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Review Article

Rho-GTPase Signalling in the Pathogenesis of Nephrotic Syndrome

Richard Robins and Tomoko Takano

Division of Nephrology, McGill University Health Centre, 3775 University Street, Room 236, Montreal, QC, Canada H3A 2B4

Correspondence should be addressed to Tomoko Takano; tomoko.takano@mcgill.ca

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Nephrotic syndrome (NS) is characterized by heavy proteinuria, hypoalbuminemia, and edema. The underlying causes of NS are diverse and are tied to inheritable and environmental factors. A common diagnostic marker for NS is effacement of podocyte foot processes. The formation and maintenance of foot processes are under the control of many signalling molecules including Rho-GTPases. Our knowledge of Rho-GTPases is based largely on the functions of three prototypic members: RhoA, Rac1, and Cdc42. In the event of podocyte injury, the rearrangement to the actin cytoskeleton is orchestrated largely by this family of proteins. The importance of maintaining proper actin dynamics in podocytes has led to much investigation as to how Rho-GTPases and their regulatory molecules form and maintain foot processes as a critical component of the kidney's filtration barrier. Modern sequencing techniques have allowed for the identification of novel disease causing mutations in genes such as *ARHGDIA*, encoding Rho-GDIα. Continued use of whole exome sequencing has the potential to lead to the identification of new mutations in genes encoding Rho-GTPases or their regulatory proteins. Expanding our knowledge of the dynamic regulation of the actin network by Rho-GTPases in podocytes will pave the way for effective therapeutic options for NS patients.

1. Introduction

The kidneys perform a number of essential functions in vertebrates including hormone secretion [1], blood pressure regulation [2], maintenance of glucose homeostasis [3], and urine formation. The latter process begins at the level of the glomerulus, the most proximal portion of the nephron, the kidney's functional unit [4]. Reports estimate the number of nephrons per kidney as one million [4, 5]. Our perception of the glomerulus has evolved over time from that of a static structure to a highly dynamic signalling hub that is capable of integrating intracellular cues from its individual structural components [4]. In humans, kidney development begins at five weeks gestation and the full complement of glomeruli is attained by 34 weeks [6]. Individual glomeruli begin development *in utero* following reciprocal interactions between the ureteric bud and metanephric mesenchyme. Mesenchymal cells are induced to coalesce and undergo a mesenchymal to epithelial transition into structures known as renal vesicles. The renal vesicles undergo a series of morphologic changes, maturing in developmental order to the comma-shape body, S-shape body, precapillary invasion stage, and finally the mature glomerulus [4, 7, 8]. Importantly, the glomerulus has evolved such as to impart size- and charge-selective properties necessary to properly filter plasma.

Glomeruli are susceptible to diseases induced by genetic mutations, infection, atherosclerosis, hypertension, diabetes, and additional causes. Diseases of the glomerulus take various forms including acute nephritic syndrome, atherosclerotic nephropathy, and nephrotic syndrome (NS). NS is a disease of glomerular filtration and the molecular mechanisms underlying its pathogenesis will be the focus of this review. During urine formation, the glomerular filtration barrier (GFB) freely allows the passage of water and electrolytes to the urinary space, while restricting the passage of high molecular weight proteins such as albumin [9], anticoagulation factors [10], and immunoglobulins [11]. The GFB is a three-layered structure consisting of capillary endothelium, an acellular basement membrane, and podocytes with the interposed slit diaphragm [12, 13]. Glomerular capillaries

filter 120–180 liters of plasma per day [14]. A defect in any single component of the GFB will predispose to plasma protein spillage into the urine (proteinuria). The daily urinary excretion of protein in healthy adults is less than 150 mg and higher and persistent proteinuria may predict the progression to kidney disease [15]. 8–10 mg of the daily protein excretion represents albumin [16] and is considered normal. Proteinuria is toxic to glomeruli and is a catalyst towards the progression to kidney disease. The mechanisms whereby proteinuria contributes to disease have been reviewed by Abbate et al. [17].

The development and maintenance of functional glomeruli are essential for survival. This is emphasized by the fact that, although the kidneys possess minor regenerative capacity, they cannot form new nephrons and therefore injured glomeruli cannot be replaced [18]. GFB injury with subsequent proteinuric disease can arise as a consequence of genetic [19] or environmental factors [20]. This review will focus specifically on the mechanisms of proteinuria induced by cytoskeletal derangements in glomerular podocytes that arise from abnormal regulation of the Rho-GTPase family of proteins. We will review our studies surrounding this topic as well as related and important findings from other researchers in the field.

1.1. Nephrotic Syndrome. Heavy proteinuria with subsequent hypoalbuminemia and edema from the loss of osmotic pressure [9] comprise the defining clinical manifestations of NS. The onset of disease is highly variable and is classified as follows; congenital NS is defined as the onset of symptoms within the first three months of life, while infantile NS develops between the ages of 4 and 12 months [21]. Beyond these time frames, disease onset requires classification as childhood, juvenile, or adult NS [21]. Diagnosis may be further classified based on the initial responsiveness to steroid treatment (i.e., prednisone) [22]. Steroid sensitive nephrotic syndrome (SSNS) is the most prevalent disease of the glomerulus encountered in children. Histologically, it presents as minimal change disease (MCD). The frequency of relapse is high in these patients, although renal prognosis is generally favourable [23]. In contrast, achieving a state of remission for patients with steroid resistant nephrotic syndrome (SRNS) is a challenge. SRNS comprises 10% of cases of idiopathic childhood NS [24]. SRNS may present as MCD, mesangial proliferative glomerulonephritis, or focal segmental glomerulosclerosis (FSGS) [25]. A significant percentage of SRNS has hereditary mutations in genes whose encoded products maintain the integrity of the GFB such as *NPHS1* (nephrin) and *NPHS2* (podocin) [19]. Congenital NS is steroid resistant [26] and 85% of the cases are known to be caused by mutations of one of the five genes: *NPHS1* (nephrin), *NPHS2* (podocin) [27], *WT1* (Wilm's tumor 1), *LAMB2* (Laminin-β2) [28], and *PLCE1* (phospholipase C epsilon) [29]. A number of additional disease causing mutations have been reported to cause SRNS with variable time of onset (congenital to adult) and histological features (diffuse mesangial sclerosis and FSGS) [21, 30]. Much has been learned from these mutations on the physiology and pathophysiology of the GFB. Below, we will discuss congenital NS caused by mutations in the gene, *ARHGDIA*, which encodes for Rho-GDIα, a negative regulator of Rho-GTPase signalling.

1.2. Architecture and Function of Podocytes. The podocyte layer of the GFB is critical for its function. Podocytes, which are sometimes referred to as visceral glomerular epithelial cells (GEC), are made up of a unique architecture comprising a cell body, major processes, and foot processes (FPs) [31]. The podocyte cell body is large, causing it to bulge into the urinary space. Podocyte cell bodies contain prominent nuclei and well-developed organelles including several lysosomes and mitochondria. The high organelle density within the cell body suggests a site of active metabolism [32]. Protruding from the cell body are several major processes rich in tubulin which further divide into FPs, which are indispensable for proper plasma filtering. FPs contain long bundles of actin fibres that run cortically and contiguously, thereby linking the FPs from adjacent cells [33]. FPs physically wrap around the glomerular capillaries [34] and interdigitate with those from adjacent cells. Interdigitating FPs are connected by a multiprotein signalling complex known as the slit diaphragm, which represents the final barrier to urinary loss of protein [31]. The loss of FPs is termed "effacement" and is almost invariably observed in patients with NS [35]. The process of FP effacement involves reorganization of the actin network, under the control of Rho-GTPases [36]. Actin filaments, which are normally in the form of parallel contractile bundles change, forming a dense network of stress fibers [37]. Mature podocytes are terminally differentiated cells with limited proliferative capacity. This implies that podocyte insults are highly limited in reversibility. A reduction in the number of podocytes (podocytopenia) has been indeed recognized as a cause of glomerular disease [38].

Research into actin originated following its discovery in muscle tissue by Straub in 1942 [39]. The expression of actin is now known to be nearly ubiquitous among eukaryotic cells. The actin cytoskeleton mediates several cellular processes including mitotic division, adhesion, and migration [40]. The actin pool within cells is comprised of F- (filamentous) and monomeric G- (globular) actin. G-actin polymerizes into F-actin in multistep processes involving numerous actin binding proteins [41]. F-actin is the form necessary to drive the aforementioned cellular processes. The importance of the actin cytoskeleton in podocyte biology will be highlighted within the context of Rho-GTPases and the molecules that regulate their activity. The importance of maintaining actin cytoskeletal dynamics in podocytes is best exemplified under conditions of proteinuric disease, where FPs are effaced. The degree of observable FP effacement is variable among proteinuric diseases but FP effacement remains a useful diagnostic tool for podocyte injury [42].

2. Rho-GTPases in Podocytes

The mammalian Rho guanine triphosphatases (GTPases) comprise a subdivision of the Ras superfamily of proteins [43]. Together they comprise more than 20 intracellular signalling molecules that are best documented for their roles

in dynamic organization of the actin cytoskeleton [44]. Ras-GTPases including Rho proteins represent a subclass of hydrolase enzymes that specifically bind to and hydrolyse GTP to GDP [45]. RhoA, Rac1, and Cdc42 are the prototypic Rho-GTPases. The function of these proteins pertaining to the physiology of the podocyte has been a primary interest of our laboratory for years. Here, we will discuss current knowledge of the functions of Rho-GTPases in podocytes.

2.1. RhoA in Podocytes. RhoA (encoded by *RHOA*) is a 23 kDa protein that is critically involved in the formation of actin stress fibres [46]. It acts via several effector molecules [47] including Rho-associated kinase (ROCK) [48]. Podocyte viability is dependent on proper regulation of RhoA activity, as stress fibre loss is typically associated with poor podocyte health [31]. As cultured podocytes differentiate *in vitro*, stress fibres increase in number which correlates with increased RhoA activity [49]. In podocytes, RhoA-dependent stress fibre formation is regulated by synaptopodin [49]. Asanuma and colleagues executed gene silencing of synaptopodin which culminated in the loss of stress fibres and established the link between synaptopodin and RhoA [50]. In a complimentary set of experiments, they demonstrated that podocyte levels of RhoA could be increased with the introduction of ectopic synaptopodin, which also promoted stress fibre formation. Furthermore, synaptopodin-dependent stabilization of RhoA levels was independent of RhoA gene expression; rather synaptopodin prevented proteasome-dependent protein degradation of RhoA [49]. While these data indicate the importance of RhoA in podocyte health, it has been also observed *in vitro* that excessive RhoA activation may derange podocyte morphology and function. For example, transient transfections with constitutively active RhoA (CA-RhoA) caused mouse podocytes to lose processes and lamellipodia [36, 51].

We addressed the impact of RhoA activation in podocytes using genetically engineered mice. To achieve this, we employed a double transgenic system which allows doxycycline-inducible and podocyte-specific gene expression (developed by the laboratory of Dr. Jeffrey B. Kopp at the NIH) [52]. We generated a transgenic mouse line that expressed a constitutively active RhoA mutant (L63) that was conjugated to the flag epitope (flag-CA-RhoA) and was under the control of the tetracycline response element [51, 52]. We crossed this line with transgenic mice expressing rtTA under the control of the podocin promoter [51]. This strategy allowed double transgenic mice fed doxycycline to express CA-RhoA exclusively in podocytes. Urinalysis (i.e., albumin to creatinine ratio) revealed that RhoA activation induced significant proteinuria after 4 weeks of doxycycline treatment. The degree of proteinuria correlated with the expression level of the transgene, flag-CA-RhoA. Doxycycline withdrawal experiments showed that the proteinuria was completely or partially reversible depending on the degree of proteinuria. When we analyzed double transgenic kidneys from doxycycline-fed mice by electron microscopy, the degree of FP effacement correlated with the levels of albuminuria. In the group of mice termed "low responders" (albuminuria below a cut-off level), FP effacement was segmental and reversible. In contrast, the kidneys from "high responders" displayed widespread FP effacement. Following a prolonged treatment with doxycycline, the high responding mice developed histological changes consistent with FSGS [51]. Although not detailed in the paper, the onset of proteinuria was as early as 2-3 days after the initiation of doxycycline. These observations are consistent with the notion that a mild RhoA activation in podocytes induces rapid and reversible FP effacement likely via cytoskeletal reorganization while more severe and sustained RhoA activation induces additional irreversible changes (Figure 1). RhoA activation occurs in podocyte injury in response to external stimuli. The complement C5b-9 membrane attack complex is well-established as an underlying cause of proteinuria in various experimental models [53]. Culturing rat podocytes in the presence of complement significantly increased RhoA activity and was due to assembly of the membrane attack complex. We have also previously shown that RhoA activity was increased in glomeruli from rats subjected to the passive Heymann nephritis (PHN) model of membranous nephropathy [54].

A subsequent report confirmed our findings. Wang et al. employed an analogous doxycycline-inducible, double transgenic system to observe the effects of RhoA hyperactivation in podocytes. Mice expressing the constitutively active mutant RhoA (V14) became significantly proteinuric at two weeks on doxycycline and the degree of albuminuria continued to rise until the 6-week time point [55]. While the results were consistent with our findings, the degree of proteinuria in these mice was much milder, compared with our study. The reason for this difference is not clear but could be attributed to different mouse backgrounds (mixed in our study versus FVB/NJ in [55]). Of interest, they also found that a dominant negative RhoA mutant (N19) causes albuminuria, which peaked at two weeks and then began to abate. This is somewhat contradictory to the findings that podocyte-specific knockout mice for RhoA did not show any kidney phenotype [37], although the latter results could be explained by compensation by other Rho isoforms (Figure 1). Proteinuria induced by both active and dominant negative RhoA was associated with loss of the podocyte protein synaptopodin [55]. However, active but not dominant negative RhoA caused loss of nephrin and podocyte apoptosis [55]. Thus, RhoA hyperactivation and inactivation are both detrimental to podocyte physiology possibly via distinct mechanisms and both predispose to the development of proteinuria (Figure 1). These findings emphasize the need for a precise regulation of RhoA activity in podocytes.

Additional recent studies implicate RhoA in the pathogenesis of proteinuric disease. Babelova et al. observed that the ROCK inhibitor, SAR407899, prevented albuminuria as well as loss of podocin and nephrin in the 5/6 nephrectomy model of chronic kidney disease [56]. In cultured podocytes, RhoA modulated apoptotic pathways induced by high-glucose [57] and the ROCK inhibitor, fasudil, was protective against albuminuria in diabetic mice [58]. Furthermore, RhoA is involved in mediating the response to

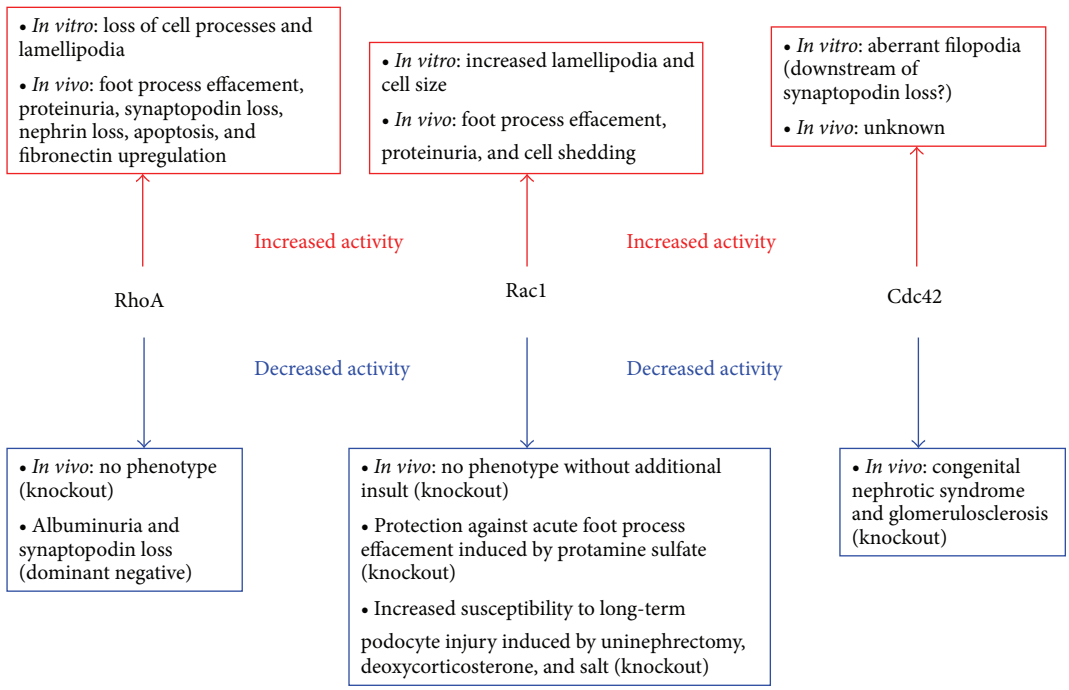

Figure 1: Summary of functional and pathogenic consequences of Rho-GTPase activity imbalances within podocytes. The activities of RhoA, Rac1, and Cdc42 within podocytes must remain under tight regulatory control in order to maintain foot process architecture and cellular function to prevent the occurrence of proteinuric disease.

podocyte injury *in vitro* induced by puromycin aminonucleoside (PAN) [59]. These results suggest that, in addition to the acute impact on the actin cytoskeleton, sustained RhoA activation causes chronic and irreversible changes in podocytes. In support of this, we have demonstrated that RhoA activation in podocytes triggers the activation of the transcription factor, nuclear factor of activated T-cells (NFAT), that contributes to fibronectin upregulation [60].

2.2. Rac1 in Podocytes. Rac1 (encoded by *RAC1*) is a 21 kDa protein that is expressed ubiquitously in mammals [61]. Ridley et al. pioneered our understanding of Rac1 as it pertains to the actin cytoskeleton. Their 1992 publication identified Rac1 as a key regulator of lamellipodium formation in response to growth factor stimulation in fibroblasts [62]. Lamellipodia are sheet-like extensions found in migrating cells. The formation of lamellipodia is complex, requiring initial nucleation of actin polymerization which is followed by controlled branching and cross-linking. These structures are believed to contribute to the forward movement of cells [63]. Transfecting cultured podocytes with constitutively active Rac1 mutants has reproducibly been demonstrated to cause an increase in cell size and membrane ruffling [36, 64, 65] (Figure 1), consistent with the findings in other cell types [66]. When immortalized mouse podocytes were differentiated *in vitro*, we observed that Rac1 activity increased after 1 week without a corresponding increase in total Rac1, but the activity returned to the baseline after 2 weeks of differentiation [64]. Using similar cells, Akilesh and colleagues reported the highest Rac1 activity in undifferentiated cells and a decline of the activity with differentiation [67]. The reason for the differences observed in these time-course studies is unclear; nonetheless both studies agree that fully differentiated podocytes show a low level of Rac1 activity. This likely suggests that, in normal mature glomeruli, Rac1 activity in podocytes is controlled at a very low level and that Rac1 hyperactivity in mature podocytes could be pathogenic. On the other hand, it is interesting to note that Rac1 activation can be triggered by nephrin-mediated signalling [64, 65, 68]. This raises a possibility that Rac1 activation in podocytes may be required in the process of development or recovery from injury.

The first *in vivo* studies to address the role of Rac1 in podocytes involved targeted deletion of Rac1. Using Cre-lox methodology, Scott et al. [37] and Blatner et al. [69, 70] both demonstrated that deletion of Rac1 in podocytes does not adversely affect the health or function of these cells (Figure 1). While a possibility remains that the redundant functions of Rac2 and Rac3 may have compensated for the loss of Rac1, these results appear to suggest that Rac1 is dispensable for podocyte development and maintenance of podocyte architecture [71, 72]. Interestingly, these mice were protected against acute protamine sulphate induced FP effacement, while being more susceptible to long-term podocyte injury in the model of uninephrectomy, desoxycorticosterone, and salt (UNX/DOCA-salt treatment) [69] (Figure 1). The latter observation suggests that Rac1 may be important for a repair process from podocyte injury. Consistent with this notion, we also observed that Rac1 activation in the glomerulus of rats treated with PAN was most prominent in the recovery phase [64].

A potential pathogenic role of Rac1 hyperactivation in podocytes was further supported by a recent report by Yu and colleagues [36]. They generated mice expressing EGFP tagged CA-Rac1 under the control of either the podocin (*NPHS2-rtTA*) or nephrin (*Nphs1-rtTA-3G*) promoters [52, 73]. When the transgenic mice were under the control of the podocin promoter, only a small percentage of podocytes expressed CA-Rac1. Nonetheless, this caused rapid and transient proteinuria with segmental foot process effacement (Figure 1). The uneven expression of CA-Rac1 within glomeruli was possibly explained by epigenetic silencing mechanisms. To circumvent the issue of uneven expression, they crossed their EGFP-CA-Rac1 line with mice expressing the *Nphs1-rtTA-3G* transgene (the 3G component confers heightened sensitivity to doxycycline [74]). In these mice, CA-Rac1 expression was detected in a much larger percentage of podocytes but was still not 100% penetrant. The degree of proteinuria of the nephrin-driven mice was more pronounced, as compared with podocin-driven mice, but nonetheless it remained transient; it peaked at 4 days and subsequently markedly decreased. The transgene (EGFP-CA-Rac1) expression detected by enumerating glomeruli at 4 weeks was negligible. The authors concluded that activation of Rac1 caused podocytes to detach and shed into the urine and this loss was repaired or compensated, although the mechanisms for this repair process were not proposed [36]. Together with the studies on podocyte-specific Rac1 knockout mice, it appears that Rac1 activation contributes to acute/transient FP effacement and proteinuria. However, from this study, it is not possible to conclude the impact of more sustained/chronic Rac1 activation on podocytes since CA-Rac1 expressing podocytes were lost relatively quickly. We are presently investigating further the consequences of *in vivo* Rac1 hyperactivation in podocytes, also using the doxycycline-inducible system. Our unpublished data suggests that proteinuria is maintained for at least one month in doxycycline-fed double transgenic mice. Furthermore, it would be interesting to study the impact of Rac1 activation when it is turned on in the setting of chronic podocyte injury such as the UNX/DOCA-salt model.

There is further evidence to support the notion that excessive Rac1 activity is pathogenic to podocytes. Shibata et al. demonstrated amelioration of proteinuria in the *ARHGDIA*$^{-/-}$ mice treated with a specific Rac1 inhibitor [75]. Clinical evidence for the adverse effects of Rac1 hyperactivation in podocyte health was documented in patients with mutations in *ARHGDIA* [22, 76] and *ARHGAP24* (which acts preferentially on Rac1) [67]. These clinical findings will be elaborated on below.

2.3. Cdc42 in Podocytes. Cdc42 (encoded by *CDC42*) is a 21 kDa protein known largely for its involvement in filopodia formation in conjunction with Arp2/3 actin nucleation complex and WASp [77, 78]. Cdc42 has thus far attracted less attention than RhoA or Rac1 in regard to podocyte biology [79]. It is likely that recent interest in Cdc42 spawned following the observation by Wei et al. that Cdc42 is activated in podocytes downstream of signalling events from the urokinase receptor (uPAR), which is upregulated in proteinuric disease [80]. Glomerular lysates prepared from wild-type and uPAR knockout mice (*plaur*$^{-/-}$) treated with or without LPS revealed the link between uPAR and Cdc42. The LPS-mediated increase in Cdc42 activity (and Rac1 activity) was blunted in uPAR knockout mice. uPAR mediates physiological and pathophysiological processes such as cell migration [81], hemostasis [82], and malignancy [83]. Wei and colleagues subsequently identified the soluble form of urokinase plasminogen activator (suPAR) as a potential circulating factor in idiopathic FSGS [84].

While studies on Cdc42 are yet limited, several recent reports highlight the emerging and important roles of this protein in podocytes. In contrast to RhoA and Rac1 which are dispensable for podocyte development, the effect of knocking out Cdc42 in podocytes is severe. Scott et al. were the first to show, using Cre-recombinase technology, that mice with floxed *Cdc42* alleles were proteinuric at birth. In addition, the proteinuria rapidly increased in severity, and mice died within two weeks due to renal failure [37] (Figure 1). Ultrastructural analysis of podocytes from these mice at birth revealed broadened FPs with widespread effacement. The glomeruli of Cdc42 conditional knockout mice were deficient in both nephrin and podocin as assessed by immunofluorescence analysis. However, synaptopodin levels remained unchanged. Blattner et al. subsequently reported a similar phenotype of Cdc42 podocyte-specific knockout mice. This resulted in severe glomerular disease characterized by glomerulosclerosis, tubular dilatation, and vacuolated podocytes. Nephrin and podocin mRNA levels were reduced in these animals [69] which was in direct agreement with the findings of Scott and colleagues. Whether Cdc42 is also required for the maintenance of normal podocyte function in adults remains to be answered.

2.4. GAPs and GEFs in Podocytes. Rho-GTPases are commonly referred to as "molecular switches," a term which describes their reversibility between GTP-bound (active) and GDP-bound (inactive) states [17]. Cycling is under tight regulation by 3 families of proteins. Rho guanine nucleotide exchange factors (GEFs) activate Rho-GTPases by facilitating the exchange of GDP to GTP. Rho-GTPase activating proteins (GAPs), on the other hand, facilitate the inactivation of Rho-GTPases by enhancing their intrinsic GTPase activity. Rho-GDIs sequester Rho-GTPases in their inactive conformation [85].

The first Rho-GAP was discovered in 1989 by Garrett and colleagues and there are now over 70 characterized members of this protein family [86]. Current knowledge on the expressions and activities of the numerous Rho-GAPs in podocytes is limited. Akilesh et al. reported the importance of Arhgap24 (gene: *ARHGAP24*), which preferentially suppresses Rac1 activity, in podocytes. They identified a mutation in Arhgap24, Q158R, which was associated with adult onset and familial FSGS. The mutation was located within the GAP domain of Arhgap24, conferring a deficiency in GAP activity which resulted in Rac1 hyperactivity. Within podocytes, the levels of the *ARHGAP24* transcript (both *in vivo* and *in vitro*)

and encoding protein (*in vitro*) increased as the cells differentiated. Analyses in murine kidneys revealed enrichment of the Arhgap24 signal within glomeruli. Arhgap24 colocalized with synaptopodin, confirming its expression within podocytes. These results are consistent with the notion that Arhgap24 is upregulated as podocytes mature, keeping the Rac1 activity low, and that a loss of function mutation of Arhgap24 causes aberrant Rac1 hyperactivation that leads to proteinuria and FSGS. Interestingly, knockdown of Arhgap24 increased the activity of Cdc42 in addition to Rac1. The significance of the increase in Cdc42 activity remains a question for further research [67].

On a par with the GAP family, over 80 known GEFs in humans have been identified [87]. Current knowledge of GEFs in relation to proteinuric disease is similarly limited. To our knowledge, there are presently no clinical reports tying GEFs to proteinuric disease [79]. The Rac specific GEFs Dock1 and Dock5 were recently scrutinized for potential involvements in development of the GFB. The origins of this investigation can be traced to slit diaphragm orthologs expressed by nephrocytes, podocyte-like cells from *Drosophila melanogaster* (and other insects) [88]. The nephrin and Neph1 orthologs (sns and hbs, resp.) recruit and signal via the GEF, mcb, which activates Rac. Proper expression and function of sns, hbs, and mcb are necessary for the filtration of hemolymph in these organisms. Experimental analysis of this system in mammals revealed that podocyte-specific loss of Dock1 (mammalian ortholog of mcb) produced no adverse effects on podocyte development or kidney function. These animals were not proteinuric. Additional loss of Dock5 which is functionally redundant to Dock1 by crossing Dock1 conditional knockout with systemic Dock5 knockout mice also failed to perturb kidney function. These results were surprising since systemic double knockout of Dock1 and Dock5 was embryonic lethal [89]. Thus, GEFs responsible for Rac1 activation in podocytes remain an open question.

We recently reported that GEF-H1 (aka ArhGEF2) could be activated in cultured podocytes by complement C5b-9 dependent mechanisms [90]. GEF-H1 has been reported to activate RhoA [91] and Rac1 [92] in different biological systems. In cultured rat podocytes, complement C5b-9 induced RhoA activation and this was abrogated by GEF-H1 knockdown. We also observed GEF-H1 and RhoA activation in glomeruli with rats with PHN [90]. GEF-H1 therefore contributes to RhoA activation by complement in podocytes and may represent an additional target to rescue derangements to the actin cytoskeleton.

2.5. Rho-GDIα in Podocytes. In mammals, the Rho-GDI protein family consists of three members: GDIα, β, and γ. Tissue distribution is isoform specific and GDIα (gene: *ARHGDIA*) is the only member with ubiquitous expression. GDIs were initially identified as negative regulators of Rho-GTPases. GDIs extract them from membranes and sequester them in the cytosol. They also inhibit nucleotide exchange and hydrolysation [85]. GDIα knockout mice ($ARHGDIA^{-/-}$) were viable when being young but developed heavy proteinuria which progressed to renal failure and death within one year [93]. Podocytes were injured with disruption of FPs. These mice were also defective in aspects of their reproductive capacity. Systemic knockout of GDIα produced no compensatory elevation in the other GDIs (i.e., GDIβ) [93].

We recently reported the case of siblings diagnosed with congenital NS, where the cause of disease was identified as a loss of function mutation in *ARHGDIA*. The proband (older sibling) presented at three weeks of age with the classical symptoms of NS including generalised edema, hypoalbuminemia, and proteinuria. The younger sibling presented similarly at 16 days of age. The proband's renal histology revealed abnormal glomerular changes including collapsed capillary tufts with cuboidal, undifferentiated podocytes. As previously mentioned, 85% of cases of congenital NS arise as a consequence of monogenic defects in one of five genes whose products affect the integrity of podocytes or podocyte-GBM interactions [26]. These patients were negative for mutations in the aforementioned genes. Therefore, we turned to whole exome sequencing [94] to identify the underlying cause of disease. The introduction of WES was an important advancement for medical genomics, allowing for the identification of rare genetic abnormalities in monogenic disorders [95]. The bioinformatic analysis revealed a homozygous deletion that encodes for 3 consecutive aspartic acid residues (D183, D184, and D185). As it was not possible to determine which residue was deleted, the mutation was termed ΔD185. When transfected in HEK293T cells, ΔD185 failed to bind to RhoA, Rac1, or Cdc42. Skin fibroblasts obtained from the proband demonstrated increased levels of active (GTP-bound) RhoA, Rac1, and Cdc42, as compared with control fibroblasts. Thus we concluded that ΔD185 is a loss of function mutation [76].

A subsequent clinical study reported additional disease causing mutations in *ARHGDIA* from patients with childhood or congenital NS. The first mutation, G173V, caused childhood NS in 3 siblings who became symptomatic in 1-2 years. The next case was congenital. The single patient became symptomatic at 14 days of age from a truncating mutation in *ARHGDIA* (R120X) [22]. In some of these patients, extrarenal manifestations were also present. However, NS was the most consistent clinical feature. This was likely because podocytes are more sensitive to mutations that affect cytoskeletal architecture compared with other cell types [96]. Analogous examples include mutations in alpha-actinin-4 (necessary for actin polymerization) [97], CD2-associated protein (involved in actin fiber cross-linking) [98], and inverted formin 2 (accelerates actin polymerization) [99], all of which are FSGS-associated. In agreement with our findings, these new mutations abrogated molecular interactions between GDIα and Rho-GTPases (Rac1 and Cdc42; RhoA was unaffected) and thus were considered to be loss of function mutations. Both reports studied cell motility in GDIα knockdown podocytes, since deranged motility of podocytes, either increased or decreased [100], is believed to contribute to the pathogenesis of proteinuria [101]. When GDIα was knocked down in mouse podocytes, we observed hypomotility [102]. This was consistent with

the hypomotility of the patient's fibroblasts [102]. In contrast, Gee et al. observed increased cell motility in GDIα knockdown podocytes [22]. A likely explanation for the apparent conflict could be different degrees of GDIα knockdown; in our study, knockdown in podocytes was nearly 100% with hyperactivation of RhoA, Rac1, and Cdc42 whereas Gee et al. showed modest GDIα knockdown with hyperactivation of Rac1 and Cdc42 but not RhoA. Rac1/Cdc42 hyperactivation unopposed by RhoA may have resulted in hypermotility. In addition, Gee et al. stimulated cells with serum whereas we studied unstimulated cells. These differences in experimental conditions could account for different results. Nonetheless, both studies agree that loss of function of GDIα results in deranged podocyte motility, which likely contributes to the deranged podocyte function and pathogenesis of NS [22].

3. Conclusion and Future Directions

There is at present much research into the biology of the podocyte, a field which has been steadily growing for over a decade since the discovery of nephrin in 1998 [103, 104]. A general survey of the literature on Rho-GTPases reveals a large body of work centered on the three prototypical family members discussed herein; RhoA, Rac1, and to a lesser degree, Cdc42. It will be interesting to learn how the additional members of the Rho-GTPase family contribute to podocyte biology in terms of regulating the actin network and other processes known to be under their control, such as endocytosis [105] and cell survival [106]. The observation that podocyte-specific ablation of Cdc42 causes rapid renal failure and death should make the study of Cdc42 in podocytes a priority among the Rho-GTPases.

The functional redundancy between Rho-GTPase isoforms is an interesting topic. Podocyte-specific loss of RhoA or Rac1 produced no effect on development of these cells [37] suggesting the existence of functional redundancy or as of yet unidentified compensatory mechanisms. Rac1 and Rac2 function in a redundant manner in the development of T-cells [71]. Also documented are functional redundancy and isoform specificity of Rho-GTPases in murine embryonic fibroblasts, where RhoA is uniquely required for cell division but dispensable for the regulation of actomyosin, a function which was redundantly dependent on RhoB and RhoC [107]. In other tissues, RhoA and Rac1 are required for development. RhoA is necessary for B-lymphocyte development within the spleen [108] and Rac1 is required for the development of precursor cells of the forebrain [106]. The isoform specific functions of Rho-GTPases in podocytes will require further investigations.

While there are a large number of Rho-GTPase regulatory proteins, that is, GAPs, GEFs, and GDIs, their specificity and regulation are largely unexplored, in particular in the context of podocyte biology. Also not explored to any significant degree in podocytes is the cross-talk between the different Rho-GTPase family members. The cross-talk concept was first proposed by Ridley et al. in 1992 [62] and likely has an important role in maintaining the healthy balance of various Rho-GTPase members as well as in pathological process in podocyte injuries. Therefore, understanding how Rho-GTPase members communicate with each other via their regulatory proteins will likely shed light on the mechanisms of FP effacement and deranged podocyte function. The tertiary structures of small Rho-GTPases are globular and lacking in surface pockets, which make them less useful as therapeutic targets for chemical inhibitors [109]. Therapeutic manipulation of Rho-GTPase activity within podocytes will therefore require a more thorough understanding of GAPs and GEFs within these cells. The large number of GEFs and GAP relative to Rho-GTPases allows for tissue-specific regulation of GTPase activity [109] and drugs targeting them should minimize nonspecific effects. For example, the compound Rhosin was recently found to specifically inhibit RhoA activity by blocking its interaction with RhoA-GEF [110]. The compound Y16 also dose-dependently inhibits RhoA activity by binding to and inhibiting Leukemia associated RhoGEF (LARG), a Rho-specific GEF, thereby inhibiting breast cancer cell tumorigenicity [109].

The importance of the next generation sequencing technology should also be underscored. The research on podocyte biology flourished after discoveries of many important podocyte proteins by linkage analysis of familial cases. More recently, however, an increasing number of gene mutations have been identified utilizing whole exome sequencing, such as mutations in *ARHGDIA* [22, 76]. The sequence technologies continue to improve rapidly as the cost is coming down significantly. It is likely that additional gene mutations will be found in Rho-GTPase members or their regulatory proteins, and the results will further our knowledge on the role of Rho-GTPases in podocyte function and NS. The Rho-GTPase signalling network and actin cytoskeleton are perturbed in patients with inheritable [22, 76] and acquired forms of NS [111]. A further comprehension as to how Rho-GTPases orchestrate the reorganization of podocyte architecture will allow more effective and specific therapeutic interventions in patients with NS.

Abbreviations

FP: Foot process
FSGS: Focal segmental glomerulosclerosis
GBM: Glomerular basement membrane
MCD: Minimal change disease
ROCK: Rho kinase
PAN: Puromycin aminonucleoside
PHN: Passive Heymann nephritis
NS: Nephrotic syndrome
SRNS: Steroid resistant nephrotic syndrome
SSNS: Steroid sensitive nephrotic syndrome.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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