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*JCI Insight*. 2019. [https://doi.org/10.1172/jci.insight.130515](https://doi.org/10.1172/jci.insight.130515).

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Sonic hedgehog connects podocyte injury to mesangial activation and glomerulosclerosis

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Running title: Sonic hedgehog independently promotes glomerulosclerosis

Abstract: 194

Word count: 5,994

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**Abstract**

Glomerular disease is characterized by proteinuria and glomerulosclerosis, two pathologic features caused by podocyte injury and mesangial cell activation, respectively. However, whether these two events are linked remains elusive. Here, we report that sonic hedgehog (Shh) is the mediator that connects podocyte damage to mesangial activation and glomerulosclerosis. Shh was induced in glomerular podocytes in various models of proteinuric chronic kidney diseases (CKD). However, mesangial cells in the glomeruli, but not podocytes, responded to hedgehog ligand. In vitro, Shh was induced in podocytes after injury and selectively promoted mesangial cell activation and proliferation.

In a mini-organ culture of isolated glomeruli, Shh promoted mesangial activation but did not affect the integrity of podocytes. Podocyte-specific ablation of Shh in vivo exhibited no effect on proteinuria after adriamycin injection but hampered mesangial activation and glomerulosclerosis. Consistently, pharmacologic blockade of Shh signaling decoupled proteinuria from glomerulosclerosis. In humans, Shh was upregulated in glomerular podocytes in patients with CKD and its circulating level was associated with glomerulosclerosis but not proteinuria. These studies demonstrate that Shh mechanistically links podocyte injury to mesangial activation in the pathogenesis of glomerular diseases. Our findings also illustrate a crucial role for podocyte-mesangial communication in connecting proteinuria to glomerulosclerosis.

**Key words:** Sonic hedgehog, proteinuria, glomerulosclerosis, CKD, podocyte-mesangial communication.
Introduction

Glomerular disease accounts for the vast majority of chronic kidney diseases (CKD) that eventually progress to end-stage renal failure (1-3). Despite different etiologies, most glomerular diseases are manifested clinically by proteinuria and glomerulosclerosis, two pathologic features caused by podocyte injury and mesangial cell activation, respectively (4, 5). Common glomerular diseases such as focal and segmental glomerulosclerosis (FSGS) and diabetic kidney disease (DKD) begins with podocyte damage leading to proteinuria, but often ends up with glomerulosclerosis, which is initiated by mesangial cell activation, matrix overproduction and scar formation (6, 7). However, whether and how podocyte damage links to mesangial cell activation remains elusive.

The relationship between proteinuria and glomerulosclerosis in the clinical setting is complex and not always straightforward (8, 9). In most circumstances, heavy proteinuria is one of the most well recognized independent risk factors for CKD progression and poor prognosis (10, 11). However, patients with proteinuria are not necessarily destined to renal deficiency characterized by functional decline and tissue fibrosis. This perplexity makes it difficult and not acceptable to use proteinuria as a surrogate marker for monitoring an outcome of clinical trials (12, 13). In this context, delineation of the molecular mechanisms underlying the possible connection between proteinuria and glomerulosclerosis will not only provide novel insights into the patho-mechanisms of CKD, but also is instrumental in designing effective treatments.

One hypothesis explaining for the potential coupling of proteinuria with glomerulosclerosis is that the injured podocytes may secrete pathogenic factors, which cause mesangial cell activation through mediating podocyte-mesangial communication. While this speculation appears plausible and attractive, the identity of such a factor remains to be unraveled. In the present study, we investigated the possibility that sonic hedgehog (Shh) may act as a unique mediator that links podocyte injury to mesangial cell activation and glomerulosclerosis in proteinuric CKD.

Hedgehog signaling is an evolutionarily conserved, developmental signal cascade that regulates a diverse array of biological processes in embryonic development, tissue homeostasis and injury repair (14, 15). Among the three hedgehog ligands, Shh is the most widely studied and best
characterized. Shh is synthesized as a precursor and then undergoes autocatalytic processing and post-translational modifications in the producing cells, which are important for its proper trafficking, secretion and receptor interaction. We and others recently reported that Shh is induced in renal tubular epithelium in obstructive and ischemic nephropathy (16-20), but targets to interstitial fibroblasts, leading to their activation and matrix production (17). These observations prompted us to test whether Shh also plays a role in coupling podocyte injury to glomerulosclerosis through mediating podocyte-mesangial communication.

Here we report that Shh is induced specifically in the glomerular podocytes in various animal models of proteinuric CKD and in patients. Genetic ablation or pharmacologic inhibition of Shh represses mesangial activation and alleviates glomerulosclerosis but exhibits no effects on proteinuria. Our studies illustrate that podocyte injury is intricately linked to mesangial activation and glomerulosclerosis via Shh ligand.

**Results**

*Shh is induced in podocytes but selectively targets mesangial cells in vivo*

We first investigated Shh expression in various models of proteinuric CKD by immunohistochemical staining. As shown in Figure 1A, little or no Shh expression was found in the glomeruli of normal adult mice. However, a marked induction of Shh protein was evident in the glomeruli of injured kidney in various models of CKD, including adriamycin (ADR) nephropathy, anti-glomerular basement membrane (GBM) disease, and DKD (Figure 1, A and B). Based on cell morphology and localization in the glomeruli, it appears that the Shh+ cells were podocytes (Figure 1A, arrows). To further clarify this, we performed double staining for Shh and nestin, a podocyte-specific marker (21). As shown in Supplemental Figure 1, Shh was co-localized with nestin in the glomerular podocytes of diseased kidney. We then sought to confirm the functional activity of Shh signaling *in vivo*. To this end, we examined renal expression of Gli proteins, the transcriptional regulators that are downstream mediators and read-out of hedgehog signaling. As shown in Figure 1C, quantitative real-time PCR (qPCR) assay revealed that both Gli1 and Gli2 were significantly induced in the kidneys after ADR
injury.

To identify the upstream signal responsible for Shh induction in podocytes, we examined the potential role of Wnt ligands using cultured podocytes in vitro, because earlier studies showed that this signal cascade is activated in podocytes after injury (22, 23). As shown in Figure 1, D and E, multiple Wnt ligands, either alone or in combination, induced Shh expression in cultured mouse podocytes. Notably, conditioned medium collected after transfection with multiple Wnt expression vectors dramatically stimulated Shh expression in vitro (Figure 1, D and E).

To delineate which cell responds to Shh stimulation in the glomeruli in vivo, we used the Gli1\textsuperscript{lacZ} reporter mice, which harbor a β-galactosidase (β-Gal) knock-in mutation. Under the control of native Gli1 promoter, lacZ expression in these mice authentically recapitulates the expression of endogenous Gli1 mRNA (17). As shown in Figure 1, F and G, X-Gal staining illustrated β-Gal + cells localized in the mesangial area at 1 week after ADR injection or 2 weeks after angiotensin II infusion. To ascertain the identity of the X-Gal + cells, we further carried out double immunostaining for endothelial cell marker CD31 and podocyte marker podocalyxin. As shown in Supplemental Figure 2, there was no co-localization of X-Gal with either CD31 or podocalyxin, suggesting that Shh is likely to target non-podocyte and non-endothelial glomerular cells in vivo.

**Shh specifically promotes mesangial cells activation and proliferation in vitro**

Since mesangial cells were identified as the possible target of podocyte-derived Shh (Figure 1), we then investigated the actions of Shh in mesangial cells by using an in vitro system. To this end, human mesangial cells were incubated with different doses of recombinant human Shh for various periods of time as indicated. As expected, Shh was able to induce its downstream Gli1 and Gli2 expression in mesangial cells (Figure 2A). Similarly, Shh also induced Patched 1 (Ptc1) receptor in mesangial cells but did not affect Smoothened (Smo) expression (Supplemental Figure 3). However, Shh had little effects on the expression of Gli1, Gli2 (Figure 2B), Ptc1 and Smo (Supplemental Figure 3) in mouse podocytes.

We observed an appreciable increase in mesangial cell densities after Shh treatment, as illustrated in the phase-contrast microscopy (Figure 2C). Cell counting revealed that Shh
substantially increased the number of mesangial cells in a time- and dose-dependent manner (Figure 2, D and E), suggesting Shh as a potent mitogen promoting mesangial cell proliferation. Similar results were obtained by using a quantitative colorimetric [3-4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 2, F and G). However, Shh did not affect the growth of podocytes (Figure 2, H and I) or endothelial cells (24). We further assessed the ability of Shh to promote mesangial cells to enter cell cycle and undergo DNA synthesis, which was reflected by bromodeoxyuridine (BrdU) incorporation. As shown in Figure 2J and Supplemental Figure 4, BrdU+ cells were increased in the mesangial cells after incubation with different doses of Shh, compared to the controls.

To elucidate the mechanism underlying Shh promoting mesangial cell activation and proliferation, we next assessed the expression of numerous proliferation-related genes. As shown in Figure 2K, proliferating cell nuclear antigen (PCNA), cyclin D1, c-fos, and c-Myc were induced in mesangial cells after incubation with Shh. Meanwhile, Shh also promoted mesangial cell activation, as manifested by the induction of fibronectin, platelet-derived growth factor receptor-β (PDGFR-β), and α-smooth muscle actin (α-SMA) (Figure 2, K and L, Supplemental Figure 4 and 5). In addition, Shh also induced Wnt ligands in mesangial cells (Figure 2K), which could further induce Shh expression in podocytes (Figure 1), forming a positive feedback loop.

Shh selectively activates mesangial cells in mini-organ cultured glomeruli ex vivo

To further corroborate the specificity of Shh action on mesangial cells, we employed an ex vivo approach by using mini-organ culture of glomeruli. To this end, glomeruli were isolated from normal rat kidneys and incubated with Shh protein. As shown in Figure 3A, transmission electron microscopy (TEM) exhibited little changes in the ultrastructure of podocyte foot process and slit diaphragm after Shh treatment, compared to the controls. Consistently, there was little or no difference in the expression of several podocyte-specific proteins such as nephrin, podocalyxin, α-actinin-4 and Wilms tumor protein 1 (WT1), as illustrated Western blot analyses (Figure 3, B and C). However, Shh induced PDGFR-β, fibronectin and α-SMA expression in the isolated glomeruli, compared to the controls (Figure 3, D and E), suggesting that Shh specifically promotes mesangial
cell activation. Consistently, immunofluorescence staining gave rise to similar results (Figure 3, F-I). Immunostaining for nestin and PDGFR-β revealed a marked mesangial expansion in the cultured glomeruli (Figure 3, H and I). In agreement with Wnt induction (Figure 2), Shh also induced β-catenin expression in glomerular mini-organ culture (Figure 3, D and E). Together, these findings suggest that podocyte-derived Shh selectively targets to mesangial cells and promotes their activation.

*Podocyte-specific ablation of Shh uncouples proteinuria with mesangial activation in vivo*

To investigate the relevance of Shh to proteinuric CKD in vivo, we next generated conditional knockout mice with podocyte-specific ablation of Shh by using Cre-LoxP system. The breeding, genotyping and characterization of these mice, designated as Podo-Shh-/-, were presented in Supplemental Figure 6. Under physiologic conditions, Podo-Shh-/- mice were phenotypically normal in kidney morphology and function.

We then challenged these Podo-Shh-/- mice with ADR to induce proteinuric CKD (Figure 4A). As shown in Figure 4B, ablation of Shh in podocyte inhibited renal Gli1 expression at 1 week after ADR injection, compared to the age- and sex-matched control littermates. Remarkably, despite the podocyte-specific induction of Shh in this model (Figure 1), ablation of Shh did not affect the severity of albuminuria at different time points after ADR, as shown in Figure 4, C and D. qPCR also confirmed that there was little change in the expression of podocyte-specific proteins such as nephrin, podocin, CD2AP, podocalyxin and WT-1 between control and Podo-Shh-/- mice at 1 week after ADR (Figure 4E). The abundance and distribution of nephrin and α-actinin-4 proteins were not affected in Podo-Shh-/- kidneys, compared to the controls (Figure 4F and Supplemental Figure 7A). There was no difference in podocyte ultrastructure and foot process effacement after ADR between two groups (Figure 4F). In a separate experiment, pharmacologic inhibition of Shh signaling by cyclopamine (CPN) also did not affect albuminuria at 4 and 7 days after ADR (Supplemental Figure 7B).

We further analyzed mesangial cell activation in control and Podo-Shh-/- mice at 1 week after ADR. As shown in Figure 4, G and H, loss of Shh in podocytes inhibited the expression of α-SMA, but not PDGFR-β (Figure 4, G and H). Consistently, double immunostaining confirmed that loss of podocyte-originated Shh inhibited mesangial cell activation (α-SMA) but not their abundance.
(PDGFR-β) in this short period after ADR (Figure 4, I-K). Because of the short duration after ADR, there was no sclerotic lesion in the glomeruli yet as shown by Periodic acid-Schiff (PAS) staining and Periodic Schiff-Methenamine (PASM) staining (Figure 4I).

Podocyte-specific ablation of Shh uncouples proteinuria with glomerulosclerosis in vivo

We investigated the role of podocyte-derived Shh in the chronic progression of glomerular diseases. To this end, we examined ADR nephropathy in mice for 5 weeks, a long period when both proteinuria and glomerulosclerosis develop in this model (25). As shown in Figure 5A, albuminuria levels displayed little differences between control and Podo-Shh-/- mice throughout the period of experiment, indicating that podocyte-derived Shh has no impact on podocyte function. Consistently, there was little difference in the mRNA levels of podocyte markers including nephrin, podocin, CD2AP, and WT1 (Figure 5B). No significant change was found in α-actinin-4 and podocalyxin proteins at 5 weeks after ADR (Figure 5, C and D). Of note, Shh mRNA level was repressed at 5 weeks in Podo-Shh-/- mice (Supplemental Figure 8A). However, there was no change in renal function as assessed by serum creatinine and blood urea nitrogen (BUN) between control and Podo-Shh-/- mice (Supplemental Figure 8, B and C).

Despite no change in proteinuria, we observed marked difference in glomerular sclerotic lesions between control and Podo-Shh-/- mice at 5 weeks after ADR. As shown in Figure 5E, loss of Shh in podocytes inhibited the mRNA expression of several fibrosis-related genes such as α-SMA, fibroblast-specific protein 1 (Fsp1), collagen I and collagen III. Likewise, protein levels of fibronectin, β-catenin, PDGFR-β and α-SMA were decreased in Podo-Shh-/- mice at 5 weeks after ADR, compared to the controls (Figure 5, F-J, and Supplemental Figure 9). Glomerulosclerosis was evident in kidneys sections of control mice after PAS and Masson’s trichrome staining (MTS), which were mitigated in Podo-Shh-/- mice (Figure 5, J and K).

Pharmacologic inhibition of Shh signaling selectively mitigates glomerulosclerosis

To validate the role of Shh signaling in glomerular diseases in vivo, we used pharmacological approach by inhibiting Shh signaling with CPN in ADR nephropathy. As presented in Figure 6A,
CPN was given immediately after ADR for 5 weeks. Inhibition of Shh signaling by CPN was able to repress renal Gli1 expression (Figure 6B). However, treatment with CPN for 5 weeks exhibited little effect on albuminuria (Figure 6C), as well as renal expression of nephrin, podocin, CD2AP and WT1 (Figure 6D). ADR caused marked reduction of α-actinin-4 expression, which was not affected by CPN treatment (Figure 6, E-H).

Consistent with the findings in genetic Podo-Shh-/- model, pharmacologic inhibition of Shh signaling by CPN was able to abolish the induction of several fibrosis-related proteins such as PDGFR-β, fibronectin, α-SMA and β-catenin (Figure 6, I-K). Immunostaining also revealed that CPN reduced PDGFR-β and α-SMA expression specifically in the mesangial area at 5 weeks after ADR (Figure 6, L and M). Of interest, CPN appeared not to inhibit α-SMA expression in podocytes (Figure 6L, yellow arrows). Consistently, MTS exhibited a marked reduction of collagens deposition by CPN in the glomeruli (Figure 6, L and N).

**Blockade of Shh signaling attenuates glomerulosclerosis in diabetic kidney disease**

To generalize the finding of Shh on glomerulosclerosis, we also investigated the effect of Shh inhibition on DKD, the most common form of CKD (26, 27). To this end, we used db/db mice, the classic model of type 2 diabetes characterized by obesity and dyslipidemia due to deficiency of leptin receptor (28). At age of 24 weeks old, db/db mice were administered with CPN or vehicle for 2 weeks. As shown in Figure 7, A and B, CPN neither ameliorated albuminuria nor affected the expression of nephrin, podocin and CD2AP in this model. Of note, CPN also did not affect blood glucose and serum creatinine levels in this model (Supplemental Figure 10).

Interestingly, administration of CPN for 2 weeks inhibited renal expression of several fibrosis-related genes such as fibronectin, collagen I and α-SMA in db/db mice (Figure 7C). Although CPN did not affect podocyte-specific α-actinin-4 protein abundance, it repressed PDGFR-β, β-catenin and α-SMA in the kidney of DKD (Figure 7, D and E). Double immunofluorescence staining confirmed that CPN selectively inhibited PDGFR-β and α-SMA expression in glomerular mesangial area, whereas it had little effect on nephrin expression and distribution (Figure 7, F-I). Furthermore, staining with PAS, PASM and MTS demonstrated that CPN alleviated glomerulosclerotic lesions in
the kidney of db/db mice (Figure 7, G and K).

**Shh is correlated with glomerulosclerosis in human CKD**

To establish the clinical relevance of Shh to human CKD, we investigated its expression in human kidney biopsies from patients diagnosed with various glomerular diseases. As shown in Figure 8A, immunohistochemical staining revealed that Shh protein was barely detectable in normal human glomeruli (3 cases tested). However, Shh protein was clearly induced in the glomeruli of diseased kidneys in all 10 biopsy specimens from patients with various glomerular diseases including IgA nephropathy (IgAN), focal and segmental glomerulosclerosis (FSGS) and membranous nephritis (MN) (Supplemental Table 1). Shh protein was predominantly localized in podocyte in human diseased glomeruli (Figure 8A, arrows in the enlarged boxes), whereas glomerular mesangial cells were virtually negative for Shh staining. In patients with proteinuric CKD, circulating Shh level was elevated in serum, compared to healthy subjects (Figure 8B) (Supplemental Table 2). Of note, Shh protein in urine samples was extremely low or undetectable (data not shown). Consistent with animal models, serum Shh was correlated with neither total urinary protein nor albuminuria levels, as shown in Figure 8, C and D. However, circulating Shh level was associated with glomerular histologic lesions such as adhesion to Bowman’s capsule and glomerulosclerosis in CKD patients (Figure 8E). Figure 8F shows representative micrographs of the glomerulosclerotic lesions revealed by MTS and PASM staining in two patients with different levels of serum Shh, suggesting that Shh is associated with glomerulosclerosis, but not proteinuria, in patients as well. Taken together, we conclude that Shh plays a crucial role in mediating podocyte-mesangial crosstalk (Figure 8G) and mechanistically links proteinuria to glomerulosclerosis.

**Discussion**

Proteinuria and glomerulosclerosis are two principal pathologic features of a wide variety of glomerular diseases (29). In this study, we present evidence for an intrinsic connection between these two processes through a novel podocyte-mesangial communication mediated by Shh ligand. This
conclusion is supported by several lines of evidence, including i) Shh is specifically induced in the glomerular podocytes in various animal models of CKD; ii) Shh selectively targets glomerular mesangial cells and induces their activation and proliferation in vitro, in vivo, and ex vivo; iii) podocyte-specific ablation of Shh uncouples mesangial cell activation and glomerulosclerosis from proteinuria after injury; iv) pharmacologic inhibition of Shh signaling by CPN selectively suppresses mesangial activation and ameliorates glomerulosclerosis in ADR nephropathy and DKD; v) Shh is also induced in the glomerular podocytes in patients with proteinuric CKD and its circulating levels are correlated with glomerulosclerotic lesions. Taken together, these studies have identified Shh as the long-sought mediator that couples proteinuria to glomerulosclerosis in the pathogenesis of glomerular diseases. To the best of our knowledge, the present study represents the first report that links podocyte injury to mesangial activation, thereby providing novel mechanistic insights into the development and progression of proteinuric CKD.

Proteinuria, as the clinical manifestation of an impaired glomerular filtration barrier, is an early marker in the pathogenesis of glomerular disease. Extensive studies have shown that podocyte injury often causes the destruction of the fine ultrastructure of podocyte processes and slit diaphragm and plays a fundamental role in the onset of proteinuria (5, 30, 31). Activation of several key signal pathways such as Wnt/β-catenin, TGF-β1 and renin-angiotensin system (RAS) has been implicated in causing podocyte dysfunction, leading to a defective glomerular filtration and proteinuria (32-36). Consistently, strategies to inhibit these signaling protect podocyte integrity and mitigate proteinuria (25, 37). In this study, we show that podocyte injury is also accompanied by Shh induction in a wide range of proteinuric CKD including DKD, ADR nephropathy, anti-GBM, IgA nephropathy and FSGS, underscoring that podocyte-specific upregulation of Shh ligand is a common pathologic feature of many proteinuric CKD. Surprisingly, such an induction of Shh does not affect podocyte integrity and function, as Shh neither impairs podocyte slit diaphragm and fine ultrastructure nor changes the expression of podocyte-specific proteins such as nephrin, podocalyxin, α-actinin 4 and WT1 ex vivo (Figure 3). Accordingly, podocyte-specific ablation of Shh in mice does not affect proteinuria after ADR injury (Figures 4 and 5). These findings are also substantiated by the observation that podocytes do not respond to hedgehog stimulation in the Gli1-LacZ knock-in reporter mice after glomerular
injury (Figure 1). Although more studies in other proteinuric CKD models are needed, the present study indicates that Shh is a marker of podocyte injury, but itself has no real influence on podocyte dysfunction and the severity of proteinuria. In this regard, unlike TGF-β1, Wnts and RAS, Shh is quite unique in that it does not exert autocrine actions in podocytes.

The most interesting finding of the present study is that Shh connects podocyte damage to mesangial activation and glomerulosclerosis by mediating a unique podocyte-mesangial communication via a paracrine fashion. This conclusion is supported by the observations that Shh is able to promote mesangial cell activation in different model systems including in vitro, ex vivo and in vivo, but does not affect the proliferation of podocytes (Figure 2) or endothelial cells (24). Furthermore, genetic ablation or pharmacologic inhibition of Shh decouples podocyte injury from glomerulosclerosis. Although it has been speculated for a long time that proteinuria and glomerulosclerosis are somehow connected mechanistically, the mediator responsible for such connection remained elusive. In this context, the present study is quite significant in that it clearly establishes the podocyte-derived Shh as the mediator that orchestrates podocyte-mesangial communication.

Shh, as a morphogen, plays an especial role in mediating intercellular communication via paracrine mechanism (14). Shh transmits its signaling through binding to the receptor Ptch1 which resulting in the de-repression of Smo. Activated Smo then moves from an intracellular vesicle to the cell membrane, where it activates the Gli family of transcription factors in Shh responding cells (14, 38, 39). We previously reported that tubule-derived Shh selectively targets interstitial fibroblasts and induces their activation in CKD, leading to renal fibrosis (16, 17). Like its role in tubulo-interstitial compartment, we show here that Shh induces mesangial cell activation in the glomeruli. Therefore, Shh in different renal compartments (podocyte and tubular epithelium) confers similar actions by activating mesangial cells and fibroblasts, respectively. It should be pointed out that podocyte-mesangial communication is quite unique in that podocytes may physically contact with mesangial cells directly without the barricade of glomerular basement membrane (40), making it easy to crosstalk via a paracrine fashion. Notably, the podocyte-mesangial communication is most likely to be reciprocal and bidirectional, as podocyte-derived Shh induce Wnt ligands in mesangial cells
(Figure 2), which in turn can target podocytes and induce podocyte injury (23, 32), thereby creating a vicious cycle.

The results presented in this study appear to have clinical relevance to human CKD as well. Podocyte induction of Shh is a common finding in human kidney biopsies from patients with various glomerular diseases including IgA nephropathy, FSGS and membranous nephropathy. The circulating levels of Shh is closely associated with glomerular sclerotic lesions, but not with proteinuria (Figure 8), suggesting that Shh may serve as a surrogate biomarker predicting glomerular sclerotic lesions. It should be noted that the clinical data presented in this study may have its limitation because of small sample sizes. Nevertheless, it provides a proof-of-principle that Shh links podocyte injury to glomerulosclerosis in humans as well. Further clinical studies using large sample sizes are warranted in the future. It is worthwhile to point out that in the advanced stage of CKD when progressive loss of podocytes occur (6, 41), the overall production of Shh by glomerular podocytes could be decreased.

In summary, we herein demonstrate that Shh is the long-sought mediator that connects podocyte injury with mesangial activation, thereby mechanistically coupling proteinuria to glomerulosclerosis. We show that one can decouple podocyte damage from glomerulosclerotic lesions by targeted inhibition of Shh signaling. Although more studies are needed, these findings provide novel insights into the mechanism linking proteinuria and glomerulosclerosis and suggest Shh signaling as a therapeutic target for ameliorating glomerulosclerosis in clinical setting.

**Methods**

**Animal models**

Mouse model of podocyte injury and proteinuria was established by intravenous injection of ADR as described previously (23). Male BALB/c mice weighing 20–22 g and 24 weeks old $db/db$ mice were obtained from the Envigo (Somerset, NJ). For pharmacologic inhibition experiments, CPN was intraperitoneally injected daily at a dose of 5 mg/kg body weight after ADR or 2 weeks before sacrifice in $db/db$ mice. CPN was complexed with 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma). A
CPN/HBC stock solution was prepared by suspending 1 mg CPN in 1 ml 45% HBC in sterile PBS and stirring at 65°C for 60 min. Three groups of BALB/c mice were used: (i) Sham control (n = 5-6), (ii) ADR mice injected with vehicle (n = 6), and (iii) ADR mice treated with CPN (n = 6). ADR (doxorubicin hydrochloride; Sigma-Aldrich, St. Louis, MO) was administered by a single intravenous injection at 10 mg/kg body weight. At 1 and 5 weeks after ADR injection, all mice were euthanized. At 2 weeks after CPN injection, db/db mice were killed. Urine, blood, and kidney tissues were collected for various analyses.

Mouse genetic models

The Gli1-LacZ reporter mice, which harbor a β-galactosidase knock-in mutation that also abolishes endogenous Gli1 gene function, were obtained from the Jackson Laboratories (Stock #008211; Bar Harbor, ME). All animals were born normally at the expected Mendelian frequency, and they were normal in size and did not display any gross physical or behavioral abnormalities.

Homozygous Shh floxed mice (C57BL/6J background) were also purchased from the Jackson Laboratories (Stock #004293). Transgenic mice expressing Cre recombinase under the control of a 2.5-kb fragment of the human podocin promoter are described previously (23). By mating Shh floxed mice with podocin-Cre transgenic mice, conditional knockout mice in which Shh gene was specifically disrupted in podocyte (genotype Shh\(^{fl/fl}\), Cre\(^{+/-}\)) were created. These mice were crossbred with homozygous Shh floxed mice (genotype Shh\(^{fl/fl}\)) to generate offspring with 50% podocin-Shh\(-/-\) mice and 50% control mice (podocin-Shh\(+/+\)) within the same litters. A routine PCR protocol was used for genotyping of tail DNA samples with the following primer pairs: Cre transgene, 5’-AGG-TGT-AGA-GAA-GGC-ACT-TAGC-3’ and 5’-CTA-ATC-GCC-ATC-TTC-CAG-CAG-G-3’, which generated a 411-bp fragment; and Shh genotyping, 5’-ATG-CTG-GCT-CGC-CTG-GCT-GTGGAA- 3’ and 5’-GAA-GAG-ATC-AAG-GCA-AGC-TCT-GGC- 3’, which yielded 483-bp band for the floxed alleles.

Human kidney biopsies specimens and serum samples

Human kidney specimens were obtained from diagnostic renal biopsies performed at two centers in
China, the Affiliated Hospital of Nanjing University of Chinese Medicine and Nanfang Hospital, Southern Medical University. Non-tumor kidney tissues from the patients who had renal cell carcinoma and underwent nephrectomy were used as the normal controls. Paraffin-embedded human kidney biopsy sections (2.5-mm thickness) were prepared using a routine procedure. The serum samples were obtained from the Affiliated Hospital of Nanjing University of Chinese Medicine.

Cell culture and treatment

Mouse podocytes were described previously (22). Human mesangial cells were kindly provided by Dr Chuanyue Wu (University of Pittsburgh, Pittsburgh, PA). Human kidney proximal tubular cell line (HKC-8) was provided by Dr. L. Racusen of the Johns Hopkins University (Baltimore, MD). The Wnts enriched conditioned medium (Wnts-CM) was prepared by transfecting HKC-8 cells with multiple Wnt expression vectors, as reported previously (42). Podocytes were transfected with different Wnts-expressing vectors by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Whole-cell lysates were prepared and subjected to Western blot analyses.

Glomerular mini-organ culture

Glomeruli were isolated by differential sieving technique from male Sprague–Dawley rats (Envigo, East Millstone, NJ). Briefly, kidneys were excised and pressed with a spatula through stainless steel screens through differential sieves (60, 100, and 200 meshes) and washed with cold PBS/1% BSA. The glomeruli were suspended in PBS/1% BSA. After centrifugation at 200g for 10 min, glomeruli were collected for cultivation. The purity of glomeruli was about 95% using this approach. Isolated glomeruli were cultured in RPMI1640/10% FBS medium at 37°C on non-coated six-well plates in the absence or presence of recombinant Shh protein (1324-WN-010; R&D Systems) for further analyses.

Determination of albuminuria, serum creatinine, blood urea nitrogen and blood glucose

Urine albumin was measured by using a mouse Albumin ELISA Quantitation Kit according to the manufacturer’s protocol (Bethyl Laboratories, Inc., Montgomery, TX). Urine creatinine was determined by a routine procedure as described previously (22). Urinary albumin was standardized to
urine creatinine and expressed as milligrams per milligram urinary creatinine. Serum creatinine and BUN levels were determined by use of QuantiChrom creatinine and BUN assay kits, according to the protocols specified by the manufacturer (BioAssay Systems, Hayward, CA). The levels of serum creatinine and BUN were expressed as milligrams per 100 ml (dl). Blood glucose levels were checked by using the Glucose Level Monitoring Device (Accu-Chek®).

Sonic hedgehog enzyme-linked immunosorbent assay
Sonic hedgehog human ELISA kit was purchased from Abcam (ab100639; Abcam, Cambridge, MA). This assay employed an antibody specific for human Shh coated on a 96-well plate. Standards and samples were pipetted into the wells and Shh present in a sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human Shh antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. After the wells were washed again, TMB substrate solution was added to the wells and color developed in proportion to the amount of Shh bound. The stop solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm.

Cell proliferation assay
Cell proliferation was assessed by two approaches: cell counting and MTT assay. Cell numbers were counted by using a hemocytometer. Cell proliferation was also determined quantitatively by an MTT assay. Briefly, podocyte and mesangial cells were seeded into 96-well plates at a density of 1×10^3/well. After adherence of cells, the cultures were changed to the serum-free medium and incubated for 24 hours followed by treatment with or without Shh at different concentrations for various periods of time as indicated. MTT (5 mg/ml) was added to the medium at 10 µl/well followed by incubation at 37°C for 4 h. After the medium was removed, cells were lysed with 100 µl dimethyl sulfoxide. Absorbance of each well was measured by a microplate reader at 490-nm wavelength.

BrdU incorporation assay
The effect of Shh on human mesangial cell DNA synthesis was evaluated by BrdU incorporation.
Briefly, cells were seeded onto 24-well plates and treated with various concentrations of Shh for 48 h, and then they were pulsed with BrdU (10 mM) for 24 h. Cells were fixed with ice-cold 70% ethanol for 20 min, and DNA was denatured by incubation with 2.5 N HCL for 20 min followed by neutralization with 0.1M boric acid. Endogenous peroxidase activity was quenched by incubating the cell with 3% H$_2$O$_2$ in PBS for 20 min, and nonspecific binding was blocked by incubating the cells with 10% donkey serum for 10 min at room temperature. Incorporated BrdU was detected with a mouse monoclonal anti-BrdU antibody (B2531; Sigma-Aldrich) followed by incubation with cyanine Cy3-conjugated, affinity-purified secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Stained cells were mounted with VectaShield anti-fade mounting media by using SYTO-Green to visualize the nuclei. Stained samples were viewed under an Eclipse E600 epifluorescence microscope equipped with a digital camera (Nikon, Melville, NY).

**Reverse transcriptase (RT) and real-time PCR**

Total RNA isolation and quantitative, real-time PCR (qPCR) were carried out by the procedures described previously (22). Briefly, the first strand cDNA synthesis was carried out by using a Reverse Transcription System kit according to the instructions of the manufacturer (Promega, Madison, WI). qPCR was performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR reaction mixture in a 25-µl volume contained 12.5 µl 2x SYBR Green PCR Master Mix (Applied Biosystems), 5 µl diluted RT product (1:10) and 0.5 µM sense and antisense primer sets. PCR reaction was run by using standard protocols. After sequential incubations at 50°C for 2 min and 95°C for 10 min, respectively, the amplification protocol consisted of 40 cycles of denaturing at 95°C for 15 sec, annealing and extension at 60°C for 60 sec. The standard curve was made from series dilutions of template cDNA. The mRNA levels of various genes were calculated after normalizing with β-actin. Primer sequences used for amplifications were presented in Supplemental Table 3.

**Western blot analysis**

Kidney tissues were lysed with radioimmunoprecipitation assay (RIPA) buffer containing 1% NP-40,
0.1% SDS, 100 μg/ml PMSF, 1% protease inhibitor cocktail, and 1% phosphatase I and II inhibitor cocktail (Sigma) in PBS on ice. The supernatants were collected after centrifugation at 13,000×g at 4°C for 15 min. Protein expression was analyzed by Western blot analysis as described previously (22). The primary antibodies used were as follows: anti-Shh (sc-9024), anti-PCNA (sc-56), anti-c-fos (sc-52), anti-c-Myc (sc-764), anti-WT1 (sc-7385), PDGFR-β (sc-432) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-catenin (#610154; BD Transduction Laboratories, San Jose, CA), anti-Wnt1 (ab15251), anti-Wnt2 (ab109222) (Abcam, Cambridge, MA), anti-cyclin D1 (RB-9041-P0; Neomarkers, Fremont, CA), anti-nephrin (20R-NP002; Fitzgerald Industries International, Acton, MA), anti-podocalyxin (MAB1556, R&D Systems, Minneapolis, MN), anti-α-actinin 4 (ALX-210–356-c050; Enzo Life Sciences, Farmingdale, NY), anti-α-SMA (A2547), anti-fibronectin (F3648), anti-α-tubulin (T9026) (Sigma, St. Louis, MO), anti-actin (MAB1501; EMD Millipore, Burlington, MA).

Histology and immunohistochemical staining

Paraffin-embedded mouse kidney sections (3-μm thickness) were prepared by a routine procedure (23). The sections were stained with periodic acid–Schiff (PAS) staining or Masson’s trichrome staining (MTS) reagents by standard protocol. Immunohistochemical staining was performed according to the established protocol as described previously.(16) The antibodies against Shh (sc-9024; Santa Cruz Biotechnology, Santa Cruz, CA) and α-SMA (ab5694; Abcam, Cambridge, MA) were used.

Immunofluorescence staining and confocal microscopy

Kidney cryosections were fixed with 3.7% paraformaldehyde for 15 min at room temperature. HKC-8 cells cultured on coverslips were fixed with cold methanol:acetone (1:1) for 10 min at -20°C. After blocking with 10% donkey serum for 1 h, the slides were immunostained with primary antibodies against nephrin (20R-NP002; Fitzgerald Industries International, Acton, MA), podocalyxin (MAB1556, R&D Systems, Minneapolis, MN), α-actinin4 (ALX-210–356-c050; Enzo Life Sciences, Farmingdale, NY), CD31 (#550274; BD Pharmingen, San Jose, CA), α-SMA (ab5694; Abcam,
Cambridge, MA), nestin (#4760), PDGFR-β (#3169) (Cell Signaling Technology, Danvers, MA). These slides were then stained with Cy2- or Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and were mounted with Vectashield anti-fade mounting media using DAPI (4′, 6-diamidino-2-phenylindeole, HCl) to visualize the nuclei (Vector Laboratories, Burlingame, CA). The stained slides were viewed under a Leica TCS-SL confocal microscope equipped with a digital camera (Buffalo Grove, IL).

Electron microscopy

Electron microscopy of kidney samples was carried out by routine procedures as described previously (22, 23). Briefly, mouse kidneys were perfusion fixed with 2.5% glutaraldehyde in phosphate buffered saline by left cardiac ventricular injection and post-fixed in aqueous 1% OsO4. Specimens were dehydrated through an ethanol series, infiltrated in a 1:1 mixture of propylene oxide/polybed 812 epoxy resin (Polysciences, Warrington, PA, USA), and then embedded. Ultrathin sections were stained with 2% uranyl acetate, followed by 1% lead citrate. Sections were observed and photographed using a JEOL JEM 1210 transmission electron microscope (Peabody, MA, USA) at the Center for Biologic Imaging at the University of Pittsburgh.

X-Gal staining

For detecting functional β-galactosidase activity, OCT-embedded kidneys from the Gli1-LacZ knock-in reporter mice were cryosectioned into 7-μm sections and fixed with paraformaldehyde solution and then stained with standard 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for 2 days at 37°C. To quantify LacZ+ cell number, 100X images were taken of the entire kidney from at least three different mice. The X-Gal staining protocol was generously provided by Dr. Ben Humphreys at the University of Washington in St. Louis, and reported previously (43).

Statistics

All data were expressed as mean ± SEM. Statistical analysis of the data was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparison between groups was
made using one-way ANOVA, followed by the Student-Newman-Keuls test. Comparison between two groups was made using nonpaired 2-tailed Student’s \( t \) test. Pearson’s correlation was applied for analyzing the associations between serum Shh level and urinary protein, ACR or glomerulosclerotic lesions. \( P < 0.05 \) was considered significant.

Study approval

*Animal studies.* All animal studies were performed according to the NIH Guide for the Care and Use of Laboratory Animals and by use of the procedures approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

*Human samples.* Studies involving human serum and kidney sections were approved by the Institutional Ethics Committee at the Affiliated Hospital of the Nanjing University of Chinese Medicine and the Nanfang Hospital at Southern Medical University, as well as the Institutional Review Board at the University of Pittsburgh.
Author Contributions

DZ and YL conceived and designed the study; DZ, HF, SL, LZ and LL carried out experiments; DZ, DS, FH and YL analyzed the data; DZ made the figures; DZ and YL drafted and revised the paper; all authors approved the final version of the manuscript.

Acknowledgements

This work was supported by the National Institutes of Health (NIH) grants DK064005 and DK106049. D.Z is supported by NIH grant 1K01DK116816.

Conflict of interest statement

The authors have declared that no conflict of interest exists.
References


Figure 1

A. Immunohistochemical staining showing Shh expression in different groups: Control, ADR, Anti-GBM, and DKD.

B. Bar graph showing the number of Shh+ podocytes per glomerulus across different groups: Ctrl, ADR, α-GBM, and DKD.

C. Graph depicting the Glil mRNA level in Ctrl and ADR 5w groups.

D. Western blot analysis showing Shh and α-Tubulin protein levels across different conditions: Ctrl, Wnt1, Wnt3, Wnt4, Wnt-Mix, and Wnt-CM.

E. Graph illustrating Shh protein levels across different treatments: Ctrl, Wnt1, Wnt3, Wnt4, Wnt-mix, and Wnt-CM.

F. Histological sections stained with X-Gal showing differences between Ctrl and ADR 7d.

G. Staining with X-Gal and PAS reveals notable changes between Ctrl, ADR 7d, and Ang II 14d.
Figure 1. Shh is induced in glomerular podocytes and selectively targets mesangial cells after injury. (A) Representative micrographs show that Shh was induced in glomerular podocytes in various models of proteinuric kidney diseases including adriamycin nephropathy (ADR), anti-glomerular basement membrane glomerulonephritis (anti-GBM), and diabetic kidney disease (DKD). Boxed areas are enlarged and presented. Arrows indicate positive staining. Scale bar, 25 µm. (B) Quantitative determination of Shh-positive podocytes in the glomeruli of different models. Shh-positive podocytes were counted on 20 glomerular cross-sections per mice, 5 mice per group. **P < 0.01 versus controls (n=5, Student-Newman-Keuls test). (C) Quantitative, real-time PCR (qPCR) analyses reveal that Gli1 and Gli2 mRNA were induced at 5 weeks after ADR administration. *P < 0.05 versus shams (n=6, t test). (D, E) Western blot analyses show that Shh was induced at 48 h after incubation with various Wnt ligands in cultured podocyte. Cell lysates were immunoblotted with antibodies against Shh and α-tubulin. Wnt mix: a combination of Wnt1, Wnt3 and Wnt4 proteins. Wnt-CM: conditioned media of HKC-8 cells transfected with expression vector pHA-Wnt1 and pHA-Wnt4. Representative Western blot (D) and quantitative data (E) are shown. *P < 0.05 versus controls (n=3, Student-Newman-Keuls test). (F, G) Identification of the hedgehog-responding cells in the glomeruli after kidney injury using Gli1-LacZ reporter mice. Gli1-LacZ reporter mice were injected with a single dose of ADR or chronic infusion of angiotensin II (Ang II). At 7 days after ADR or 14 days after Ang II, kidney sections were subjected to X-Gal staining. Arrows indicate β-Gal-positive cells located at the mesangium (F, G). Images with X-Gal staining combined with PAS staining (G) are also presented. Dotted lines denote the border of glomerulus. Scale bar, 25 µm.
Figure 2
Figure 2. Shh selectively promotes mesangial cell activation and proliferation *in vitro*. (A) qPCR shows that Shh induced Gli1 and Gli2 expression in cultured mesangial cells in a dose-and time-dependent manner. *P < 0.05, **P < 0.01 versus controls (n=3, Student-Newman-Keuls test). (B) Shh did not have significant effect on Gli1 and Gli2 expression in cultured podocytes (n=3, Student-Newman-Keuls test). (C) Representative micrographs show the phase-contrast images of glomerular mesangial cells after incubation with different doses of Shh for 48 h. Scale bar, 10 µm. (D-E) Cell counting demonstrates that Shh promoted mesangial cell proliferation in a dose- and time-dependent manner. Glomerular mesangial cells were incubated with a fixed dose of Shh (100 ng/ml) for various periods of time (D), or with different concentrations of Shh for 3 days (E). Cell numbers (×10^4 per well) were counted and presented. *P < 0.05, **P < 0.01 versus controls (n=3, Student-Newman-Keuls test). (F, G) Colorimetric MTT assay shows that Shh promoted mesangial cell proliferation in a time- (F) and dose-dependent fashion (G). *P < 0.05, **P < 0.01 versus controls (n=3, Student-Newman-Keuls test). (H, I) Shh did not affect podocyte proliferation, as assessed by MTT assay. (J) Representative micrographs show that Shh promoted mesangial cell DNA synthesis as demonstrated by BrdU incorporation. Mesangial cells were incubated with 25 and 50 ng/ml Shh for 2 days, respectively. Cells were immunostained with mouse anti-BrdU antibody (red). SYTO-Green was used to visualize the nuclei. Arrows indicate BrdU-positive cells. Scale bar, 20 µm. (K) Western blots show that Shh induced the expression of numerous proliferation-related genes in cultured mesangial cells. Cell lysates were subjected to Western blot analyses for PCNA, cyclin D1, c-fos, c-Myc, fibronectin (FN), PDGFR-β, α-SMA, Wnt1, Wnt2, β-catenin, and α-tubulin, respectively. (L) Representative micrographs show that Shh activated mesangial cells by inducing α-SMA expression after incubation for 2 days. Scale bar, 20 µm.
Figure 3
Figure 3. Shh selectively promotes mesangial cell activation in glomerular mini-organ culture \textit{ex vivo}. (A) Representative micrographs show that Shh did not significantly affect podocyte integrity and ultrastructure \textit{ex vivo}. Isolated glomeruli from normal rats were incubated in the absence or presence of Shh (50 ng/ml) for 24 h. Transmission electron microscopy (TEM) demonstrates that podocyte foot processes and slit diaphragm were largely preserved after Shh incubation. Arrows indicate slit diaphragm. Dotted lines indicate the boundary of glomerular basement membrane. Scale bar in upper panel, 25 µm. Scale bar in lower panel, 500 nm. (B, C) Western blots show that Shh did not affect the abundance of podocyte-specific proteins such as nephrin, podocalyxin, α-actinin-4 and WT1 \textit{ex vivo}. Representative Western blot (C) and quantitative data (D) are presented (n=3, \(t\) test). (D, E) Western blots show that Shh induced mesangial cell activation in glomerular mini-organ culture. Isolated glomeruli were incubated with Shh (50 ng/ml) for 24 h, and glomerular lysates were immunoblotted with antibodies against PDGFR-β, fibronectin, α-SMA, β-catenin and α-tubulin, respectively. Representative Western blot (D) and quantitative data (E) are presented. *\(P < 0.05\) versus controls (n=3, \(t\) test). (F, G) Immunofluorescence staining shows that Shh did not change the expression and localization of podocyte-specific proteins \textit{ex vivo}. Isolated glomeruli were incubated without or with Shh for 24 h, and glomerular sections were immunostained for nephrin, podocalyxin, and α-actinin-4 proteins. Representative micrographs (F) and quantitation of immunofluorescence (IF) intensity (arbitrary unit) (G) are shown. Data were obtained from three glomeruli per experiment, three independent experiments per group. Arrows indicate positive staining. Scale bar, 20 µm. (H, I) Double staining show that Shh selectively promoted mesangial cell activation. Isolated glomeruli were incubated without or with Shh for 24 h and subjected to double immunostaining for podocyte marker nestin (green) and mesangial cell marker PDGFR-β (red). Representative micrographs (H) and quantitation of IF intensity (arbitrary unit) (I) are shown. *\(P < 0.05\) versus controls, \(t\) test. Data were obtained from three glomeruli per experiment, three independent experiments per group. White arrows indicate PDGFR-β\(^+\) mesangium, whereas yellow arrows denote nestin\(^+\) podocytes. Scale bar, 20 µm.
Figure 4
Figure 4. Genetic ablation of podocyte Shh does not affect proteinuria but inhibits mesangial cell activation after injury. (A) Generation of conditional knockout mice with podocyte-specific ablation of Shh. (B) qRT-PCR reveals that loss of podocyte Shh repressed Gli1 expression at 1 week after ADR injection. *P < 0.05 versus controls (n=5-7, t test). (C) Podocyte-specific ablation of Shh did not affect albuminuria at day 4 and day 7 after ADR injection (n=12-17). (D) SDS-PAGE analysis shows the abundance and composition of urinary proteins in different groups of mice at 1 week after ADR. Urine samples after normalization to creatinine were analyzed on SDS-PAGE. The numbers (1 and 2) indicate each individual animal in a given group. Asterisk indicates the position of albumin. (E) qRT-PCR reveals that loss of Shh in podocyte did not affect nephrin, podocin, CD2AP, podocalyxin and WT1 expression at 1 week after ADR, compared with the controls (n=5-7, t test). (F) Representative micrographs show that loss of Shh in podocytes affected neither nephrin and α-actinin-4 expression nor podocyte ultrastructure at 1 week after ADR. Scale bar, 20 µm. Scale bar in TEM images, 500 nm. (G, H) Western blot analyses demonstrate that loss of Shh in podocyte had little impact on α-actinin-4 and PDGFR-β expression, but inhibited α-SMA induction at 1 week after ADR. Representative Western blots (G) and quantitative data (H) are presented. Numbers (1 through 4) indicate each individual animal in a given group. *P < 0.05 (n=7, t test). (I) Representative micrographs show that ablation of Shh in podocytes did not affect mesangial abundance but inhibited mesangial activation at 1 week after ADR. Kidney sections were subjected to PAS and PASM staining, or double immunofluorescence staining for podocalyxin (green) and PDGFR-β (red), or podocalyxin (green) and α-SMA (red). Arrows indicate positive staining. Scale bar, 20 µm. (J) Quantitation of IF intensity (arbitrary unit) of PDGFR-β in the glomeruli of control and podo-Shh-/- mice at 7 days after ADR. (K) Quantitation of α-SMA-positive cells in the glomeruli of control and podo-Shh-/- mice. *P < 0.05 versus controls, t test.
Figure 5
Figure 5. Podocyte-specific deletion of Shh decouples proteinuria with glomerulosclerosis at 5 weeks after ADR injection. (A) Podocyte-specific ablation of Shh did not affect albuminuria at different time points after ADR injection (n=5-14). Urines were collected from mice at 1, 2, 3, 4 and 5 weeks after ADR injection. (B) qPCR analyses revealed that podocyte-specific loss of Shh did not affect nephrin, podocin, CD2AP and WT1 expression at 5 weeks after ADR injection (n=13-15). (C, D) Ablation of Shh did not affect podocyte-specific protein expression. Representative Western blots (C) and quantitative data (D) showed the expression of α-actinin-4 and podocalyxin at 5 weeks after ADR. (E) qPCR analyses revealed that loss of podocyte Shh repressed α-SMA, FSP-1, collagen type I, and collagen type III expression at 5 weeks after ADR injection. *P < 0.05, **P < 0.01 versus controls (n=13-15, t test). (F) Representative micrographs showed the expression of actinin-4, nephrin (red) and PDGFR-β (green) in the glomeruli of control and Podo-Shh-/- mice. Scale bar, 25 μm. (G-I) Podocyte-specific ablation of Shh repressed protein expression of fibronectin, α-SMA, β-catenin and PDGFR-β at 5 weeks after ADR injection. Representative Western blots (G) and quantitative data (H, I) are presented. Numbers (1 through 4) indicate each individual animal in a given group. *P < 0.05 versus controls (n=10, t test). (J) Representative micrographs showed a decreased glomerulosclerotic lesion and reduced α-SMA expression at 5 weeks after ADR injection in Podo-Shh-/- mice, compared to controls. Glomerulosclerotic lesions were assessed by PAS and Masson’s trichrome staining (MTS). Arrows indicate positive staining. Scale bar, 20 μm. (K) Quantitative determination of glomerulosclerotic score. The percentage of sclerotic area was assessed in 20 glomeruli. *P < 0.05 versus controls, t test.
Figure 6
Figure 6. Pharmacologic inhibition of Shh signaling decouples proteinuria from glomerulosclerosis. (A) Experimental design. Red arrow, ADR injection. Green bar, CPN treatment. (B) qPCR analyses reveal that pharmacologic inhibition of Shh signaling repressed Gli1 expression at 5 weeks after ADR injection, compared with the vehicles. *P < 0.05 (n = 5-6, Student-Newman-Keuls test). (C) Inhibition of Shh signaling by CPN did not affect albuminuria at 5 weeks after ADR. *P < 0.05 (n = 5-6, Student-Newman-Keuls test). (D) qPCR analyses revealed that inhibition of Shh signaling by CPN did not affect nephrin, podocin, CD2AP, podocalyxin, and WT1 expression at 5 weeks after ADR injection, compared with the vehicles (n = 4-5, Student-Newman-Keuls test). (E, F) Western blot analyses showed that CPN did not affect α-actinin-4 expression at 5 weeks after ADR injection. Representative Western blot (E) and quantitative data (F) are presented (n=5-6). (G, H) Representative micrographs and quantitation of IF intensity (arbitrary unit) showed α-actinin-4 expression and localization in the glomeruli of mice after various treatments as indicated. Scale bar, 20 µm. *P < 0.05 versus controls, Student-Newman-Keuls test. (I-K) Western blot assays showed that CPN inhibited the expression of fibrosis-related genes including PDGFR-β, fibronectin, α-SMA and β-catenin at 5 weeks after ADR injection. Representative Western blot (I) and quantitative data (J, K) are presented. *P < 0.05 versus controls, †P < 0.05 versus ADR alone (n = 5-6, Student-Newman-Keuls test). (L) Representative micrographs showed that CPN did not preserve podocyte integrity but inhibited mesangial activation and glomerulosclerosis. Kidney sections were immunostained for nephrin (red), PDGFR-β (green) and α-SMA or subjected to Masson’s trichrome staining. Arrows indicate positive staining. Yellow arrow denotes α-SMA⁺ podocytes. Scale bar, 25 µm. (M) Quantitation of IF intensity (arbitrary unit) of PDGFR-β in the glomeruli at 5 weeks after ADR in the absence or presence of CPN. *P < 0.05 versus controls, †P < 0.05 versus ADR alone, Student-Newman-Keuls test. (N) Quantitative determination of glomerulosclerotic score. The percentage of sclerotic area was assessed in 20 glomeruli. *P < 0.05 versus controls, †P < 0.05 versus ADR alone, Student-Newman-Keuls test.
Figure 7

A. Bar graph showing albuminuria (mg/mg Ucr) with groups Lean, db/db + Veh, and db/db + CPN. * indicates statistical significance, n.s. indicates non-significance.

B. Bar graph showing mRNA levels of Nephrin, Podocin, and CD2AP with groups Lean, db/db + Veh, and db/db + CPN. * indicates statistical significance, n.s. indicates non-significance.

C. Bar graph showing mRNA levels of Fibronectin, Collagen I, and α-SMA with groups Lean, db/db + Veh, and db/db + CPN. * indicates statistical significance, † indicates trend.

D. Western blot analysis showing protein levels of α-Actinin 4, PDGFR-β, β-Catenin, α-SMA, and α-Tubulin with groups Lean, db/db, and db/db + CPN.

E. Western blot analysis showing protein levels of α-Actinin 4, PDGFR-β, β-Catenin, and α-SMA with groups Lean, db/db, and db/db + CPN. * indicates statistical significance, † indicates trend.

F. Immunofluorescence images showing expression of Nephrin and PDGFR-β with groups Lean, db/db + Veh, and db/db + CPN.

G. Dot plot showing Nephrin (% area) with groups Lean, db/db, and db/db + CPN. * indicates statistical significance, n.s. indicates non-significance.

H. Dot plot showing PDGFR-β (% area) with groups Lean, db/db, and db/db + CPN. * indicates statistical significance, † indicates trend.

I. Dot plot showing α-SMA+ cells (per glomerulus) with groups Lean, db/db + Veh, and db/db + CPN. * indicates statistical significance, † indicates trend.

J. Hematoxylin and eosin (H&E) stain images showing PAS positivity with arrows indicating areas of interest. Scale bar = 50 μm.

K. Dot plot showing Glomerulosclerosis with groups Lean, db/db + Veh, and db/db + CPN. * indicates statistical significance, † indicates trend.
Figure 7. Blockade Shh signaling attenuates glomerulosclerosis in diabetic kidney disease. (A) Urinary albumin levels of db/db mice at different time points after CPN treatment. *P < 0.05 versus lean mice (n = 3-4, Student-Newman-Keuls test). (B, C) qPCR showed that CPN did not significantly affect podocyte-specific genes such as nephrin, podocin and CD2AP expression (B) but inhibited numerous fibrosis-related genes including fibronectin, collagen I and α-SMA (C). *P < 0.05 versus lean mice; †P < 0.05 versus vehicle (n = 3-4, Student-Newman-Keuls test). (D, E) Western blot analysis showed that CPN has little effects on podocyte-specific α-actinin-4 expression in db/db mice, but it inhibited α-SMA, PDGFR-β and β-catenin expression at 2 weeks after CPN treatment. Representative Western blot (D) and quantitative data (E) are presented. *P < 0.05 versus lean mice, †P < 0.05 versus vehicle, Student-Newman-Keuls test. (F) Representative micrographs reveal that CPN inhibited glomerular PDGFR-β and α-SMA expression but did not affect nephrin. Arrows indicate nephrin expression, while arrowheads denote PDGFR-β- and α-SMA-positive staining. Scale bar, 25 µm. (G-I) Quantitative determination of nephrin (G), PDGFR-β (H) and α-SMA (I) staining in the glomeruli of different groups of mice as indicated. *P < 0.05 versus lean mice; †P < 0.05 versus vehicle, Student-Newman-Keuls test. (J) Representative micrographs revealed that CPN attenuated glomerulosclerosis in db/db mice. Kidney sections were subjected to periodic acid Schiff (PAS), PAS-Methenamine (PASM) and Mason’s trichrome staining (MTS), respectively. (K) Quantitative determination of glomerulosclerosis in different groups of mice as indicated. *P < 0.05 versus lean mice; †P < 0.05 versus vehicle, Student-Newman-Keuls test.
Figure 8
Figure 8. Podocyte-specific induction of Shh is associated with glomerulosclerosis in humans.

(A) Representative immunohistochemical staining showed a podocyte-specific induction of Shh in human biopsies from various proteinuric CKD such as IgA nephropathy (IgAN), focal and segmental glomerulosclerosis (FSGS), and membranous nephritis (MN), compared with the healthy controls. Boxed areas are enlarged and presented. Arrows in the enlarged box indicate Shh+ podocyte. Scale bar, 50 µm.  

(B) Serum Shh levels in CKD patients diagnosed with different glomerular diseases, compared with healthy adults. **P < 0.01 (n=27-35, t test).  

(C-E) Pearson’s correlation analyses revealed Shh has no correlation with proteinuria (C) (n=22) and albuminuria (D) (n=16) levels, but it closely correlated with the extent of glomerulosclerosis (E) (n=9).  

(F) Representative micrographs of MTS and PASM staining show collagen deposition in the glomeruli of CKD patients with different levels of serum Shh. The serum Shh levels were presented on the top of representative micrographs. Arrow indicates collagen deposition in human kidney biopsies.  

(G) Schematic diagram shows that Shh couples podocyte injury to mesangial activation and glomerulosclerosis.