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Role and Mechanisms of Tyro3 in Podocyte Biology and Glomerular Disease

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Abstract
Background: Podocyte loss occurs in both primary and secondary glomerular diseases, leading to the progression of kidney disease. A large body of evidence suggests that apoptosis and detachment are the mechanisms mediating the reduction in podocyte numbers in glomerular diseases. Recent studies demonstrate a renal protective effect of protein S (PS) through the activation of Tyro3, one of the TAM receptors. Tyro3 is predominantly expressed in podocytes within the kidney, and its expression increases in early diabetic kidney disease (DKD) but decreases in patients with progressive DKD and focal segmental glomerulosclerosis (FSGS). Glomerular expression of Tyro3 also correlates with the progression of DKD and predicts the progression of primary glomerular diseases. High glucose increases Tyro3 expression, while TNF-α suppresses the expression of PS and Tyro3. PS has anti-inflammatory and antiapoptotic effects in podocytes, likely via the activation of the Akt pathway and the inhibition of NF-kB activation. In vivo, the knockout of PS or Tyro3 exacerbates podocyte loss and glomerular disease, while the overexpression of PS and Tyro3 attenuates the injury in mice with DKD and FSGS. Tyro3 agonists have also been shown to protect podocytes from injury in these animal models. ***Summary:*** Tyro3 plays a critical role in podocyte biology and glomerular disease. Tyro3 agonists could potentially be developed as a new therapy for glomerular disease. ***Key Message:*** The aim of this review article was to summarize the role and mechanisms mediating the protective effects of Tyro3 in podocyte biology and glomerular disease. Additionally, we discuss the possibility of developing Tyro3 agonists as potential treatment for glomerular diseases.

Introduction

Primary glomerular disease includes minimal change disease, focal segmental glomerulosclerosis (FSGS), and membranous nephropathy, while diabetic kidney disease (DKD) is the most common secondary glomerular disease. Podocytes constitute the final barrier of the glomerular filtration unit, and the loss of podocytes plays a pivotal role in both primary and secondary glomerular diseases, resulting in proteinuria and disease progression

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[1]. In the normal kidney, podocytes are highly differentiated and quiescent cells with limited proliferation and regeneration capacity. To maintain quiescence, podocytes activate several cell cycle arrest genes such as WT-1, p53, and p21 [2]. Consequently, the depletion or reduction of podocytes, termed podocytopenia, contributes to the development of glomerular diseases [3, 4]. Clinically, the decrease in podocyte density in kidney histology strongly predicts progressive DKD [5, 6], and the degree of podocyte reduction correlates directly with the magnitude of proteinuria [7, 8]. The potential mechanisms for podocyte loss include apoptosis, necroptosis, and detachment from the glomerular basement membrane [1, 9–13]. Nevertheless, the precise mechanisms of podocyte loss remain elusive. Numerous attempts have been made to rescue podocytes from loss as a potential therapy for glomerular diseases. Recent studies have shown that anti-PD-1 antibody treatment improved proteinuria by preventing podocyte loss in experimental FSGS models [14, 15]. However, treatments specifically targeting podocyte loss are still lacking in clinical trials.

Podocyte injury is an early event in DKD. In our investigation, we conducted a proteomic analysis of glomeruli isolated from streptozotocin (STZ)-induced diabetic rats with early-stage DKD, identifying a list of proteins with significant expression changes in these diabetic rats. Among the top 10 proteins with altered expression, protein S (PS) was prominently featured.

PS, encoded by Pros1, is a plasma glycoprotein known for its role as a negative regulator of blood coagulation [16]. Structurally, PS bears similarity to Gas6, which has demonstrated significant involvement in kidney diseases [17]. Both PS and Gas6 interact with TAM receptors (Tyro3, Axl, and Mer), a family of receptor tyrosine kinases that regulate processes such as immune response/inflammation, clearance of apoptotic cells, platelet aggregation, cell proliferation, survival, adhesion, and migration [18]. The roles of TAM have been studied in the immune, reproductive, hematopoietic, vascular, and nervous systems [19]. Due to the cell survival role, TAM receptors have been also extensively studied in cancer [20].

While structurally akin, PS and Gas6 exhibit distinct binding affinities for individual TAM receptors [21, 22] and exert divergent functions [23]. Gas6 binds mostly to Axl, while PS binds mostly to Tyro3 [18, 24]. Gas6 primarily binds to Axl, mediating mesangial cell proliferation and glomerular hypertrophy in early-stage DKD [25, 26]. The interaction between Gas6 and Axl can also promote inflammation in glomerulonephritis [27, 28]. Conversely, PS is considered a negative regulator of immune and inflammatory responses, participating in the clearance of apoptotic cells [29–31]. These anti-inflammatory properties of PS, in contrast to the pro-inflammatory attributes of Gas6, suggest a potential protective role for PS in the progression of kidney diseases. Over the past several years, our research has consistently demonstrated that PS, through its interaction with Tyro3, shields podocytes from injury in glomerular diseases, including DKD and FSGS. In this review, we aimed to summarize our work and that of others on the critical role of the PS-Tyro3 pathway in podocyte biology and glomerular diseases. Additionally, we discuss the possibility of developing Tyro3 agonists as potential drugs for the targeted treatment of glomerular diseases with a focus on podocyte injury.

Structure of Tyro3 and Its Ligands

The TAM receptors consist of an extracellular domain containing two immunoglobulin-like and two membranous proximal fibronectin III motifs, a transmembrane region, and an intracellular tyrosine kinase domain [32, 33]. Human Tyro3 is synthesized as an 890-amino acid protein. Although the predicted molecular weight of Tyro3 is 97 kD, the extracellular domain includes sites for NH2-linked glycosylation, resulting in Tyro3 proteins ranging in size from 100 to 140 kD due to potential posttranslational modifications [34]. The extracellular domain of Tyro3 serves as the ligand-binding region. Previous research has indicated that TAMs can undergo proteolytic cleavage by metalloproteases, leading to the formation of soluble TAMs, which are considered potential decoy receptors capable of inhibiting TAM-mediated signaling pathways [35, 36]. Tumor necrosis factor-α-converting enzyme (TACE; also known as ADAM17) has been identified as a key protease responsible for Mer cleavage in macrophages [37]. ADAM17 plays a role in the progression of chronic kidney disease through its pro-inflammatory and pro-fibrotic effects [38]. Recent studies have also demonstrated that TAM receptors can be cleaved into soluble intracellular fragments by ADAM10 and the γ-secretase [39]. Furthermore, soluble TAM receptors have been detected in the circulation and considered as biomarkers for autoimmune disorders [40–42]. Intriguingly, studies have shown that plasma and urinary soluble Tyro3 levels are higher in diabetic patients with macroalbuminuria compared to those with normoalbuminuria and are associated with renal injury [43]. We hypothesize that the elevated levels of soluble Tyro3 in DKD may result from

increased metalloprotease activity, leading to Tyro3 cleavage in podocytes and possibly other cell types. Soluble Tyro3 may function as a decoy receptor, inhibiting the Tyro3 pathway's protective effects. The loss of Tyro3's protective effects contributes to increased podocyte injury and DKD progression.

PS and GAS6 are recognized as the primary ligands for Tyro3, sharing approximately 40% sequence identity [44]. Both molecules feature an N-terminal Gla domain rich in glutamic acid, followed by four consecutive EGF-like domains, and a C-terminal sex hormone-binding globulin domain composed of two laminin G domains [45]. These laminin G domains play a crucial role in TAM binding and activating downstream signaling pathways. When the sex hormone-binding globulin-like domain at the ligands' C-terminus binds to the Ig domains of the receptors, it leads to Tyro3 receptor dimerization, forming homodimers and activating its protein tyrosine kinase domain through autophosphorylation of tyrosine residues in the cytoplasmic domain. This, in turn, provides docking sites for downstream signaling molecules, including proteins containing Src homology-2 and other phosphotyrosine-binding domains [34].

Both GAS6 and PS1 undergo constitutive γ-carboxylation on glutamic acid residues within their N-terminal Gla domains, a process dependent on vitamin K-dependent γ-carboxylase. This γ-carboxylation appears to influence their interaction with TAM receptors [46]. The bridging of GAS6 and PS1 to apoptotic cells is essential for fully activating TAM signaling in macrophages [22, 47].

In recent years, several additional TAM ligands, such as Tubby, tubby-like protein 1 (Tulp-1), and Galectin-3, have been discovered. While Tulp-1 can interact with Tyro3 in macrophages [48], galectin-3 and tubby exclusively bind to Mer [49]. Our prior research, along with other studies [50, 51], has demonstrated that PS serves as an endogenous ligand for Tyro3 in podocytes, whereas GAS6 predominantly interacts with Axl in mesangial cells. However, whether other endogenous TAM ligands, such as Tulp-1, also interact with Tyro3 in podocytes, remains unknown. The specificity of ligand-receptor binding for TAM receptors determines distinct downstream biological effects. For instance, the interaction between Gas6 and Axl induces mesangial cell proliferation and glomerular hypertrophy in early DKD [25, 26]. Additionally, Gas6-Axl interaction has been implicated in promoting inflammation in glomerulonephritis [27, 28]. Conversely, PS exerts an anti-inflammatory effect and plays a role in the clearance of apoptotic cells [29–31]. As we discuss in more detail below, our research has shown that the PS-Tyro3 interaction exerts anti-inflammatory and antiapoptotic effects in podocytes, thereby impeding the progression of glomerular disease [50, 51].

PS and Tyro3 Expression

While PS is predominantly found in plasma and primarily synthesized in the liver, it has also been established that PS synthesis occurs in cells and tissues beyond the liver, including endothelial cells and smooth muscle cells [29]. Earlier studies suggested an increase in serum PS levels in diabetes patients [52, 53]. However, more recent research indicates that serum PS levels remain relatively stable between normal controls and diabetic patients, whether with or without diabetic nephropathy (DN) [43]. Our investigations reveal an increase in both Pros1 mRNA and PS protein levels within the glomeruli of diabetic rats. Interestingly, there is no significant difference in PS levels in the plasma among these groups [50]. This suggests that PS expression is likely regulated locally within glomerular cells. Through immunostaining, we have observed increased PS expression in glomerular cells, particularly in podocytes, in kidney biopsy samples from diabetic patients exhibiting microalbuminuria. Conversely, PS expression decreases in diabetic patients with macroalbuminuria when compared to normal kidney tissue samples. This suggests that PS expression increases in the early stages but decreases in the later stages of DKD. Additionally, our findings show that glomerular endothelial cells express PS, although at lower levels than podocytes.

Conversely, GAS6 functions as an autocrine growth factor for mesangial cells. Double-staining immunohistochemistry demonstrates that most Gas6 and Axl are primarily co-localized in mesangial cells. The binding of Gas6 to its receptor Axl plays a critical role in mesangial cell proliferation [54].

Regarding the expression of TAM receptors, Axl and Mer are ubiquitously expressed in various cell types, including immune sentinel cells, vascular endothelial cells, professional phagocytes of the immune system, and multiple epithelial cell types. In contrast, Tyro3 is most abundantly expressed in the nervous system [55]. It is also expressed to a higher degree in the lungs, breast, pancreas, adipose tissue, skin, retina, kidneys, and several hematopoietic cell lines, including dendritic cells, monocytes/macrophages, and platelets [56, 57].

In human kidneys, we have discovered that Tyro3 expression is primarily localized in podocytes, as

evidenced by immunostaining. However, Tyro3 expression is markedly suppressed in the glomeruli of patients with progressive DKD [51]. Tyro3 mRNA levels are also significantly reduced in the glomeruli of progressive DKD patients, with a positive correlation noted with the estimated glomerular filtration rate (eGFR) in these individuals [51, 58]. Interestingly, Tyro3 expression increases in the glomeruli of type 2 diabetic Pima Indians with early and mild DKD, which aligns with our observations in human kidneys and diabetic mice [51]. Together, these data suggest that Tyro3 expression increases in the early stage of DKD but is suppressed in those with progressive DKD, similar to what we observed for PS. These data are consistent with a potential renal protective role of PS and Tyro3.

Furthermore, a substantial reduction in Tyro3 expression has been observed in the glomeruli of patients with FSGS. This reduction in Tyro3 mRNA is consistent in Tg26 mice, a model of HIV-associated nephropathy with collapsing FSGS [59]. In collaboration with the Nephrotic Syndrome Study Network Consortium (NEPTUNE), we have found that glomerular Tyro3 expression is reduced in patients with primary glomerular disease significantly correlated with eGFR, even after adjusting for age, gender, and race. This association remains significant when baseline eGFR and protein/creatinine ratio are considered.

Recent single-cell transcriptomic analysis studies in diabetic mice and humans support our findings, confirming that Tyro3 expression is almost exclusive to podocytes, while Mer and Axl are more widely expressed in kidney cells and immune cells [60, 61]. These studies suggest that Tyro3 is a specific marker for podocytes within the kidney.

In terms of regulation by the diabetic milieu, we have discovered that high glucose can upregulate both PS and Tyro3 expression in cultured human podocytes. Interestingly, in diabetic mice, the induction of Tyro3 expression is suppressed in podocyte-specific PS knockout (KO) mice, suggesting that Tyro3 expression is upregulated by its own ligand PS through a positive feedback loop in early DKD. To delve into the mechanism of Tyro3 suppression in progressive DKD, we performed in silico promoter analysis of Tyro3, revealing potential binding sites for NF-κB. In cultured human podocytes, we confirmed that TNF-α stimulation reduces Tyro3 expression, an effect alleviated by the NF-κB inhibitor, BAY 11-7082. Given the central role of NF-κB in progressive DKD [62–64] and other glomerular diseases [65], the suppression of Tyro3 expression in progressive kidney disease may, in part, be mediated by the activation of the NF-κB pathway. Loss of podocytes may also contribute to the low glomerular expression of Tyro3 in patients with advanced DKD.

Effects of PS and Tyro3 in Cultured Human Podocytes and in Experimental Animal Models of Glomerular Disease

PS, a plasma glycoprotein, is well established as an essential cofactor for activated protein C (APC), which inhibits coagulation factors FVa and FVIIIa in a thrombomodulin-dependent manner. In diabetic kidneys, thrombomodulin-dependent APC formation has been found to mediate cytoprotection by preventing apoptosis in glomerular endothelial cells and podocytes [66].

Our research demonstrates that PS safeguards podocytes from injury through its binding to and activation of Tyro3. In cultured human podocytes, we observed that shRNA-mediated silencing of PROS1, when incubated with high glucose, leads to an increased expression of BAX, suppression of BCL-2 expression, and a higher rate of cell death, as measured by caspase-3 activity [50]. As PS has a negative regulatory effect on inflammatory responses [67] and TNF-α-induced NF-κB signaling plays a pivotal role in DN [62, 68], we investigated this pathway. We found that PS can effectively suppress both high glucose- and TNF-α-induced NF-κB luciferase reporter activity, as well as the expression of several pro-inflammatory genes mediated by NF-κB [50].

To determine which of TAM receptors mediates the antiapoptotic and anti-inflammatory effects of PS, we conducted shRNA silencing experiments. Our findings confirm that the silencing of Tyro3, but not Mer or Axl, significantly mitigates the anti-inflammatory effects of PS in podocytes, underscoring the crucial role of Tyro3 in mediating the anti-inflammatory effects of PS.

To further confirm the roles of PS and Tyro3 in experimental models of glomerular disease, we generated a series of transgenic and KO mice for both PS and Tyro3. Initially, we created podocyte-specific Pros1 KO (PS-KO) mice by crossing Pros1 floxed mice (provided by Dr. G. Lemke) with podocin-Cre (Pod-Cre) transgenic mice. These mice exhibited normal viability, fertility, and were indistinguishable from wildtype (WT) littermates. Upon induction of diabetes in these mice through low-dose STZ injections, we observed a significant increase in albuminuria,

glomerular hypertrophy, mesangial expansion, podocyte loss, and injury in diabetic PS-KO mice compared to diabetic WT controls. To investigate whether increased PS expression could mitigate diabetic kidney injury in vivo, we employed intrarenal arterial injection of Pros1-expressing rAAV9 (rAAV9-PS) into the left kidneys of OVE26 mice, another genetic model of DKD [23, 24]. This led to a significant reduction in mesangial expansion and podocyte loss in the rAAV9-PS-injected left kidneys compared to the uninjected right kidneys. Additionally, injecting rAAV9-PS into both kidneys of diabetic OVE26 mice resulted in a significant reduction in albuminuria [50]. Similarly, we observed that STZ-induced Tyro3 KO mice developed increased albuminuria, glomerular hypertrophy, mesangial expansion, and podocyte injury compared to WT mice. The loss of Tyro3 also exacerbated albuminuria and podocyte injury in mice with adriamycin-induced nephropathy, a model of FSGS. We also generated a tetracycline-inducible podocyte-specific Tyro3 overexpression mouse model and found that inducing Tyro3 expression in podocytes ameliorated albuminuria, glomerular injury, and podocyte loss in three different animal models including diabetic OVE26 mice, adriamycin-induced nephropathy mice, and HIV-1 transgenic mice [51]. These in vivo results strongly support a protective role of PS-Tyro3 against podocyte injury in both primary and secondary glomerular diseases.

Mechanisms Mediating the Protective Effects of PS-Tyro3 in Podocytes

TAM receptors are known to trigger various signal transduction pathways, including PI3K/Akt, MAP kinase, NF-κB, and STAT [69]. However, most studies on these pathways have focused on Axl and Mer in immune cells [19, 69]. Nevertheless, numerous investigations have linked the PI3K pathway to Tyro3, emphasizing the critical roles of PI3K and its downstream effector Akt in protecting against apoptosis [70, 71].

In the context of glomerular cell growth, Gas6 functions as a growth factor, particularly influencing the Akt/mammalian target of rapamycin pathway [26, 72]. The binding of Gas6 to Axl induces Axl phosphorylation, activating the PI3K/Akt pathway, which exhibits pro-survival and antiapoptotic effects [73]. Upregulating Gas6/Axl signaling has proven protective, reducing tubulointerstitial apoptosis and slowing chronic kidney disease progression [74].

Our research indicates that PS exerts antiapoptotic and anti-inflammatory effects in podocytes by binding to Tyro3, likely activating the Akt pathway and inhibiting the NF-κB pathway [50]. Tyro3 is activated through autophosphorylation, subsequently binding to the p85 subunit of PI3K via phosphorylated Tyro3. This activation suppresses apoptosis and enhances cell proliferation, differentiation, and survival [75]. Nevertheless, the direct interaction between Tyro3 and PIK3R1 in podocytes remains unclear, as this was previously described only in NIH3T3 cells [76]. While it is known that TAM receptors regulate the NF-κB pathway, the specific mechanism by which Tyro3 inhibits NF-κB in podocytes is yet to be determined. Additionally, Tyro3 may interact with other signaling molecules, activating various downstream pathways in podocytes.

Apoptosis plays a pivotal role in podocyte loss in glomerular diseases, and the PI3K/Akt signaling pathway is crucial for podocyte protection [77]. In cultured podocytes, angiotensin II has been reported to promote apoptosis by inhibiting Akt phosphorylation [78], while PI3K/Akt activation can inhibit kidney cell apoptosis and reduce interstitial fibrosis [79]. This pathway activates downstream molecules like glycogen synthase kinase 3β (GSK3β), mammalian target of rapamycin, and actin-related proteins [80], all of which are critical for podocyte biology and glomerular disease. Fully active Akt regulates various cellular processes, including the cell cycle, protein synthesis, transcription, and cell migration [81, 82]. CD2AP, highly expressed in podocytes, interacts with the p85 regulatory subunit of PI3K to recruit PI3K to the plasma membrane, stimulating PI3K-dependent Akt signaling in podocytes, maintaining Akt activation and cell survival [83]. Tyro3 likely contributes to maintaining normal Akt activity, ensuring podocyte survival. Consequently, the loss of Tyro3 leads to podocyte loss and the progression of glomerular disease.

Therapeutic Role of Tyro3 Agonists in Glomerular Disease

Our findings highlight the protective role of PS-Tyro3 against podocyte injury in DKD and FSGS [50, 51]. However, using PS as a drug is challenging due to its significant effect on coagulation. To overcome this, there is a need to develop new Tyro3 agonists that can treat DKD and FSGS without affecting coagulation.

Designing agonists is more complex than antagonists, and the absence of Tyro3's crystal structure presents additional challenges. Our recent study [61] used

homology modeling by focusing on amino acid sequences at allosteric interaction points. By studying the structures of two known Tyro3 ligands (Pros1 and Gas6), we identified unique amino acid sequences specific to PS. PS's amino-terminal Gla domain, which requires gamma-carboxylation with vitamin K for activation, displayed significant differences between Gas6 and Pros1. This domain became our focal point for developing more specific Tyro3 agonists. Additionally, we explored Tyro3's tyrosine autophosphorylation site, crucial for receptor dimerization and activation. Our analysis revealed a unique sequence (Y804INI) for Tyro3, distinct from Axl and Mer, offering potential targets for designing specific Tyro3 compounds. From these efforts, we designed 12 compounds, identifying three pharmacophore groups.

Biological activity screening identified C-8 and C-10 as potential Tyro3 agonists. Notably, these compounds induced Tyro3 and Akt phosphorylation and suppressed NF-κB target gene expression in podocytes. C-10 emerged as a strong candidate, as it induced Tyro3 phosphorylation and Akt activation specifically in Tyro3-overexpressing cells. Subsequent in vivo studies in mice with adriamycin-induced nephropathy and diabetic db/db mice demonstrated C-10's ability to attenuate proteinuria, glomerular injury, and podocyte loss. These protective effects were Tyro3-dependent because C-10 loses its renal protective effects in Tyro3 KO mice, validating C-10 as a selective Tyro3 agonist with therapeutic potential for glomerular disease. Importantly, no apparent toxicity was observed during the treatment period.

Challenges and Considerations

Using Tyro3 agonists as a therapy for glomerular disease presents concerns. Recent studies have implicated TAM receptors in cancer progression, leading to the development of TAM receptor inhibitors for certain cancers. Tyro3, specifically, has been associated with tumor cell survival, proliferation, metastasis, and chemotherapy resistance [70]. Overexpressed Tyro3 has been linked to decreased overall survival in patients with various cancers [70]. While the exact role of Tyro3 in tumorigenesis remains unclear, it is evident that Tyro3's cell survival and anti-inflammatory effects, beneficial in podocytes, may have contrasting consequences in cancer. The similar observation was reported for other molecules such as Yes-associated protein. Yes-associated protein, a downstream gene of Hippo kinase is a cell survival protein for podocytes but may promote tumorigenesis [84]. The challenge is to mitigate potential tumorigenesis risks while using Tyro3 agonists to treat glomerular disease.

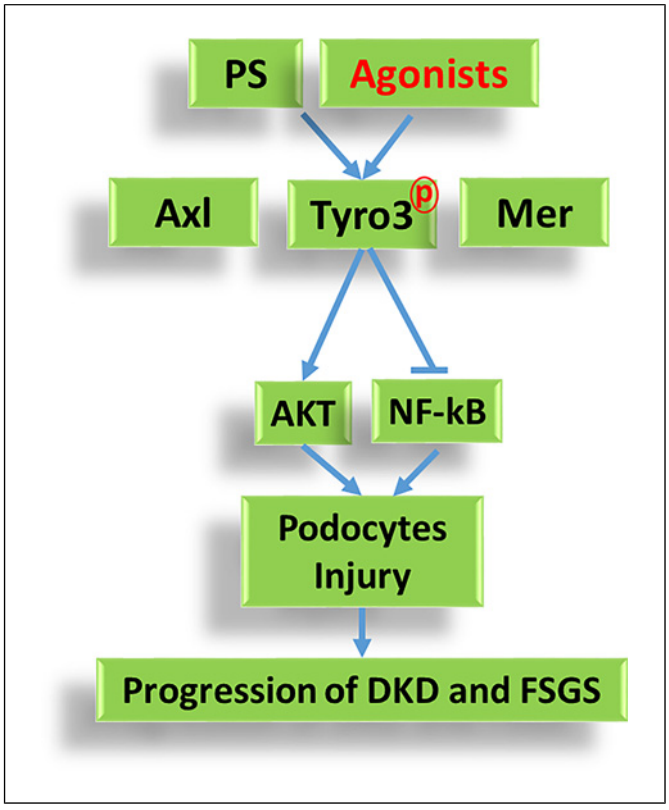

Fig. 1. Summary of the renal protective effects of PS and Tyro3 in the progression of DKD and FSGS.

Preliminary investigations found that C-10 did not induce cell proliferation in a gastric cancer cell line at concentrations significantly higher than those required for Tyro3 phosphorylation in podocytes. Moreover, long-term treatment with C-10 in mice did not lead to cancer development. Nevertheless, further studies are needed to assess potential cancer risks. Additionally, low-dose Tyro3 agonist treatment and targeted drug delivery strategies to podocytes or glomeruli should be explored to minimize systemic side effects [85–88].

Conclusion

Tyro3 is highly expressed in podocytes and plays a pivotal role in podocyte biology (Fig. 1). Its expression correlates with glomerular disease progression, making it a potential biomarker for podocyte injury and glomerular disease progression. PS, via Tyro3 activation, exerts anti-inflammatory and antiapoptotic effects, likely through Akt pathway activation and NF-κB pathway inhibition. In

vivo studies confirm the crucial roles of PS and Tyro3 in both primary and secondary glomerular diseases. Tyro3 agonists have been developed and shown to attenuate podocyte injury and glomerular disease in animal models. However, potential tumorigenesis risks must be carefully considered when developing Tyro3 agonists as a therapy for glomerular disease.

Conflict of Interest Statement

The authors declare that they have no competing financial interests.

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Author Contributions

L.M., Y.C., F.Z., L.G., K.L., and J.C.H. participated in the writing and revising the manuscript.

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