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The β isoform of GSK3 mediates podocyte autonomous injury in proteinuric glomerulopathy

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Abstract

Converging evidence points to glycogen synthase kinase (GSK) 3 as a key player in the pathogenesis of podocytopathy and proteinuria. However, it remains unclear if GSK3 is involved in podocyte autonomous injury in glomerular disease. In normal kidneys, the β isoform of GSK3 was found to be the major GSK3 expressed in glomeruli and intensely stained in podocytes. GSK3 β expression in podocytes was markedly elevated in experimental or human proteinuric glomerulopathy. Podocyte specific somatic ablation of GSK3 β in adult mice attenuated proteinuria and ameliorated podocyte injury and glomerular damage in experimental adriamycin (ADR) nephropathy. Mechanistically, actin cytoskeleton integrity in podocytes was largely preserved in GSK3 β knockout mice following ADR insult, concomitant with a correction of podocyte hypermotility and lessened phosphorylation and activation of paxillin, a focal adhesion associated adaptor protein. In addition, GSK3 β knockout diminished ADR induced NF κ B RelA/p65 phosphorylation selectively at serine 467, suppressed *de novo* expression by podocytes of NF κ B dependent podocytopathic mediators, including B7-1, cathepsin L and MCP-1, but barely affected the induction of NF κ B target prosurvival factors, such as Bcl-xL. Moreover, the ADR-elicited podocytopenia and podocyte death was significantly attenuated in GSK3 β knockout mice, associated with a protection against podocyte mitochondrial damage and reduced phosphorylation and activation of cyclophilin F, a structural component of mitochondria permeability transition pores. Overall, our findings suggest that the β isoform of GSK3 mediates autonomous podocyte injury in glomerulopathy by integrating multiple podocytopathic signalling pathways.

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Keywords

inducible gene knockout; glycogen synthase kinase 3 β ; podocyte; adriamycin; proteinuria; glomerulopathy

Introduction

Regardless of the original aetiology, the pathogenic basis of persistent proteinuria is generally attributable to podocyte dysfunction or injury [1]. Converging evidence recently points to glycogen synthase kinase (GSK) 3 as a novel therapeutic target for podocytopathy and proteinuria [2-4]. In accordance with this, blockade of GSK3 has been shown to attenuate proteinuria, podocyte injury and glomerulosclerosis in a number of experimental glomerular diseases, including diabetic nephropathy [5], lupus nephritis [6], and podocytopathy elicited by adriamycin (ADR) or LPS [2-4]. GSK3 is a highly conserved, ubiquitously expressed, and constitutively active multitasking serine/threonine protein kinase, situated at the nexus of a multitude of essential pathways in the complex cell signalling network and acting on a multitude of cognate substrates, including NF κ B, β -catenin, paxillin, cyclophilin F (Cyp-F), and cyclin D1 [3,4,7-11]. GSK3 exists as two isoforms, GSK3 α (51 kDa) and GSK3 β (47 kDa), which are encoded by separate genes that produce highly homologous proteins with significant difference only in their N- and C-terminal regions [12]. Although displaying 84% structural homology, GSK3 α and GSK3 β are not functionally interchangeable, and each possesses unique and distinct biological actions [13-17]. The two isoforms exert similar or distinct roles in diverse cellular processes, including glycogen metabolism, cell cycle progression, cytoskeletal organization, inflammation and immunity, and mitochondria permeability transition, and have been recently identified as a putative target for the treatment of disorders including diabetes, cancer, Alzheimer's disease and glomerulopathy [17-21].

Despite burgeoning evidence suggesting a role for GSK3 in podocyte injury, previous data are not conclusive due to the following limitations. First, most studies relied exclusively on the use of small molecule inhibitors of GSK3 β , which acts in a concentration-dependent way and may possess additional unknown properties, such as off-target activities. Secondly, chemical inhibitors developed so far all possess a poor selectivity between the isoforms of GSK3, even though it remains unknown if the two isoforms of GSK3 are equally expressed in glomerular podocytes or play the same role in the pathogenesis of podocyte injury. Thirdly, podocyte injury and proteinuria in most glomerular diseases result from both podocyte autonomous and non-autonomous pathogenic processes. Thus, with systemic use of chemical inhibitors of GSK3 β *in vivo* it is impossible to discriminate a direct podocyte effect from an effect secondary to systemic actions, such as immunomodulation [22]. Genetic knockout of GSK3 β would be a conclusive approach with great targeting selectivity, but unfortunately has caused embryonic lethality [23]. To circumvent these issues, inducible deletion of GSK3 β in mature glomerular podocytes offers an ideal and preferred approach to avoid potential congenital defects of the kidney. In this study, mice with the doxycycline-inducible podocyte specific somatic ablation of GSK3 β were exposed to ADR injury and the

role of GSK3 β in podocyte injury and proteinuria was delineated in knockout mice *in vivo* and in primary podocytes *in vitro*.

Materials and Methods

Animal studies

Animal studies were approved by the institution's Animal Care and Use Committee and they conformed to the United States Department of Agriculture regulations and the National Institutes of Health guidelines for humane care and use of laboratory animals.

Mice with the floxed *GSK3 β* gene (*GSK3 β ^{fl/+}*), on a genetic background of C57/B6J, were used previously [15,24] and backcrossed for 10 generations onto an FVB/N congenic background. To generate mice with doxycycline-inducible podocyte-specific GSK3 β KO genotype (*NPHS2^{rtTA}/TRE^{Cre}/GSK3 β ^{fl/fl}*), *GSK3 β ^{fl/+}* mice were respectively crossed with *TRE^{Cre}* mice and *NPHS2^{rtTA}* mice on a genetic background of FVB/N (Jackson Laboratory, Bar Harbor, ME). All animals were born normally at the expected Mendelian frequency. For the induction of podocyte-specific *GSK3 β* deletion, 8- to 10-week-old mice with KO genotype received oral doxycycline (TCI, Tokyo, Japan; 2mg/ml with 5% sucrose, protected from light) for 2 wk and were subsequently designated as KO mice. Littermates with the same KO genotype but not treated with doxycycline served as control (Ctrl). Littermates with the *NPHS2^{rtTA}/GSK3 β ^{fl/fl}* genotype were treated with doxycycline for 2 wk and served as *Cre*-negative control (*Cre*-Ctrl). A routine PCR protocol was performed (Figure S1) for genotyping tail DNA samples using transgene-specific primers (Supplementary Table S1).

KO mice, Ctrl and *Cre*-Ctrl littermates aged 10 to 12 wk were randomly assigned to receive ADR (25 mg/kg) or vehicle treatment *via* tail vein injection on day 0. Spot urine was collected and mice were sacrificed on post injury days 0, 2, 6, and 14. Six mice were randomly assigned to each group for each observed time point. For additional experiments, male Balb/c mice (Jackson Laboratory) aged 8 wk received a low dose of ADR (10mg/kg) or vehicle *via* tail vein injection and were sacrificed 4 wk later.

Patients

This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of Shanghai Tenth People's Hospital of Tongji University School of Medicine. Written informed consent was obtained from each subject. Archived formalin-fixed paraffin-embedded kidney biopsy tissues were randomly chosen for analysis from three patients diagnosed with each of the following renal diseases, including idiopathic focal segmental glomerulosclerosis (FSGS), class V lupus nephritis [LN(V)] and tubulointerstitial nephritis (TIN). Morphologically normal tissues from the unaffected areas of renal tumour nephrectomy specimens were used as controls.

Urinary and serum analyses

Urine samples were subjected to SDS-PAGE followed by Coomassie Brilliant Blue (Sigma) staining. Urine albumin concentration was measured using a mouse albumin ELISA quantitation kit (Bethyl Laboratories Inc., Montgomery, TX, USA). Urine and serum

creatinine concentration was measured by a creatinine assay kit (BioAssay Systems, Hayward, CA, USA).

Histology and immunohistochemical staining

Formalin-fixed mouse kidneys were embedded in paraffin and 3 μ m-thick sections prepared. Sections were processed for PAS staining or immunohistochemical staining. A semiquantitative glomerular damage index [25-28] was used to evaluate the degree of glomerular damage. The detailed protocol is provided in the Supplementary Methods.

Glomerular isolation and primary podocyte culture

Glomeruli were isolated from mouse kidneys by a magnetic beads based approach as described previously [3]. Primary podocytes were prepared from the isolated glomeruli as reported before [29-31]. A detailed protocol is provided in the Supplementary Methods.

Western immunoblot analysis and immunoprecipitation

Cultured cells were lysed and animal tissues homogenized for immunoblot analysis or immunoprecipitation. Detailed protocols are provided in the Supplementary Methods.

Measurement of cell apoptosis

Fixed cells were subjected to TUNEL staining by using a commercial kit (Roche Molecular Biochemicals, Mannheim, Germany) as described previously [32].

Statistical Analysis

For immunoblot analysis, bands were scanned and the integrated pixel density was determined using a densitometer and the ImageJ analysis program. All data are expressed as means $\pm$ SD. Data from two groups were compared by the *t* test. For multiple comparisons of values, ANOVA was performed followed by the Student-Newman-Keuls test or the Scheffé test; *p* < 0.05 was considered significant.

Results

GSK3 β is the major isoform of GSK3 expressed in podocytes

The two isoforms of GSK3, GSK3 α and GSK3 β , have been recently shown to have distinct distributions in distal nephrons [33]. In mouse kidney, both the α and the β isoforms were evidently expressed in mouse renal parenchyma as shown by immunoperoxidase staining (Figure 1A). In glomeruli, the β rather than the α isoform was mainly detected and was located within the mesangium and the periphery of glomerular tufts, consistent with a podocyte distribution. Immunoblot analysis demonstrated that GSK3 β was expressed in much greater abundance than GSK3 α in isolated glomeruli, primary podocytes and an immortalized podocyte cell line (Figure 1B). In contrast, in whole kidney homogenates, GSK3 α was detected at a level almost equal to GSK3 β (Figure 1B). Likewise, in normal human kidney specimens, immunostaining also revealed predominant GSK3 β expression in glomeruli, with intense staining clearly localized within podocytes at the periphery of glomerular tufts (Figure 1C). Computerized morphometric analysis (Figure 1D, E) precisely

quantified the integrated pixel densities of the staining and indicated that the level of GSK3 β expression was 1.5-fold greater than GSK3 α in glomeruli of human kidney specimens (Figure 1E).

Podocyte expression of GSK3 β is amplified in proteinuric glomerulopathy

Increased expression of GSK3 β in renal tubules has been implicated in the pathogenesis of progressive chronic kidney disease [34]. Whether glomerular expression of GSK3 β alters in glomerular disease was explored next. We first assessed experimental FSGS in ADR-injured Balb/c mice [35,36]. Four wk after ADR injury, representing the chronic stage of ADR nephropathy, glomerular staining of GSK3 β was markedly increased with intense immunostaining located in podocytes at the periphery of glomerular tufts (Figure 2A). To determine if the elevated glomerular expression of GSK3 β also occurs in human proteinuric glomerular disease, kidney biopsy tissues were examined. As shown in Figure 2B, the staining of GSK3 β in glomeruli was substantially enhanced in two proteinuric glomerulopathies, FSGS and LN(V), with strong staining localised to podocytes at the periphery of glomerular tufts, compatible with the findings in experimental ADR nephropathy. In contrast, only a weak level of induced glomerular expression of GSK3 β was noted in TIN. The morphological findings were further corroborated by the computerized morphometric analysis, which demonstrated 1.6 fold induction in mouse ADR nephropathy as compared with control mice (Figure 2C), and 1.1, 2.6 and 3.1 fold induction respectively in TIN, FSGS and LN(V) as compared with normal human kidney tissues (Figure 2D).

Podocyte specific ablation of GSK3 β attenuates proteinuria, podocyte injury and glomerular damage in experimental ADR nephropathy

To further substantiate a possible role of GSK3 β in the pathogenesis of proteinuria and podocyte injury, the doxycycline-inducible Cre/*loxP* mediated gene targeting strategy was adopted to ablate GSK3 β selectively in mature podocytes (Figure S2A). Following doxycycline treatment, KO mice exhibited no detectable abnormalities in development, kidney gross morphology, function (Figure S3) or histology (Figure 3) as compared with Ctrl littermates. Dual colour immunofluorescent staining revealed striking loss of GSK3 β immunoreactivity specifically in synaptopodin-positive podocytes in KO mice (Figure S2A), inferring a successful knockout. To confirm this finding, primary podocytes were isolated and cultured from glomeruli prepared from WT, KO and Ctrl mice and subjected to immunoblot analysis (Figure S2B) and fluorescent immunostaining (Figure S2C). GSK3 β was drastically ablated, whereas GSK3 α expression was barely affected (Figure S2B) in primary KO podocytes that were characterized by the expression of typical podocyte markers, including podocin, Wilms' tumor 1 (WT1) and synaptopodin (Figure S2C). Of note, GSK3 β knockout seemed barely to affect β -catenin signalling, a major downstream pathway of GSK3, because the levels of total β -catenin and nuclear translocation of β -catenin were comparable between KO podocyte and Ctrl or WT podocytes, as determined by immunoblot analysis (Figure S2B).

To examine the effect of GSK3 β knockout on podocyte injury *in vivo*, ADR nephropathy was developed in KO, Ctrl and *Cre*Ctrl mice. In *Cre*Ctrl and Ctrl mice, ADR injury was equally effective at eliciting a heavy proteinuria that peaked on day 6 and partially receded

on day 14, as estimated by urine electrophoresis and quantified by urine albumin to creatinine ratios (Figure 3A, B). This was associated with prominent renal histological injury on PAS staining (Figure 3C), featured by large protein casts in tubules, glomerular synechiae, mesangial extracellular matrix accumulation and progressive glomerulosclerosis (Figure 3C). In addition, ultrastructural injuries in podocytes, such as extensive podocyte foot process effacement, were evident from transmission electron microscopy images (Figure 3D). These morphological findings were further corroborated by the semiquantitative morphometric measurements of glomerular damage index (Figure 3E) and by absolute count of the number of foot process per unit length of glomerular basement membrane (Figure 3F). In KO mice, the ADR induced proteinuria was drastically attenuated (Figure 3A, B), concomitant with lessened histological injury in glomeruli and tubulointerstitium (Figure 3C, E) and improved podocyte injury and foot process effacement (Figure 3D, F). In parallel, focal lesions of fibrosis and inflammatory infiltration in the kidney 14 d after ADR injury were ameliorated in KO mice, as shown by immunofluorescent staining (Figure 3G) and immunoblot analysis of kidney homogenates (Figure 3H) for fibronectin and the leukocyte common antigen CD45.

Loss of podocyte GSK3 β improves podocyte focal adhesions and cytoskeleton integrity *in vivo* and *in vitro* upon ADR injury

Disruption of actin cytoskeleton integrity is the molecular basis for podocyte foot process effacement [37-40]. To determine if the attenuated podocyte foot process effacement found in KO mice after ADR injury is attributable to any improvement in actin cytoskeleton in podocytes, kidney specimens procured from animals on day 6 were subjected to both phalloidin labelling for filamentous actin (F-actin) and immunofluorescence staining for synaptopodin (Figure 4A). Confocal fluorescence microscopy demonstrated that intense F-actin staining was present extensively all over the glomerular tufts of normal kidneys from KO and Ctrl mice. Podocyte specific F-actin was highlighted by co-localization of F-actin signals (red) with synaptopodin expression (green) in glomeruli. ADR injury in Ctrl mice resulted in a remarkable reduction in F-actin and synaptopodin co-localization, indicative of disruption of actin cytoskeleton integrity in podocytes. This injurious effect of ADR was significantly abrogated in KO mice. To corroborate the morphological findings, computerized morphometric analysis was performed and revealed that the ratio of integrated pixel densities between the signal of podocyte specific F-actin (yellow signal) and the signal of podocytes (green signal) was decreased in Ctrl mice but considerably preserved in KO mice following ADR injury (Figure 4B). Evidence suggests that GSK3 β dictates the phosphorylation and activation of paxillin [3,41], which is a core structural and regulatory component of focal adhesions, the crucial determinant of cellular actin cytoskeleton dynamics and motility. In consistency, knockout of GSK3 β dramatically abolished the ADR elicited paxillin hyperphosphorylation in glomeruli, shown by immunoblot analysis of the isolated glomeruli (Figure 4C).

To understand if the protective effect on podocyte cytoskeleton observed in KO mice may be ascribed primarily to a direct effect on podocytes, primary podocytes were prepared from Ctrl or KO mice and examined. Podocytes are motile cells with considerable constitutive motility [42]. Indeed, as shown by a traditional cell migration assay, podocytes derived from

Ctrl mice possessed a substantial migratory capacity under basal conditions that lessened the distances between the leading edges of the migrating podocyte sheets (Figure 4D). This basal migratory capacity was slightly reduced in podocytes isolated from KO mice, as quantified by morphometric analysis of cell migration areas (Figure 4E). ADR injury strikingly accelerated the closure of the gap between the invading fronts of podocytes from Ctrl mice, indicative of podocyte hypermotility. This effect of ADR was markedly abrogated in podocytes from KO mice, suggesting that loss of GSK3 β impedes podocyte hypermotility. The difference seen in the motility assay was unlikely to be attributable to distinct proliferative ability, because, as terminally differentiated cells, primary podocytes exhibited a low proliferative ability that was comparable between KO and WT podocytes as shown by immunoblotting for proliferating cell nuclear antigen (Figure S4). Focal adhesion turnover is a prerequisite for cell spreading and migration [43-45]. As a key adaptor and structural component of the focal adhesions, paxillin was hyperphosphorylated in Ctrl podocytes upon ADR injury, as measured by immunoblot analysis. This effect was markedly mitigated in KO podocytes (Figure 4F). To visualize the changes in actin cytoskeleton, fluorescent immunocytochemistry for paxillin and phalloidin labelling for F-actin was carried out in primary cells. ADR injury in Ctrl podocytes caused a typical disruption of actin cytoskeleton, manifested as increased distribution of cortical filaments, diminished ventral stress fibres, more transverse arcs, and sporadic short dorsal stress fibres that were connected to focal adhesions only at one end, marked by colocalization of F-actin with paxillin. In contrast, KO podocytes largely preserved F-actin stress fibres following ADR and retained the pattern of focal adhesion distribution (Figure 4G).

Loss of podocyte GSK3 β diminishes the ADR elicited proinflammatory NF κ B activation and *de novo* expression of NF κ B dependent podocytopathic mediators in podocytes *in vivo* and *in vitro*

Evidence suggests that podocyte *de novo* expression of multiple podocytopathic mediators, such as B7-1 [46] and cathepsin L [47], is implicated in podocyte cytoskeleton disorganization and foot process effacement. Of note, expression of these podocytopathic molecules is under the control of NF κ B [48,49]. In agreement, ADR injury in Ctrl mice elicited marked expression of a multitude of NF κ B target molecules, including monocyte chemoattractant protein-1 (MCP-1), B7-1, cathepsin L and the prosurvival protein Bcl-xL, concomitant with an induced phosphorylation of NF κ B RelA/p65 at all serine residues (serine 276, 467 and 536) as assessed by immunoblot analysis of the isolated glomeruli (Figure 5A). The expression of these molecules in glomeruli was predominantly localised to podocytes that were positive for synaptopodin or WT1, as revealed by fluorescent immunohistochemistry, implying a podocyte specific induction of NF κ B dependent molecules involved in both podocytopathy and prosurvival (Figure 5B). Our latest data indicate that the serine 467 residue of RelA/p65 lies within the consensus motif for GSK3 β phosphorylation and serves as a cognate substrate of GSK3 β [9]. In podocytes, GSK3 β fine tunes NF κ B activation and directs the expression of selective NF κ B target molecules, including podocytopathic mediators, but not Bcl-xL [2]. Congruously in this study, the ADR elicited expression of podocytopathic factors, like MCP-1, B7-1 and cathepsin L, was considerably abrogated in KO mice, but the Bcl-xL induction in podocytes was largely preserved, associated with a diminished phosphorylation of NF κ B RelA/p65 selectively at

serine 467. To verify if loss of GSK3 β affects proinflammatory NF κ B activation and *de novo* expression of podocytopathic mediators in a podocyte autonomous fashion, primary podocytes were examined next. Shown in Figure 5C, ADR injury induced RelA/p65 phosphorylation at all serine residues in Ctrl podocytes and elicited the expression of diverse NF κ B target molecules, including both prosurvival Bcl-xL and podocytopathic mediators, like MCP-1, B7-1 and cathepsin L, as measured by immunoblot analysis. In contrast in KO podocytes, the ADR induced RelA/p65 phosphorylation was selectively mitigated at serine 467, associated with minimal induction of podocytopathic mediators, like MCP-1, B7-1 and cathepsin L, but a preserved up-regulation of prosurvival Bcl-xL. Fluorescent immunocytochemistry demonstrated prominent *de novo* expression of B7-1 and cathepsin L in Ctrl podocytes but minimal expression in KO podocytes following ADR injury (Figure 5D).

GSK3 β knockout mitigates the ADR elicited mitochondria dysfunction and podocyte loss *in vivo* and *in vitro*

As a prototypical pro-oxidant, ADR is known to cause podocyte loss by inducing oxidative stress and podocyte mitochondrial dysfunction, ensued by podocyte loss and podocytopenia [50]. In concordance with this fluorescence immunohistochemistry (Figure 6A) and immunoblot analysis of isolated glomeruli for WT1 (Figure 6B) indicated that ADR injury resulted in striking podocytopenia in Ctrl mice on day 6, associated with evident mitochondria damage in podocytes, characterized by decreased number of mitochondria, mitochondrial swelling and fragmentation, destruction of the inner mitochondrial membrane and disruption of mitochondrial cristae on electron microscopy (Figure 6C). In contrast, the ADR elicited podocytopenia and podocyte mitochondria damage was ameliorated in KO mice. To explore if GSK3 β knockout affects autonomous podocyte injury, primary podocytes were examined. Upon ADR injury, KO *versus* Ctrl podocytes developed much less apoptosis as evaluated TUNEL staining (Figure 6D) and validated by immunoblot analysis of cell lysates for cleaved caspase 3 (Figure 6E). Under basal condition, MitoTracker Red labelling of Ctrl or KO podocytes (Figure 6F) revealed a healthy complex network of mitochondria of elongated, branched and non-uniform shape spreading throughout the whole podocyte cell body and stretching out to podocyte extensions. Upon ADR injury, the mitochondria network in Ctrl podocytes was drastically disrupted into smaller mitochondria in a shape of small spheres or short rods that were mainly concentrated in the perinuclear area. This effect was dramatically attenuated in KO podocytes, suggesting a direct protective effect of GSK3 β knockout on mitochondria injury. Dual colour staining for cleaved caspase-3 and mitochondria demonstrated that mitochondrial damage preceded overt apoptosis, and both were sequentially mitigated in KO podocytes upon ADR insult, inferring that the lessened mitochondrial damage in KO podocytes is a primary effect of GSK3 β ablation rather than an effect secondary to the anti-apoptotic action. Our latest data indicated that Cyp-F, a core structural constituent of the mitochondria permeability transition (MPT) pore, is a substrate for GSK3 β [4]. In agreement, loss of GSK3 β in KO podocytes abolished the ADR elicited phosphorylation of Cyp-F, as shown by immunoblot analysis of immunoprecipitated Cyp-F for phosphorylated serine (Figure 6G).

Discussion

As a key structural constituent of the glomerular filtration barrier, podocyte dictates glomerular permselectivity and its injury has been implicated to play a central role in the pathogenesis of proteinuria and progression of chronic renal disease [1,51-53]. This study examined the effect of podocyte specific ablation of GSK3 β on podocytopathy and the underlying mechanisms. Our results suggest that GSK3 β signalling is responsible, at least in part, for podocyte autonomous injury.

The finding in this study that podocyte specific ablation of GSK3 β attenuated proteinuria and podocyte injury is consistent with the data using chemical inhibitors, like lithium or TDZD-8 [2,4]. However, it seems that pharmacologic blockade of GSK3 β attained a pronounced proteinuria-reducing efficacy. This discrepancy might be ascribed to the difference in the mechanism of action between systemic and podocyte specific GSK3 β inhibition. In addition to an effect on podocyte autonomous injury, systemic blockade of GSK3 β by chemical inhibitors is also able to regulate global immunity [22] and thus possibly corrects the ADR impaired systemic immune homeostasis, which contributes to podocytopathy and proteinuria by generating podocyte injurious immunogenic factors [35]. Alternatively, systemic inhibition of GSK3 β might protect other kidney parenchymal cells from injury, like glomerular endothelial cells and mesangial cells. Apart from podocytes, GSK3 β also plays a detrimental role in mediating injuries in other kidney cells and nephron segments [33,54]. At least, amelioration of glomerular endothelial injury has been shown to improve glomerular filtration barrier and attenuate proteinuria in experimental ADR nephropathy [55].

The glomerular filtration barrier is a highly dynamic structure. In support of this, perfusion of rat kidneys with the polycation protamine sulphate elicits foot process effacement and proteinuria within a few minutes, which can be fully reversed within 15 min by the administration of the polyanion heparin [56]. These rapid changes of podocyte foot processes architecture require foot processes to retract or elongate, hence to “move” quickly along the glomerular basement membrane. Most importantly, podocyte movement was directly observed *via* multiphoton imaging in injured glomeruli in rats with experimental FSGS, suggesting that podocyte motility occurs *in vivo* [57]. Built upon this observation, podocyte cultures have been used to study their motility patterns *in vitro* [58]. Stimuli that induce foot process effacement *in vivo* (e.g., LPS or ADR) have been found to induce hypermotility of cultured podocytes in cell migration assays. In our study, the ADR-elicited hypermotility was attenuated in KO podocytes, indicative of a pro-motility effect of GSK3 β . GSK3 β seems to exert this effect by regulating multiple structural components involved in the control of cellular motility, including cytoskeletons and focal adhesions. Indeed, GSK3 β is able to directly catalyse the phosphorylation and activation of paxillin, a focal adhesion associated adaptor protein, and thereby accelerate focal adhesion turnover, resulting in podocyte hypermotility and disruption of actin cytoskeleton integrity. Besides, GSK3 β was found to catalyse the phosphorylation of serine 467 of NF κ B RelA, which specifies the transcription of a subset of NF κ B dependent molecules involved in podocyte cytoskeleton disorganization, like MCP-1, B7-1, and cathepsin L (Figure 6H). Moreover, GSK3 β is also likely involved in MPT. Knockout of GSK3 β reduced the activation of clyclophilin F, a key

structural component of the MPT pore, resulting in a lessened mitochondrial damage and apoptosis in podocytes (Figure 6H).

In addition to the regulation of podocyte cytoskeletons, NF κ B-dependent acquisition of proinflammatory phenotypes, and MPT, GSK3 β is also a crucial element of the canonical Wnt/ β -catenin pathway, which has been recently implicated in podocytopathy [59,60]. However, a drastic inhibition of GSK3 seems to be essential to activate the β -catenin pathway. In support of this, Doble *et al.* revealed a functional redundancy of GSK3 α and GSK3 β in Wnt/ β -catenin signalling by using compound knockouts of GSK3 α and β [61]. They found that gene deletion of at least three of the four alleles of both isoforms of GSK3 α and β is required to show an appreciable change in β -catenin activation. In consistency, our study demonstrated that knockout of GSK3 β barely activate β -catenin in podocytes.

In summary, podocyte specific ablation of GSK3 β attenuated podocyte injury and ameliorated proteinuria in ADR nephropathy. Our data suggest that GSK3 β mediates podocyte autonomous injury in proteinuric glomerulopathy possibly by integrating multiple podocytopathic signalling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The β rather than the α isoform of GSK3 is predominantly expressed in glomeruli and podocytes in mouse and human kidneys

(A) Representative micrographs of peroxidase immunohistochemistry staining of mouse kidney specimens for GSK3 α or GSK3 β . Black arrowheads and white arrows indicate positive staining in tubular cells and glomerular cells, respectively. As a negative control, the primary antibody was replaced by preimmune IgG and no specific staining was noted. Scale bar = 20 μ m. (B) Whole mouse kidney homogenates, homogenates of glomeruli isolated from mouse kidneys, and lysates of immortalized podocytes originating from our previous study [3] or primary cultures of podocytes were subjected to immunoblot analysis for

GSK3 α / β using a monoclonal antibody that recognizes GSK3 α and GSK3 β equally. (C) Representative micrographs of immunoperoxidase staining of human normal nephrectomy samples for GSK3 α and GSK3 β . Black arrowheads and white arrows indicate positive staining in tubular cells and glomerular cells, respectively. As a negative control, the primary antibody was replaced by preimmune IgG and no specific staining was noted. Scale bar = 20 μ m. (D) Computerized morphometric analysis of GSK3 α or GSK3 β in glomeruli of human normal kidney specimens. (E) Relative integrated pixel density of the staining of the α or the β isoform of GSK3 in glomeruli in normal human kidney specimens, as measured by computerized morphometry; * p = 0.007 (n = 3, unpaired t -test).

Figure 2. Glomerular expression of GSK3 β is amplified in experimental and human proteinuric glomerulopathy

(A) Male 8 wk old Balb/c mice were treated with adriamycin (10mg/kg) to induce adriamycin nephropathy (ADN), or with an equal volume of vehicle to serve as controls (Ctrl). Kidneys were collected 4 wk later for immunoperoxidase staining for GSK3 β . Scale bar = 20 μ m. (B) Archived formalin-fixed paraffin-embedded kidney biopsy tissues from patients with TIN, FSGS and LN(V) were prepared for immunoperoxidase staining for GSK3 β . Archived normal kidney tissues served as control. Black arrowheads indicate positive staining in podocytes at the periphery of glomerular tufts. Scale bar = 20 μ m. (C) Computerized morphometric quantification of GSK3 β staining in glomeruli in control and ADN mice; * p = 0.002 (n=6, unpaired t -test). (D) Computerized morphometric quantification of GSK3 β staining of in glomeruli in normal and diseased human kidneys; * p < 0.01 *versus* normal controls (n=3, unpaired t -test).

Figure 3. Podocyte specific knockout of GSK3 β attenuates proteinuria and ameliorates glomerular damage and podocytopathy in experimental ADR nephropathy

(A) Doxycycline (DOX)-treated KO mice and *Cre* negative control littermates (*Cre*⁻Ctrl) together with DOX-untreated control littermates (Ctrl) received a single tail vein injection of adriamycin (ADR, 25mg/kg) or an equal volume of vehicle. Spot urine (3 μ l) collected at the indicated days was subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining. Bovine serum albumin (BSA), 5, 10 and 40 μ g, served as standard. (B) Quantification of urine albumin levels adjusted with urine creatinine concentrations. **p* < 0.05 versus all other groups except for *Cre*⁻Ctrl + ADR group (n=6, ANOVA followed by *post hoc* Scheffé test);

† $p < 0.05$ versus all other groups (n=6, ANOVA followed by *post hoc* Scheffé test). (C) PAS staining of mouse kidneys collected on indicated post-injury days. Scale bar = 20µm. (D) Electron micrographs of kidney specimens procured from animals on day 6. Scale bar = 1µm. (E) Morphometric analysis of PAS stained kidney sections on day 14 for glomerular damage index. * $p < 0.05$ versus KO+ADR groups (n=6, ANOVA followed by *post hoc* Student-Newman-Keuls test); (F) Absolute count of the number of podocyte foot processes (FP) per unit length of glomerular basement membrane (GBM) on electron micrographs of kidney specimens. * $p < 0.05$ versus KO+ADR groups (n=6, ANOVA followed by *post hoc* Student-Newman-Keuls test). (G) Kidney specimens procured on day 14 from ADR-injured mice were subjected to immunofluorescence staining for fibronectin (FN, green) and the leukocyte common antigen CD45 (red). Sections were counterstained with DAPI (blue). Scale bar = 40µm. (H) Kidney specimens procured on day 14 from ADR injured mice were homogenized for immunoblot analysis for FN, CD45, and GAPDH.

Figure 4. Loss of podocyte GSK3 β improves podocyte focal adhesions and cytoskeleton integrity *in vivo* and *in vitro* following ADR injury

KO mice and control littermates (Ctrl) were treated as elaborated in Figure 3A. (A) Kidney specimens were procured from mice on day 6 and processed for rhodamine phalloidin labelling of F-actin (red) and immunofluorescence staining for synaptopodin (SYNPO; green). The merged signals (yellow), shown by laser scanning confocal fluorescence microscopy, indicate F-actin within synaptopodin positive podocytes. Scale bar = 20 μ m. (B) Computerized morphometric analysis of the ratios of integrated pixel densities between yellow signals to green signals in immunofluorescence micrographs obtained in (A). * $p <$

0.001 *versus* all other group (n=6, ANOVA followed by *post hoc* Scheffé test). (C) Glomeruli were isolated and homogenized for immunoblot analysis for p-Paxillin, total paxillin, and GAPDH. (D) Primary podocytes were prepared from KO mice and Ctrl littermates. Cells were stimulated with 0.25µg/ml of ADR or vehicle and subsequently a scratch was made using a 10 µl pipette tip. Images were recorded immediately after scratching (0 h) and 24 h later. Original magnification: ×200. (E) Computerized morphometric assessment of the area occupied by migrating cells. * $p < 0.05$ *versus* all other groups (n=3, ANOVA followed by *post hoc* Scheffé test). (F) Primary podocytes were stimulated with 0.25µg/ml of ADR or vehicle. Cell lysates were prepared 8 hours later and were subjected to immunoblot analysis for p-Paxillin, total paxillin, and GAPDH. (G) Primary podocytes were treated as described in (F), and subsequently were fixed and subjected to dual colour staining for cytoskeletal F-actin with rhodamine phalloidin (red) and paxillin (green). Scale bar = 20µm.

Figure 5. Loss of podocyte GSK3 β diminishes the ADR elicited proinflammatory NF κ B activation and *de novo* expression of NF κ B dependent podocytopathic mediators in podocytes *in vivo* and *in vitro*

KO mice and control littermates (Ctrl) were treated as elaborated in Figure 3A. (A) Glomeruli were isolated on day 6 and prepared for immunoblot analysis for the indicated molecules. (B) Kidney specimens procured from mice on day 6 were subjected to dual colour immunofluorescence staining for synaptopodin (SYNPO; red) and B7-1 (green), monocyte chemoattractant protein-1 (MCP-1; red) and Wilms' tumour 1 (WT1; green), or SYNPO (red) and Bcl-xL (green). Scale bar = 20 μ m. (C) Primary podocytes were treated with ADR (0.25 μ g/ml) or vehicle for 24 h. Whole cell lysates and conditioned media were harvested and subjected to immunoblot analysis for indicated molecules. (D) Primary podocytes were treated as described in (C) and subsequently were fixed and processed for fluorescent immunocytochemistry staining for B7-1 (green) and cathepsin L (green). Sections were counterstained with DAPI (blue). Scale bar = 20 μ m.

Figure 6. GSK3 β knockout mitigates the ADR elicited mitochondria dysfunction and podocyte loss *in vivo* and *in vitro*

KO mice and control littermates (Ctrl) were treated as elaborated in Figure 3A. (A) Kidney specimens procured on day 6 were processed for immunofluorescence staining of WT1; Scale bar = 20μm. (B) Glomeruli were isolated on day 6 and prepared for immunoblot analysis for WT1 and GAPDH. Lower panel: Densitometric analyses of the expression of WT1 in glomeruli, presented as relative levels normalized to GAPDH levels. **p* < 0.01 *versus* all other groups (n=3, ANOVA followed by *post hoc* Scheffé test); (C) Kidney cortex tissues were obtained from animals on day 2 and prepared for electron microscopy.

Representative electron micrographs of glomeruli demonstrating mitochondrial morphology in podocytes. (D) Primary podocytes were treated with ADR (0.25µg/ml) or vehicle for 24 h, and subsequently were fixed and processed for TUNEL staining and nuclear counterstaining with DAPI. White arrows indicate the TUNEL positive cells. Scale bar = 40µm. (E) Whole cell lysates were harvested 24 h after ADR injury and subjected to immunoblot analysis for cleaved caspase 3 and GAPDH (n=3). (F) Primary podocytes were treated with ADR (0.25µg/ml) or vehicle for indicated time and incubated with MitoTracker Deep Red FM to label mitochondrial, and subsequently were fixed and stained for cleaved caspase-3 (green); Scale bar = 20µm. (G) Whole cell lysates were collected 24 h after ADR injury and subjected to immunoprecipitation for cyclophilin F (Cyp-F). Immunoprecipitates were processed for immunoblot analysis for phosphorylated serine (p-Ser) or Cyp-F. (H) Schematic depicting the postulated role of GSK3β in the pathogenesis of podocytopathy. Diverse injurious stimuli elicit over-expression or over-activation of GSK3β in glomerular podocytes, which is the major isoform of GSK3 predominantly expressed in podocytes. GSK3β facilitates podocyte autonomous injury *via* multiple mechanisms: 1) GSK3β dictates paxillin phosphorylation and activation and thereby accelerates the turnover of focal adhesions, eventuating in actin cytoskeleton disorganization and podocyte hypermotility; 2) GSK3β arbitrates NFκB RelA/p65 phosphorylation selectively at serine 467, and thereby directs proinflammatory NFκB activation and specifies the transcription of selective NFκB dependent podocytopathic mediators in podocytes, including cathepsin L and B7-1, which are implicated in podocyte cytoskeleton disorganization and foot process effacement; 3) GSK3β directs phosphorylation and activation of Cyp-F, a structural component of mitochondria permeability transition (MPT) pore, and thereby potentiates MPT and sensitizes podocyte death, resulting in podocytopenia. All of these pathways culminate in the development of podocytopathy, proteinuria and progressive glomerular damage. Podocyte specific knockout of GSK3β intercepts all of the podocytopathic pathways, attenuates podocyte injury and improves proteinuria.