reverse CKD progression and offer patients an effective option beyond dialysis and transplantation. We acknowledge the challenges of developing an innovative treatment, discuss several promising pathways, and raise questions that should be addressed by basic, translational, and clinical research.

STRENGTHS AND WEAKNESSES OF PODOCYTES IN TISSUE CULTURE

A major conceptual switch in the way CKDs such as FSGS and diabetic nephropathy are viewed occurred in 1990s with the realization that podocyte injury underlies the molecular mechanism for a number of proteinuric glomerular diseases (reviewed in Reference 4). Dysfunction of glomerular podocytes and subsequent cellular death were found to be the driving forces behind disease initiation and progression, respectively. This was a significant shift in our understanding of the pathophysiology and pathology of glomerular diseases because it directed the focus away from late-stage symptoms, such as scarring and

immunoglobulin deposition, and toward early-stage events, such as effacement of foot processes (FPs) and proteinuria (the presence of protein in the urine). The realization that CKD could be reversed by targeting these early-stage events has led to the current theory that novel therapeutics should strive to inhibit podocyte loss. It also signals a radical departure from the current anti-inflammatory and renin-angiotensin drugs that have been used for CKD patients. Novel mechanistic insights led to a large gap between the current treatments for proteinuric kidney diseases and our knowledge of CKD pathophysiology. One of the essential prerequisites to bridge this gap has been the development of conditionally immortalized podocyte (mouse and human) cell lines.

Cultured podocytes are essential research tools, but they are not without limitations. Their huge advantage is their ability to generate cell lines harboring different mutations that correspond to mutations found in FSGS patients. While there are several cell lines with diverse properties (reviewed in Reference 5), a major weakness of cultured podocytes is their two-dimensional organization in contrast to the three-dimensional form of podocytes in the glomerulus. As viewed by scanning electron microscopy, a glomerular podocyte consists of a large cell body that sends out thick primary processes, which are supported by microtubular filaments and further extend into fine secondary processes supported by the actin cytoskeleton. FPs of one podocyte interdigitate with FPs from adjacent podocytes and form a slit diaphragm structure. Under the extensions of primary (referred to as major) and secondary (referred to as foot) processes are various urinary spaces, including the subpodocyte space, that all together empty into Bowman's urinary space (6). Directly under the FPs is the glomerular basement membrane, which circles around the glomerular capillary beside the area covered by the mesangium (6). Thus, podocytes consist of three well-defined and separate cellular domains: a cell body, primary processes, and FPs. The FPs have three separate membrane platforms, each facing different environments. The basal membrane, which is attached to the glomerular basal membrane, is surrounded by the blood environment; the slit diaphragm, which is part of the lateral membrane, is affected by cell contact; and the apical membrane is surrounded by primary urine. Each of these three platforms is specialized for its unique environment, as evidenced by the different proteins that are concentrated in these membranes.

In contrast to this highly specialized cellular organization in vivo, podocytes in culture are large round cells that do not exhibit major structural characteristics of the glomerular podocytes. Indeed, the cells in culture do not form well-defined primary processes (microtubule-based membrane extensions) or FPs (actin-based extensions), and although they can form slit diaphragms, the number of these structures in culture is highly variable and in general not abundant. In addition, cultured podocytes are bathed in media, which exposes the cells to a uniform environment very different from the three unique environments found in glomerular podocytes in vivo. Given the structural and environmental differences, it is obvious that podocytes in culture differ importantly from podocytes in the glomerulus. Podocyte tissue cultures that more accurately mimic the conditions found in the glomerulus are urgently needed.

The loss of the three-dimensional structure of glomerular podocytes in culture represents a major hurdle when studying spatially (defined in distinct spaces on the membrane) and temporally regulated signaling pathways that originate at the plasma membrane, such as nephrin signaling at the slit diaphragm or the role of transient receptor potential (TRP) channels. Hence, most nephrin-signaling studies have been done in homologous systems such as NIH 3T3 cells (7) of uncertain relevance to podocyte function in vivo, and we have little detailed information about different TRP channel complexes and their regulation in podocytes.

In comparison, studies of the actin cytoskeleton have been easier to conduct and seem more relevant to *in vivo* function. The different molecular mechanisms that regulate the global organization of the actin cytoskeleton are one of the best-studied aspects of cultured podocytes (reviewed in Reference 8). One characteristic of a cultured podocyte is its well-organized actin cytoskeleton. It consists of stress fibers that can be described as dorsal, ventral, and arcs. Cultured podocytes also form cytoskeleton projections called filopodia and lamellipodia. It is generally accepted that the cortical actin cytoskeleton that drives the formation of filopodia and lamellipodia in cultured podocytes is mechanistically similar to the actin cytoskeleton that lies under the plasma membrane of FPs, and thus might be involved in driving FP effacement. Stress fibers are often associated with actin cables that span along the FPs and are usually associated with well-formed FPs. As in other cell types, the global organization of the actin cytoskeleton in podocytes is regulated by canonical small GTPases such as RhoA, Rac1, and Cdc42. In addition to these classical regulators of the actin cytoskeleton, several actin binding proteins such as formin (9), α -actinin 4 (10), and synaptopodin (11), as well as actin regulatory proteins, e.g., dynamin (12), have been implicated in regulation of the actin cytoskeleton. The list is ever expanding and it would not be surprising to find novel and perhaps unexpected molecules in the future.

PODOCYTE MOTILITY IS PODOCYTE PLASTICITY

In addition to our appreciation of podocytes' role in CKD, our view of their role in maintaining the kidney filtration barrier is dramatically changing. *In vitro* studies using cultured podocytes as well as the most recent two-photon microscopy of podocytes in live mice and rats (13) have led us to regard the kidney filtration barrier as a highly dynamic structure. There is a consensus in the field that regardless of the original cause of podocyte injury (genetic, environmental, or both), podocyte response to injury follows a similar path. Specifically, injury results in FP effacement (loss of membrane extensions) driven by the global reorganization of the actin cytoskeleton, which is accompanied by proteinuria. If these early structural changes within podocytes are not reversed, progressive and severe damage occurs, which can include podocyte detachment, obliteration of the urinary space, development of segmental glomerulosclerosis, and end-stage renal failure. Thus, the current working hypothesis suggests not only that the early stages of FP effacement are reversible, but also that the reversal of effacement is critical for maintenance of healthy podocytes within the glomerulus. This dynamic model is supported by classical experiments using perfusion of rat kidneys with the polycation protamine sulfate, where FP effacement and accompanying proteinuria develop within a few minutes, yet can be fully reversed within 15 min by the administration of the polyanion heparin (14). These rapid changes of podocyte FP architecture require FPs to retract or elongate, hence to "move" quickly along the glomerular basement membrane.

Building on this observation, we and others are using cultured podocytes to study their motility patterns *in vitro* (15). Stimuli that lead to the development of FP effacement *in vivo* (e.g., lipopolysaccharide or puromycin aminonucleoside) have been found to cause hypermotility of cultured podocytes in cell migration assays (15, 16). Based on these observations, the term podocyte motility has been coined, referring to the dynamic reorganization of the interdigitating FP structure *in vivo* and to the cell migration of cultured podocytes *in vitro*, which is seen as a surrogate for the former.

Initially, it was believed that a stationary podocyte phenotype reflects a stable FP structure with intact slit diaphragms, whereas hypermotility would manifest as FP effacement *in vivo* (8). This hypothesis was recently directly tested in mouse strains overexpressing either dominant active (Da) or dominant negative (Dn) mutants of RhoA (18). In cells, expression of the Da mutant, which lasts longer in the GTP-bound state, resulted in an increase in the

number of focal adhesions (FAs) and stress fibers, which in podocytes in vitro is associated with diminished motility. In contrast, expression of the Dn mutant, which cannot bind GTP, results in dramatic loss of stress fibers and FAs, and these losses lead to increased motility in podocytes in vitro. Based on the podocyte motility hypothesis, expression of Dn-RhoA specifically in podocytes was expected to induce FP effacement and proteinuria (due to increased motility), whereas expression of Da-RhoA was not predicted to have any profound phenotype based on the assumption that decreased motility in vitro equals stable FP in the glomerulus. Contrary to the podocyte motility hypothesis, expression of both these RhoA mutants resulted in FP effacement and proteinuria, demonstrating that hypermotility in vitro can lead to FP effacement and proteinuria as quickly and directly as hypomotility. Thus, the model, in which prominent stress fibers in cultured podocytes reflect intact FPs in vivo, whereas the loss of actin stress fibers and reorganization into cortical actin in vitro equals FP effacement in vivo, is somewhat oversimplified. This RhoA study demonstrated that the actin cytoskeleton in podocytes is tightly regulated and that dysregulation (either through gain- or loss-of-function mutations) has a direct consequence on the structure and thus the function of FPs. Researchers often use simplified concepts to model complex biological processes, but in the case of cultured podocytes, simplification might be misleading: e.g., an increase in actin stress fibers in vitro can be interpreted as a sign of podocyte damage; however, a decrease in stress fibers can be interpreted similarly.

The above findings also suggest a need to redefine the roles of signaling GTPases in podocytes. For example, currently, some researchers believe that the RhoA signaling pathway results in a stationary (healthy) podocyte phenotype, whereas activity of Rac1/Cdc42 underlies the motile (effaced) phenotype (8). It seems more probable that signaling by all three members of the small Rho-type family of GTPases is highly coordinated within podocyte FPs in a manner similar to that in cultured mouse embryonic fibroblasts (19). In addition, signaling by RhoA, Rac1, and Cdc42 has been implicated in a broad variety of other cellular processes including adhesion, proliferation, and apoptosis (20). Thus, the effects of overexpression of mutants of these GTPases in podocytes may be mediated by processes other than the reorganization of the actin cytoskeleton in FPs.

Because interpretation of the actin dynamics in cultured podocytes does not seem to be as straightforward as originally assumed, we suggest a concept of “podocyte plasticity.” Podocyte plasticity implies that podocytes have an inherent ability to efface as well as to reform their FPs, which is driven by the global reorganization of the actin cytoskeleton and the coordinated interplay between signaling GTPases. One of the major unanswered questions with respect to the development of novel therapeutics is the critical period of plasticity for effaced (injured) podocytes. The concept of podocyte plasticity also suggests two novel druggable pathways for proteinuric kidney diseases: outside-in signaling to the actin cytoskeleton and direct regulation of the actin cytoskeleton.

NOVEL THERAPEUTIC TARGETS IN PROTEINURIC CKD

Outside-In Signaling to the Actin Cytoskeleton Foot Processes

The actin cytoskeleton in FPs is linked to the glomerular basement membrane by $\alpha 3\beta 1$ and $\alpha v\beta 3$ integrin (21) as well as α -dystroglycans/ β -dystroglycans (22). Integrins are heterodimeric cell adhesion receptors whose functions are central to inflammation, immunity, tumor progression, and the development and maintenance of normal tissue architecture in mature organs. Genetic inactivation of $\alpha 3$ or $\beta 1$ integrin causes severe podocyte FP disorganization and kidney failure in newborn mice, thereby underscoring the critical role of $\alpha 3\beta 1$ integrin for the development of the glomerular filter (23). In contrast, genetic deletion of $\beta 3$ integrin or the $\alpha v\beta 3$ integrin ligand vitronectin does not cause a renal phenotype per se but instead protects against lipopolysaccharide (LPS)–mediated FP

effacement and proteinuria (16). Integrin-mediated adhesion requires the activation of integrin heterodimers, which involves conformational changes, thereby enhancing integrin affinity for ligands, a process termed integrin activation. The activation of integrins can occur from inside the cell through signaling cascades that originate from the active remodeling of the cytoskeleton (24), the membrane domain of the FP such as the slit diaphragm (25), and the podocyte FP sole plate. The latter is classically referred to as outside-in activation of integrins and ensures a constant capacity to tune the dynamic motility of the FP. Modulation of FP motility is now considered an important part of the physiological regulation of the kidney filtration barrier. An example is the regulation of $\alpha\text{v}\beta 3$ integrin, which is heavily expressed in podocytes (16). Using activation-recognizing antibodies (16, 26), investigators have found podocyte $\alpha\text{v}\beta 3$ integrin activity is low under normal conditions and can be enhanced by ligands such as the urokinase plasminogen type I activator receptor uPAR (in podocytes) (16), or its soluble form, suPAR (from the circulation) (17). Activation of $\alpha\text{v}\beta 3$ integrin in turn causes activation of small GTPases, including Rac-1. The consequence is a reorganization of the podocyte actin cytoskeleton, which in its most severe form manifests itself as FP effacement. It has been suggested that sustained activation of $\alpha\text{v}\beta 3$ integrin might lead to progressive glomerular disease such as FSGS (16, 17).

In line with this hypothesis, increased glomerular uPAR gene and podocyte uPAR protein expression are found in proteinuric diseases such as FSGS and diabetic nephropathy. Increased systemic suPAR levels are found in approximately two-thirds of FSGS patients. Manipulating the uPAR– $\alpha\text{v}\beta 3$ integrin signaling pathway through uPAR blocking antibody or small molecules such as cyclo-RGDfV alleviated proteinuria. Similar results were obtained in a study performed in 5/6 nephrectomized rats, which displayed an increase in podocyte uPAR expression and $\beta 3$ integrin activation (27). In this report, uPAR expression could be modulated by amiloride, presumably through an off-target effect of this diuretic on podocytes. In FSGS, $\alpha\text{v}\beta 3$ integrin can be pathologically activated by an increase in circulating suPAR. It is presently unclear if circulating suPAR works synergistically with cell-membrane uPAR to activate $\alpha\text{v}\beta 3$ integrin in podocytes during FSGS. Published animal experiments have shown that an increase in circulating suPAR was sufficient to cause pathological podocyte $\alpha\text{v}\beta 3$ activation and glomerular disease. Thus, removal or inhibition of suPAR presents one of the potential novel treatments for patients with high levels of suPAR that is relevant for both native and recurrent FSGS.

Direct Regulation of the Actin Cytoskeleton

Because the actin cytoskeleton plays an essential role during cell migration and division, it has been thought for some time that small molecules targeting the actin cytoskeleton of tumor cells have potential therapeutic value (28). With podocytes being terminally differentiated cells that are mostly stationary and do not proliferate, it might seem counterintuitive that regulation of the actin cytoskeleton is one of the promising druggable pathways in podocytes. Although actin cytoskeleton rearrangement in podocyte FPs serves as a common denominator during FP effacement, this commonality alone does not imply that targeting the actin cytoskeleton in podocytes is a plausible therapeutic approach. Indeed, none of the current treatments for proteinuria targets the actin cytoskeleton. Furthermore, although microtubule-targeting drugs are routinely used in cancer therapy, very few actin-targeting drugs have been characterized and none are used in the clinic. Despite these obvious challenges, we believe that the cytoskeleton is a druggable target in CKD on the basis of two key observations: the plasticity of the podocytes (see above) and identification of the regulatory GTPase dynamin as a major regulator of the actin cytoskeleton in FPs.

To dynamin aficionados, our suggestion to target dynamin in CKD must seem as counterintuitive as our suggestion to target the actin cytoskeleton. Admittedly, dynamin is

best known for its function in orchestrating clathrin-coated vesicle formation during endocytosis (29). However, a growing body of evidence suggests that dynamin plays an essential role in actin regulation (reviewed in Reference 29).

As a GTPase, dynamin exhibits a unique biochemical characteristic: its ability to self-assemble into higher-order structures such as rings and helices. Ring formation stimulates dynamin's GTPase activity (30) owing to activation of a GTPase-activating domain within dynamin (named a GED domain) that only functions when dynamin oligomerizes into higher-order structures (31). At its C terminus, dynamin contains a proline-arginine-rich domain (PRD) that binds directly to the Src homology 3 (SH3) domains of multiple actin-regulating or -binding proteins such as profilin, Nck, and cortactin (reviewed in References 32 and 33). It is through these PRD interactions that dynamin is currently believed to exert its role in regulation of the actin cytoskeleton.

Despite the numerous links between dynamin and actin, the molecular mechanism by which dynamin regulates actin dynamics is still unclear. The role of dynamin in lamellipodia formation as well as in focal adhesion disassembly has been linked to endocytosis (33, 34). It has also been suggested that dynamin acts as a scaffold protein that directly assembles multiprotein complexes to drive actin polymerization at defined places in the cell (33). In addition, GTP hydrolysis by dynamin has been implicated in the regulation of actin filament organization in vitro (35) and in cells (e.g., *Listeria* motility) (36, 37). All these studies point to a relationship between actin and dynamin.

Our studies on the molecular mechanism of dynamin and actin dynamics have answered many questions and raised several more. We recently showed that dynamin is a unique regulatory GTPase that binds actin filaments directly (38). In addition, dynamin was found to promote actin polymerization by releasing the capping protein gelsolin from the barbed ends in vitro, but only if dynamin is oligomerized into rings. Together, these results suggested that the dynamin oligomerization cycle might regulate actin cytoskeleton directly by regulating actin polymerization. What is still unanswered is whether dynamin regulates actin cytoskeleton in parallel to known small canonical GTPases, such as RhoA, Rac1, and Cdc42, or whether it does so by acting on the GTPases' downstream effectors.

Our data point to the increasing likelihood that dynamin regulates actin independently of the small canonical GTPases. We have found expression of cytosolic cathepsin L (CatL) in certain rodent models of nephrotic syndrome and in a subset of patients with FSGS and diabetic nephropathy. Presence of CatL in the cytosol results in proteolysis of dynamin (12) as well as synaptopodin (11); down-regulation of synaptopodin is associated with downregulation of RhoA:GTP and thus its signaling cascade. Importantly, mice expressing dynamin mutants that favor ring-state conformation were able to resist and reverse LPS-induced proteinuria. These data suggest that preservation of the dynamin oligomerization state might be sufficient for reformation of FPs and amelioration of proteinuria. Although preservation of RhoA signaling might show promise for reformation of the actin cytoskeleton in injured podocytes, RhoA regulates multiple downstream effectors, so it is a far less specific target. Thus, developing a small molecule that will activate RhoA in a highly specific manner in the signaling pathway of podocytes is not likely. The brilliance of targeting the dynamin pathway lies in the narrow but critical role that dynamin oligomerization plays in actin regulation. Our studies suggest that dynamin rings have two distinct functions: first, they protect dynamin from proteolysis caused by cytosolic CatL, and second, they increase actin polymerization by acting directly on actin filaments. Identification of small molecules that specifically promote dynamin oligomerization shows tremendous promise as one approach to develop novel therapeutics to treat injured podocytes.

SUMMARY

The next few years will be crucial in determining whether manipulation of the actin cytoskeleton can override the different genetic mutations and environmental influences that underlie CKD. Clinical testing will show whether removal and/or inhibition of suPAR will be beneficial to patients suffering from FSGS. We also recognize that although we have discussed and favored targeting the dynamin oligomerization cycle and suPAR inhibition, there might be other possible druggable targets. Clearly, many questions remain in our search for novel therapeutics for CKD, but what becomes exceedingly clear is that we have passed the hinge point and are now starting to see encouraging trends and better strategies on the horizon to effectively treat podocytes and thus to improve or even stop CKD.

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Glossary

FSGS focal segmental glomerulosclerosis

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