Podocyte-specific KLF4 is required to maintain parietal epithelial cell quiescence in the kidney

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Podocyte loss triggering aberrant activation and proliferation of parietal epithelial cells (PECs) is a central pathogenic event in proliferative glomerulopathies. Podocyte-specific Krüppel-like factor 4 (KLF4), a zinc-finger transcription factor, is essential for maintaining podocyte homeostasis and PEC quiescence. Using mice with podocyte-specific knockdown of Klf4, we conducted glomerular RNA-sequencing, tandem mass spectrometry, and single-nucleus RNA-sequencing to identify cell-specific transcriptional changes that trigger PEC activation due to podocyte loss. Integration with in silico chromatin immunoprecipitation identified key ligand-receptor interactions, such as fibronectin 1 (FN1)–αVβ6, between podocytes and PECs dependent on KLF4 and downstream signal transducer and activator of transcription 3 (STAT3) signaling. Knockdown of Itgb6 in PECs attenuated PEC activation. Additionally, podocyte-specific induction of human KLF4 or pharmacological inhibition of downstream STAT3 activation reduced FN1 and integrin β6 (ITGB6) expression and mitigated podocyte loss and PEC activation in mice. Targeting podocyte-PEC crosstalk might be a critical therapeutic strategy in proliferative glomerulopathies.

INTRODUCTION

Podocytes are terminally differentiated visceral epithelial cells that are critical to the maintenance of the glomerular filtration barrier. Podocyte injury leading to aberrant activation and proliferation of neighboring parietal epithelial cells (PECs) is a central event in proliferative glomerulopathies such as rapidly progressive glomerulonephritis (RPGN) and collapsing and cellular subtypes of focal segmental glomerulosclerosis (FSGS) (1, 2). Given the high morbidity and mortality of individuals with these diseases, identifying therapeutic targets that attenuate and/or reverse the progression is of high importance (3, 4). In RPGN, along with accumulation of inflammatory cells and capillary injury/necrosis, podocyte injury triggering pathologic PEC activation and proliferation is a critical component of the crescentic lesion (5). Similarly, podocyte injury in collapsing FSGS leads to segmental or global collapse of the capillary tuft with pronounced PEC hyperplasia (i.e., pseudo-crescents) (5). While these previous studies suggest that the potential crosstalk between podocytes and PECs is a major contributor to the pathogenesis of these hyperplastic lesions (6, 7), the mechanisms remain unclear.

Activation of signal transducer and activator of transcription 3 (STAT3) signaling has been previously reported to play a role in the development and progression of both RPGN and collapsing FSGS (8, 9). Podocyte-specific deletion of Stat3 attenuated crescent formation and glomerular injury in mice treated with nephrotoxic serum (NTS), an animal model of RPGN (8). In addition, global knockdown of Stat3 abrogated podocyte injury and accompanying epithelial hyperplasia in HIV-1 transgenic mice, a model of collapsing FSGS (9).

Krüppel-like factors (KLFs) are a subclass of zinc-finger family of DNA binding transcriptional regulators that are involved in a broad range of cellular processes (i.e., cell differentiation, apoptosis, and cell proliferation) (10). While several members in the KLF family have been implicated in cell differentiation (10, 11), KLF4 was first identified as a negative regulator of proliferation by inducing cell cycle arrest and restoring prodifferentiation markers in intestinal epithelial cells (12). Apart from its critical role in cell cycle regulation, we recently reported that KLF4 suppresses STAT3 transcriptional activity. Podocyte-specific knockdown of KLF4 increased STAT3 signaling leading to podocyte injury, triggering surrounding epithelial cell hyperplasia in the glomerulus with eventual kidney injury and reduced survival (13). While the protective role of KLF4 in nonproliferative glomerulopathies has been reported (10, 14, 15), our recent studies demonstrate the critical role of podocyte-specific KLF4 in maintaining podocyte homeostasis and preventing hyperplastic lesions in the glomerulus (13). Furthermore, we also showed that the activation of STAT3 signaling inversely correlated with KLF4 expression in the glomeruli of human kidney biopsies with RPGN as compared to control specimens (13). Collectively, these data suggest that podocyte-specific knockdown of Klf4 in mice serves as a robust model to investigate the mediators of podocyte-PEC cross-talk in proliferative glomerulopathies. Here, we use an integrative approach of unbiased glomerular RNA sequencing (RNA-seq), label-free quantification (LFQ), and isobaric tag for relative and absolute quantification (iTRAQ) proteomics, and single-nucleus RNA-seq (snRNA-seq) to investigate the ligand-receptor interactions, mediating podocyte-PEC cross-talk in this model of proliferative glomerulopathy.
**RESULTS**

**Podocyte-specific loss of Klf4 induces podocyte loss and triggers the activation and proliferation of PECs**

We recently reported that the podocyte-specific knockdown of Klf4 using the Cre recombinase system (Klf4ΔPod) triggered podocyte loss, pathologic glomerular epithelial cell proliferation, and FSGS, leading to kidney dysfunction and death as compared to control (Klf4fl/fl) mice (13). While immunostaining revealed a loss of podocyte markers with concurrent activation and proliferation of PECs (13), the origin of these cells remained unclear [i.e., quiescent PECs that transitioned to activated PECs (aPECs) and/or dedifferentiated podocytes expressing markers of aPECs]. To ascertain whether conditional knockdown of Klf4 in podocytes triggered the activation and proliferation of PECs, we conducted lineage tracing studies by breeding Nphps2-Cre mice with LSL-tdTomato reporter mice to permanently tag podocytes with red fluorescent protein (RFP). These mice were subsequently bred with Klf4ΔPod and Klf4fl/fl to generate Klf4ΔPod:TdT and Klf4fl/fl:TdT animals. All mice were euthanized at 12 weeks of age, with this time point reflecting previously reported severity of kidney injury (i.e., podocyte injury, FSGS, proteinuria, and kidney dysfunction) (13). Immunostaining for CD44 (marker of aPECs), EdU (5-ethyl-2′-deoxyuridine, a thymidine analog that acutely reports levels of in vivo proliferation), and RFP revealed that Klf4ΔPod:TdT mice exhibit pronounced glomerular CD44 and EdU staining with negligible levels of expression in Klf4fl/fl:TdT control mice (Fig. 1A). Conversely, RFP staining was significantly reduced with a lack of CD44 colocalization in Klf4fl/fl:TdT mice as compared with Klf4ΔPod:TdT mice, thereby suggesting that podocyte loss triggered the activation and proliferation of PECs. Collectively, these data indicate that the podocyte-specific genetic knockdown of Klf4 induces podocyte loss, which triggers the activation and proliferation of PECs.

**Podocyte-specific loss of Klf4 engages pathways involved in proliferation, cell adhesion, inflammation, and complement activation within glomeruli**

To identify the mechanisms mediating podocyte loss and PEC proliferation in Klf4ΔPod mice, we conducted bulk RNA-seq in isolated glomeruli from Klf4fl/fl and Klf4ΔPod mice at 12 weeks of age. In total, 964 genes (458 genes down-regulated and 506 up-regulated) were differentially expressed in isolated glomeruli between Klf4ΔPod and Klf4fl/fl mice (Fig. 1B). The ranked list of differentially expressed genes between the Klf4ΔPod and Klf4fl/fl mice is provided in Supplementary File 1. We subsequently performed gene pathway analysis with Enrichr (16, 17) on the list of genes differentially up-regulated in Klf4ΔPod compared with Klf4fl/fl glomerular fractions. Enrichment analysis used WikiPathways (18) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (19), revealing a significant decrease in podocyte-specific gene expression in Klf4ΔPod as compared with Klf4fl/fl mice, demonstrating podocyte loss (Fig. 1, C and D). Conversely, enrichment analysis of the up-regulated genes showed activation of pathways involved in inflammation, complement signaling, cell adhesion, and cell proliferation (Fig. 1, C and D). Several key transcripts specific to aPECs (Cd44, Cd74, Krt19, Cd9, and Egrf) were also significantly increased in Klf4ΔPod as compared with Klf4fl/fl glomerular fractions (Fig. 1E).

Because we previously demonstrated that glomerular STAT3 signaling is activated in Klf4ΔPod mice (13), we sought to determine the extent to which the differentially expressed genes are directly and indirectly controlled by KLF4 and/or STAT3 using previously reported chromatin immunoprecipitation sequencing (ChIP-seq) datasets (20). We cross-matched the differentially expressed genes from the glomerular bulk RNA-seq with genes that contain KLF4 and STAT3 binding sites in their promoter region (±1 kb from the transcription start site (TSS)). Of the 506 up-regulated genes, 218 genes contained KLF4 binding sites (P = 9.15 × 10−7) and 156 genes contained STAT3 binding sites (P = 1.03 × 10−5), with 101 genes containing both in their promoter region (Fig. 2A). Next, we performed gene list enrichment analysis by applying the tool Enrichr (16) to the list of genes differentially expressed genes in Klf4ΔPod as compared with Klf4fl/fl glomerular fractions. Enrichment analysis against the gene set libraries Wiki pathways (18) and KEGG pathways (19) reveal a significant increase in pathways involving cell-cell communication, extracellular matrix (ECM) interactions, mitogen-activated protein kinase signaling for integrins, and cell junction organization in Klf4ΔPod as compared with Klf4fl/fl glomeruli (Fig. 2, B and C). In addition, enrichment analysis of the 101 genes containing both KLF4 and STAT3 binding sites cross-referenced with a protein-protein interaction (PPI) database, revealed prominent enrichment of PPI hub proteins affecting cell cycle progression, proliferation, inflammation, and fibrosis (Npxb2, STAT3, CDK1/2, and EGFR) (Fig. 2D). We subsequently assessed the potential of these genes to be regulated by a given transcription factor (TF) using a previously established TF-gene association scoring strategy, whereby genes are given a score from 0 to 1, with a higher score indicating a greater probability that a gene is regulated by a specific TF (20). For KLF4 and STAT3, a score greater than 0.8 indicates the presence of a transcription factor binding site (TFBS) within 1 kb of the TSS. Using this strategy for the up-regulated genes, 118 of the 228 (~50%) genes with KLF4 binding sites had a binding score between 0.8 and 1.0, whereas only 26 of the 156 (~16%) STAT3 target genes had a binding score between 0.8 and 1.0 (Fig. 2E). Enrichment analysis of these 130 genes with a binding score between 0.8 and 1.0 for KLF4 and STAT3 showed a significant increase in pathways involving interferon-γ (IFN-γ) signaling, cell junction organization, and SMAD signaling in Klf4ΔPod as compared with Klf4fl/fl glomeruli (Fig. 2F). Of the 458 significantly down-regulated genes, 159 genes contained KLF4 binding sites (P = 4.78 × 10−3) and 91 genes contained STAT3 binding sites (P = 4.48 × 10−3) in their promoter region, with 61 genes containing both (fig. S1A). Using the TF-gene association scoring strategy, a significant majority of the binding sites with a binding score between 0.8 and 1.0 were enriched for KLF4 (77 of 159, 48%) as compared to STAT3 (3 of 91, 3%) (fig. S1B). Subsequent enrichment analysis of these down-regulated genes with a KLF4 and STAT3 binding score between 0.8 and 1.0 demonstrated an increase in pathways involved in semaphorin signaling and cell-cell migration (fig. S1C). Collectively, these data demonstrate that KLF4 might directly and indirectly, in part via STAT3, regulate transcripts involved in cell-cell communication, proliferation, migration, and ECM signaling in proliferative glomerulopathies.

**Proteins involved in ECM remodeling, metabolism, and growth factor signaling are shed from podocytes with KLF4 knockdown**

We recently demonstrated that knockdown of KLF4 (KLF4-shRNA) in cultured human podocytes led to reduced podocyte survival as compared with control podocytes (Scr-shRNA) under nonpermissive conditions (13). We also reported that conditioned medium harvested from KLF4-shRNA knockdown podocytes triggered the activation and proliferation of cultured mouse PECs (mPECs) compared with...
Fig. 1. RNA-seq of isolated glomeruli of Kif4ΔPod mice identifies dysregulated signaling pathways. (A) Representative images of immunofluorescence staining for RFP, CD44, EdU, and Hoechst in Kif4ΔPod/WT mice and Kif4ΔPod/ΔPod controls. n = 3 per group. Scale bars, 10 µm. Dashed white lines indicate glomerular area; solid white lines indicate area of extracapillary proliferation; inset of merged images shows lack of colocalization. (B) Heatmap representation showing 964 differentially expressed genes identified by mRNA sequencing in glomeruli from Kif4ΔPod and Kif4ΔPod mice (n = 3 per group). (C) KEGG, WikiPathway, and Gene Ontology (GO) enrichment analysis in down-regulated (458) and up-regulated (506) genes. (D) Heatmaps of differentially expressed genes in pathway analysis (podocyte-related, inflammatory and complement, cell adhesion, cell cycle, and proliferation). (E) Expression of genes implicated in PEC activation and proliferation in Kif4ΔPod and Kif4ΔPod mice. n = 3 per group; *P < 0.05, **P < 0.001, and ***P < 0.0001; unpaired t-test. FPKM, fragments per kilobase of transcript per million mapped reads.
conditioned medium from Scr-shRNA podocytes (13). To identify differentially secreted proteins from KLF4-shRNA podocytes as compared with Scr-shRNA podocytes, we performed tandem mass spectrometry on conditioned medium harvested from each of these samples under nonpermissive conditions, after removal of cell debris. Because both LFQ and isobaric tag labeling (iTRAQ) have their advantages and disadvantages (21), data were integrated across these two methods to identify differentially abundant proteins in the conditioned medium from KLF4-shRNA as compared with Scr-shRNA podocytes. Samples were run in duplicate for both LFQ and iTRAQ, and a total of 1604 proteins were identified, with 136 proteins significantly increased [fold change (FC) > 1.3 and P < 0.05] and 88 proteins significantly decreased (FC < 0.77 and P < 0.05) in the KLF4-shRNA compared with the Scr-shRNA conditioned medium (Fig. 3A and Supplementary File 2). IGFBP5, PRSS23, and migration inhibition factor (MIF) were not only abundantly expressed but were also unique enrichment analysis to the 136 up-regulated proteins following conditions (Fig. 3B). Next, we applied Gene Ontology (GO) and pathway enrichment analysis to the 136 up-regulated proteins following KLF4-shRNA knockdown as compared with the Scr-shRNA conditioned medium (Fig. 3, C and D) and identified signaling pathways enriched for ECM components, metabolism, and growth factor signaling. These functional annotations align with the pathways enriched in up-regulated genes from glomerular RNA-seq of Klf4 ΔPod mice, including those related to cell adhesion, ECM, and growth factor signaling.

Single-nucleus transcriptomics identifies changes in gene expression unique to podocytes and PECs after podocyte-specific loss of Klf4 in mice

While we observed significant changes to the glomerular transcriptome from Klf4 ΔPod mice as compared with Klf4 Δ+/+ mice (Fig. 1), these changes might be a result of podocyte loss and/or changes in gene expression in other cell types that comprise the glomerulus (i.e., PECs, endothelial cells, and mesangial cells). To identify transcriptomic changes at the single-cell level, we performed snRNA-seq on kidney cortex from Klf4 ΔPod and Klf4 Δ+/+ mice (Fig. 4). Our rationale for choosing snRNA-seq instead of single-cell RNA-seq was based on recent studies in the kidney (23). Using the 10X Genomics platform, we successfully generated transcriptomes for 63,868 nuclei (30,838 from Klf4 ΔPod mice and 33,030 from Klf4 Δ+/+ mice) that passed all quality control checks (fig. S2). Unsupervised clustering analysis generated 23 clusters (Fig. 4A). To assign cell type identity to these clusters, we manually compared top cluster marker genes with established cell type markers from several published

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**Fig. 2. Differentially expressed genes are coregulated by KLF4 and STAT3.** Significantly up-regulated genes identified in Klf4 ΔPod glomeruli compared to Klf4 Δ+/+ glomeruli were cross-matched with published ChIP-seq studies on KLF4 and STAT3 from Chen et al. (20). (A) Scaled Venn diagram showing overlap of TFBS. Of 506 significantly up-regulated genes, 218 contain KLF4 TFBS (P = 9.15 × 10^{-7}) and 156 contain STAT3 TFBS (P = 1.03 × 10^{-4}), with 101 genes containing both. Top enriched pathways from KEGG, WikiPathways, and Reactome databases of genes up-regulated in Klf4 ΔPod mice that contain (B) KLF4 TFBS (218 genes) and (C) STAT3 TFBS (156 genes). MAPK, mitogen-activated protein kinase. (D) Top enriched PPI hub proteins of 101 genes significantly up-regulated in Klf4 ΔPod glomeruli containing both KLF4 and STAT3 TFBS. (E) Bar plot showing the number of genes passing each TF-gene association score threshold. Higher TF-gene association scores indicate TFBS closer to TSS. TF-gene association scores > 0.8 indicate presence of TFBS within 1 kb of TSS. (F) Top enriched pathways from KEGG, WikiPathways, and Reactome databases of genes up-regulated in Klf4 ΔPod mice that have STAT3 or KLF4 TF-gene association score > 0.8.
datasets (23, 24). Annotated clusters included many of the most abundant cell types in the kidney including proximal tubule (PT) S1 and S2, PT S3, endothelial cells, mesangial cells, podocytes, connecting tubule, distal convoluted tubule, intercalated cells A and B, loop of Henle, juxtaglomerular apparatus cells, macrophages, and fibroblasts (Fig. 4, A and B, and Supplementary File 3). We also detected populations of proliferating PT cells and aPecs. We compared the relative abundance of the PEC/Prolif.PT cluster in the Klf4<sup>fl/fl</sup> and Klf4<sup>APod</sup> mice and found a significant increase in the number of cells grouping to the PEC/Prolif.PT cluster in the Klf4<sup>APod</sup> mice (Fig. 4C). CD44, a cell surface marker specific to aPecs (25), was enriched in the PEC cluster in the Klf4<sup>APod</sup> as compared to the Klf4<sup>fl/fl</sup> mice, confirming the presence of aPecs in Klf4<sup>APod</sup> mice as compared with the quiescent PECs in Klf4<sup>fl/fl</sup> mice (Fig. 4D). Cd44 was also expressed in other clusters in both Klf4<sup>fl/fl</sup> and Klf4<sup>APod</sup> mice, albeit to a lesser extent as compared to the PEC/Prolif.PT cluster.

In addition to our lineage tracing studies in Fig. 1A, we previously reported that podocyte-specific loss of KLF4 in cultured human podocytes results in initial podocyte cell cycle reentry but subsequent mitotic catastrophe and cell death (13). To further validate that these injured podocytes undergo cell death rather than transition to mesenchymal phenotype, we interrogated epithelial and mesenchymal markers in a cell-specific manner in all glomerular cell clusters between Klf4<sup>fl/fl</sup> and Klf4<sup>APod</sup> mice (fig. S3, A and B). We observed a reduction in epithelial markers in the podocyte cluster without significant changes in mesenchymal markers. Most changes in mesenchymal markers were in PEC/Prolif.PT and mesangial cells/fibroblast clusters (fig. S3B). In addition, while our glomerular RNA-seq demonstrated a significant increase in genes involved in inflammatory and complement-mediated pathways (Fig. 1), further analysis of these cell clusters showed that these changes were largely driven in the PEC/proliferating PT cell clusters, suggesting that these changes
Fig. 4. snRNA-seq profiles transcriptomic changes of ∆Klf4Pod mice. From kidney cortex samples of six different mice, 63,868 nuclei (30,838 from Klf4fl/fl and 33,030 from Klf4∆Pod) meeting proper quality control thresholds were sequenced. (A) Uniform Manifold Approximation and Projection (UMAP) plot illustrates all 63,868 nuclei, mapping to 23 clusters. (B) Cluster identities aligned to canonical cell types in the adult mouse kidney based on a variety of cell type–specific marker genes. (C) Relative cell-type abundance compared across different subclusters. (D) Cd44 expression plotted in UMAP space. Gray indicates no expression and purple indicates high expression. (E) Subclustering of the PEC/Prolif.PT cluster and expression of Cd44, Slc5a12, Nphs1, and Vcam1 across different subclusters. (F) Relative cell-type abundance compared between Klf4fl/fl and Klf4∆Pod mice in PEC/Prolif.PT subclusters. (G) KEGG and (H) Reactome pathway analysis with ClusterProfiler for up-regulated genes in PEC/Prolif.PT mice.

PEC/Prolif.PT subclustering

Cluster 2/3 up-regulated genes (KEGG)

Cluster 2/3 up-regulated genes (Reactome)

Signaling by receptor tyrosine kinases

Signaling by Rho GTPases

Signaling by PDGF

Signaling by ERBB2

Regulation of actin dynamics for phagocytic cup formation

Integrin cell surface interactions

Cell–cell communication

Cell junction organization

AGE-RAGE signaling pathway in diabetic complications

Leadzalo transthelial migration

Bacterial invasion of epithelial cells

Supplemental figures indicate the number of genes up-regulated in PEC/Prolif.PT subclusters 2/3. Pod, podocyte; Endo, endothelial; Mes, mesangial; Fib, fibroblast; PT, proximal tubule; LoH, loop of Henle; DCT, distal convoluted tubule; CNT, connecting tubule; CD-PC, collecting duct principal cell; IC, intercalated cell; JGA, juxtaglomerular apparatus; Ma, macrophage; GTPase, guanosine triphosphatase.
are likely a secondary event subsequent to initial podocyte injury (fig. S3C).

To further investigate the subpopulation of pathogenic aPECs in the PEC/Prolif.PT whose appearance was triggered by the podocyte-specific loss of Klf4, we isolated and subclustered the PEC/Prolif.PT cluster. Subclustering of the main PEC/Prolif.PT cluster revealed nine subclusters with most nuclei originating from Klf4−Pod mice (Fig. 4, E and F, and Supplementary File 4). We subsequently interrogated Cd44 expression across the nine subclusters, against the backdrop of markers specific to the PT (Slc5a12), podocytes (Nphs1), and mesenchymal cells (Vcam1) (Fig. 4E). This comparison yielded two subclusters containing high Cd44, low Slc5a12, and high Vcam1 expression (subclusters 2 and 3), indicating the presence of aPECs in Klf4−Pod mice. We also observed three separate subclusters with high Cd44 and high Slc5a12 expression (subclusters 7, 8, and 9), indicating the presence of proliferating PT cells. All PEC/Prolif.PT subclusters lacked expression of mature podocytes markers, such as Nphs1, confirming that these cells are not of podocyte origin (Fig. 4E). Absence of podocyte markers also suggests that podocyte-specific knockdown of Klf4 triggers the transition of PECs from the quiescent to the activated state, rather than transdifferentiation of podocytes to PECs or, conversely, PECs to podocytes. On the basis of these findings, we identified genes up-regulated in subclusters 2 and 3 and performed pathway analysis using KEGG and Reactome databases (Fig. 4, G and H). These subclusters were significantly enriched for receptor tyrosine kinase signaling, Rho guanosine triphosphatase signaling, integrin cell surface interactions, focal adhesion, and ECM-receptor interactions, which all support the increased motility and proliferation observed in aPECs. Pathway analysis of genes significantly down-regulated in subclusters 2 and 3 yielded distinct pathways relating to various metabolic processes (fig. S4, A and B). Last, we conducted KEGG and GO gene set enrichment analysis of differentially expressed transcripts from subcluster 2 and 3 to demonstrate the distribution of expression of these core genes (up and down) (fig. S4, C to F).

To explore the potential transcriptional paths bridging quiescent PECs and aPECs, we performed cell trajectory analysis using Monocle 2 (26, 27) on the nine subclusters of the main PEC/Prolif.PT cluster (Fig. 5A). Pseudotime ordering of cells yielded distinct Cd44-expressing populations at opposite ends of the pseudo-timeline, with only one end highly expressing Slc5a12 (Fig. 5B). We subsequently interrogated the top differentially expressed transcripts as a function of pseudotime between a state of low Cd44 and high Cd44/low Slc5a12 expression, which revealed a significant decrease in several genes including Dab2 and an increase in Itgav, Creb5, Runx1, and others (Fig. 5C). Pathway analysis of differentially expressed genes along pseudotime yielded significant enrichment of various pathways relating to cell signaling, motility, and migration—all of which are consistent with the in vivo behavior of the aPEC subclusters (Fig. 5, D and E). In this setting, pseudotemporal ordering allows for the identification of genes critical to the activation of PECs from their quiescent state.

Integration of glomerular RNA-seq, snRNA-seq, and podocyte secretome identifies putative ligand-receptor interactions that mediate podocyte-PEC cross-talk

To identify the mechanisms through which podocyte loss triggers PEC activation and proliferation following the conditional knockdown of Klf4 in podocytes, we initially integrated proteomics and glomerular RNA-seq data to identify ligand-receptor interactions mediating podocyte-PEC cross-talk. To start, we queried a curated database of known ligand-receptor interactions (28) to select ligands from the differentially expressed proteins in the secretome of KLF4-shRNA podocytes and the corresponding receptors from the differentially up-regulated genes in the glomerular RNA-seq (Klf4−Pod versus Klf4+/Pod) (Fig. 6, A and B). We cross-matched genes with KLF4 binding sites with previously reported KLF4 ChIP-seq (29) and identified that Fn1, Mif, and Gsp1 all enriched for KLF4 binding events within 1 kb of the TSS (Fig. 6C). To more closely link these KLF4 binding sites with the kidney, we used a recently published chromatin accessibility [assay for transposase-accessible chromatin using sequencing (ATAC-seq)] dataset (29) to demonstrate that Fn1, Mif, and Gsp1 all contained accessible promoters, which were increased in expression with Klf4 knockdown, suggesting a potential transcriptional regulatory role for KLF4 (Fig. 6C). In addition, we searched and identified other known ligand-receptor interactions in the literature that were not present in the database, such as MIF-CD44/CD74 (7). We subsequently cross-matched these ligand-receptor interactions with the snRNA-seq (Fig. 4) data to identify cell surface receptors that are uniquely enriched in aPECs (Fig. 6D).

In addition to Cd44, we observed a significant enrichment of Cd47, discoidin domain receptor tyrosine kinase 1 (Ddr1), and integrin receptors Itga1, Itgav, Itgb8, and Itgb6 in the PEC/Prolif.PT cluster (Fig. 6D). Specifically, the expression of Cd44, Cd47, and Itgav showed a significant increase in aPECs (subclusters 2/3) in Klf4−Pod as compared with Klf4+/Pod mice (Fig. 6E). Itgb6 was expressed in both in Klf4−Pod and Klf4+/Pod subclusters 2/3, suggesting that Itgb6 is expressed in both quiescent PECs and aPECs (Fig. 6E). We subsequently used the protein atlas to confirm that Cd44 is minimally expressed in quiescent PECs, whereas integrin subunits αV and β6 is expressed at baseline in quiescent PECs and in tubular compartments (fig. S5A). In addition, interrogation of previously reported expression arrays from microdissected glomeruli from human kidney biopsies deposited in NephroSeq and European Renal eDNA Bank—Kroener-Fresenius Biopsy Bank demonstrates the increased expression of ITGB6 in patients with systemic lupus erythematosus (SLE) nephritis and RPGN as compared to minimal change disease (MCD) and FSGS (fig. S5, B and C) (30). Because proliferative lesions and progressive proteinuria occur in stages 3 and 4 of SLE nephritis, we also observed that proteinuria was associated with increased ITGB6 expression in microdissected glomeruli from patients with SLE nephritis, which was confirmed from isolated glomeruli from three strains of SLE mice (NZB/W, NZM2410, and NZW/BXSB F1) with proteinuria (fig. S5, D and E) (31). In addition, expression arrays from Hodgkin et al. (32) deposited in NephroSeq confirmed the increase in ITGB6 expression in microdissected glomeruli of patients with collapsing FSGS compared to healthy control individuals and MCD (fig. S5F).

Integrin subunit β6 belongs to a family of heterodimeric integral membrane proteins composed of α and β subunits that function in cell surface adhesion and signaling. The αV subunit heterodimerizes with the β6 subunit to create a functional multimeric αVβ6 receptor for binding of ligands such as fibronectin 1 (FN1) with R-G-D sequence (33, 34). FN1, an integral ECM protein, was significantly up-regulated in isolated glomeruli of Klf4−Pod mice and conditioned medium from KLF4-shRNA podocytes. In addition, published ChIP-seq studies (20) revealed 19 STAT3 binding sites and 23 KLF4 binding sites for within 1 kb of the TSS of Fn1, hinting at the potential for direct transcriptional regulation of Fn1 by STAT3.
Fig. 5. Cell trajectory analysis identifies genes up-regulated in aPECs. Cell trajectory analysis using Monocle2 on the PEC/Prolif.PT cluster. (A) Pseudotime ordering of PEC/Prolif.PT cluster colored by PEC/Prolif.PT subcluster (0 to 8) with noted branchpoint A (Cd44+ /Scl5a12−) and branchpoint B (Cd44+/Scl5a12+). (B) Expression of Cd44 and Scl5a12 projected over cell trajectory. (C) Gene expression analysis identifies genes that are differentially expressed across pseudotime, progressing from branchpoints A to B. (D) KEGG and (E) Reactome pathway analysis with ClusterProfiler of differentially expressed genes across pseudotime branchpoints A to B.

and KLF4. To assess whether glomerular FN1 expression is increased in human proliferative glomerulopathies, we initially immunostained for FN1 in kidney biopsies with RPGN and healthy control individuals (fig. S6A). Glomerular FN1 expression was significantly increased in human RPGN as compared to healthy control individuals (fig. S6, A and B). Furthermore, glomerular FN1 expression was increased in previously reported expression arrays deposited in NephroSeq and European Renal cDNA Bank–Fresenius biopsy bank with SLE nephritis and RPGN as compared to MCD and FSGS (fig. S6, C and D) (30). We also observed that proteinuria was associated with increased FN1 expression in microdissected glomeruli from patients with SLE nephritis, which was confirmed from isolated glomeruli from three strains of SLE mice (NZB/W, NZM2410, and NZW/BXSB F1) with proteinuria (fig. S6, E and F) (31). Expression arrays from Hodgins et al. (32) confirmed the increase in FN1 expression in microdissected glomeruli of patients with collapsing FSGS compared to healthy control individuals and MCD (fig. S6G).

To assess the role of αVb6 in triggering PEC activation, we initially conducted lineage tracing studies by generating mice labeled with a PEC-specific reporter using the “tet-on” system, where the
binding of reverse tetracycline-controlled transactivator protein (rtTA) to the tet operator, as well as gene activation, only occurs in the presence of doxycycline (DOX). We bred PEC-rtTA mice with TRE-GFP reporter mice to tag PECs with green fluorescent protein (GFP) under DOX treatment. Subsequently, we used an accelerated autologous-phase NTS model, using intraperitoneal injections of sheep NTS in mice preimmunized with sheep immunoglobulin G (IgG) to induce crescentic glomerulonephritis (35). We observed that CD44 was significantly up-regulated in podocytes (fig. S7B). We subsequently isolated PECs from these mice by fluorescent-activated cell sorting for GFP⁺ cells. We validated that these GFP⁺-sorted cells were specific for PECs by measuring markers for PECs as compared to podocytes (fig. S7B). To identify markers of PEC activation, further analysis of snRNA-seq from Klf4⁺/⁻ and Klf4(SE) mice demonstrated that Creb5 and Runx1 were specifically and uniquely coexpressed with Cd44 in the PEC/Prolif.PT cluster (fig. S7C). In addition, Creb5 and Runx1 were also highly expressed in cells with high Cdh4/Cdh3 expression as compared to cells with low Cdh4 expression from the pseudotime analysis, suggesting a putative role in PEC activation (Fig. 5C). To test whether Fn1-OVβ6 might mediate PEC activation, we initially isolated these

**Fig. 6. Ligand-receptor analysis uncovers potential mediators of podocyte-PEC cross-talk.** Significantly up-regulated proteins were cross-matched to a ligand-receptor database published by Ramilowski et al. (28). (A and B) Using proteins differentially up-regulated in Klf4-shRNA conditioned medium as ligands were cross-matched with partner receptors from genes significantly up-regulated in Klf4⁺/⁻ glomeruli from glomerular RNA-seq. (C) Embryonic stem cell (ESC) KLF4 ChIP-seq dataset from Chen et al. (20) was queried for binding sites in the promoters of genes obtained from bulk and sc-RNAseq as well as proteomics analyses. KLF4 binding sites ± 1 kb of the promoters of AGRN, COL1A1, COL2A1, COL4A1, COL4A2, CTGF, FBN1, FN1, GFBP4, INHBA, MEF, PRSS23, THBS1, KLF4 TFBS, and STAT3 TFBS sites were fragmented into 1 kb bins and queried for enrichment in iPSCs and PECs. (D) Expression and percentage (Pct) of nuclei of identified receptors in all cell clusters from snRNA-seq. (E) Further stratification of receptor expression and Pct by PEC/Prolif.PT subclusters and by genotype (Klf4⁺/⁻ and Klf4(SE) mice).
aPecs and knocked down Itgb6 (Itgb6-shRNA) using a lentiviral shRNAmir system, which was validated by real-time polymerase chain reaction (PCR) and immunostaining for Integrin β6 (ITGB6) (fig. S7, D and E). CD44 expression was significantly lost in Itgb6-shRNA aPecs as compared to Scr-shRNA aPecs (fig. S7E). While Itgb6-shRNA aPecs exhibited a significant decrease in expression of these aPEC markers (Cd44, Creb5, and Runx1) as compared to Scr-shRNA aPecs, treatment with FN1 failed to restore their expression (fig. S7F). Collectively, these data suggest that FN1-αvβ6 (ITGB6) chain reaction (PCR) and immunostaining for Integrin expression (percent area) of these previous data, we initially confirmed the efficacy of S3I-201 as a STAT3 inhibitor by performing immunostaining for phospho-STAT3 (pSTAT3) (Tyr705) in Klf4ΔKlf4 mice (fig. S8A). Quantification of nuclear pSTAT3 (phosphorylated signal transducer and activator of transcription 3), using Hoechst for nuclear colocalization, revealed a decrease in pSTAT3 expression in S3I-201–treated Klf4ΔKlf4 mice compared to those treated with DMSO (fig. S8B). On the basis of these data, we initially confirmed that albuminuria was reduced in S3I-201–treated Klf4ΔKlf4 mice as compared to DMSO-treated Klf4ΔKlf4 mice (Fig. 7A). Periodic acid–Schiff (PAS) staining showed a reduction in FSGS and global sclerosis lesions, proteinaceous casts, and tubulointerstitial injury in S3I-201–treated Klf4ΔKlf4 mice as compared to DMSO-treated Klf4ΔKlf4 mice (Fig. 7B). Furthermore, immunostaining for CD44 and Nephrin demonstrates a significant decrease in CD44 expression (Fig. 7C) and an increase in Nephrin expression (Fig. 7D) in S3I-201–treated Klf4ΔKlf4 mice compared to DMSO-treated Klf4ΔKlf4 mice, suggesting that STAT3 inhibition attenuated

**The STAT3 small-molecule inhibitor S3I-201 ameliorates podocyte loss and PEC proliferation in Klf4ΔKlf4 mice**

We previously reported that KLF4 is a negative regulator of STAT3 and that the loss of KLF4 results in dysregulated STAT3 signaling leading to podocyte loss and subsequent PEC proliferation (13). We recently reported that S3I-201, a small-molecule selective inhibitor of STAT3, reduced albuminuria in Klf4ΔKlf4 mice as compared with dimethyl sulfoxide (DMSO)–treated Klf4ΔKlf4 mice (13). On the basis of these previous data, we initially confirmed the efficacy of S3I-201 as a STAT3 inhibitor by performing immunostaining for phospho-STAT3 (pSTAT3) (Tyr705) in Klf4ΔKlf4 mice (fig. S8A). Quantification of nuclear pSTAT3 (phosphorylated signal transducer and activator of transcription 3), using Hoechst for nuclear colocalization, revealed a decrease in pSTAT3 expression in S3I-201–treated Klf4ΔKlf4 mice compared to those treated with DMSO (fig. S8B). On the basis of these data, we initially confirmed that albuminuria was reduced in S3I-201–treated Klf4ΔKlf4 mice as compared to DMSO-treated Klf4ΔKlf4 mice (Fig. 7A). Periodic acid–Schiff (PAS) staining showed a reduction in FSGS and global sclerosis lesions, proteinaceous casts, and tubulointerstitial injury in S3I-201–treated Klf4ΔKlf4 mice as compared to DMSO-treated Klf4ΔKlf4 mice (Fig. 7B). Furthermore, immunostaining for CD44 and Nephrin demonstrates a significant decrease in CD44 expression (Fig. 7C) and an increase in Nephrin expression (Fig. 7D) in S3I-201–treated Klf4ΔKlf4 mice compared to DMSO-treated Klf4ΔKlf4 mice, suggesting that STAT3 inhibition attenuated
Podocyte loss and PEC activation in Klf4ΔPod mice. Because STAT3 also occupies the promoter region of FN1, we confirmed that glomerular FN1 expression was reduced S3I-201–treated Klf4ΔPod mice compared to DMSO-treated Klf4ΔPod mice (Fig. 7E). Last, we also observed a reduction in ITGB6 expression in S3I-201–treated Klf4ΔPod mice as compared to DMSO-treated Klf4ΔPod mice (Fig. 7F). These data demonstrate that pharmacological STAT3 inhibition attenuated FN1 and ITGB6 induction, podocyte loss, and PEC activation in Klf4ΔPod mice, suggesting the potential pathogenic role of KLF4-STAT3-FN1-ITGB6 complex in proliferative glomerulopathies.

DISCUSSION

We previously demonstrated that genetic knockdown of podocyte-Klf4 (Klf4ΔPod) leads to FSGS with extracapillary lesions and reduced overall survival in mice; however, the underlying mechanisms remain unexplored. In this set of studies, we demonstrate that Klf4ΔPod serves as a robust model to investigate mechanisms by which podocyte loss triggers the activation and proliferation of a distinct PEC population by using a multimomics approach consisting of glomerular RNA-seq, iTRAQ, and LFQ proteomics and snRNA-seq. We leverage these datasets through the use of a ligand-receptor–based analysis to identify putative mechanisms linking podocyte loss to aberrant PEC activation, noting a potential FN1-αVβ6 interaction to target for future therapy (Fig. 9). Using published ChIP-seq data, we further highlight a potential role for KLF4 and STAT3 in regulating expression of key podocyte-secreted ligand and receptor genes that are expressed in our captured subpopulation of aPECs. We also demonstrate the potential transcriptional changes required to mediate the switch from a quiescent to aPEC state in this model of proliferative glomerulopathy. Last, suppression of activated STAT3 signaling with a small-molecule inhibitor or podocyte-specific induction of human KLF4 abrogates these pathological events.

To begin, we used a lineage tracing system in which podocytes are permanently labeled with RFP to determine whether proliferating glomerular epithelial cells are of podocyte origin. To date, this is the first study to report that podocyte-specific knockdown of Klf4 contributes to podocyte loss, triggering the activation of a distinct quiescent PEC population, rather than transdifferentiation of injured podocytes to PECs. These data provide the rationale to interrogate the podocyte–PEC cross-talk in the setting of podocyte-specific knockdown of Klf4. While initial RNA-seq from isolated glomeruli demonstrated the expected decrease in podocyte-specific transcripts and an increase in expression of transcripts enriched for inflammatory, adhesion, and cell cycle pathways in Klf4ΔPod mice, in silico ChIP-seq highlights that these transcriptional changes might be mediated by the loss of a direct transcriptional regulatory role by KLF4 as well as the induction in pSTAT3 transcriptional activity. Future studies are required to determine the dynamics of KLF4 and pSTAT3 transcriptional antagonism, especially as they relate to effects of distant pSTAT3 to proximal KLF4 binding sites on target genes.

Mass spectrometry using both label-free and isobaric tag labeling methods identified that the proteins released by KLF4-knockdown podocytes were related to pathways of ECM formation, cell adhesion, and growth factor signaling, which together support the observed glomerulosclerosis and aberrant PEC proliferation in Klf4ΔPod mice. To ensure that the secreted proteins identified did not originate from cell debris, we performed multistep centrifugation and spin filter approaches and used the subcellular localization data base to determine whether these differentially expressed proteins are expressed.
Fig. 8. Podocyte-specific induction of KLF4 protects against NTS-induced nephritis. (A) Urine albumin-to-creatinine ratio of NTS-treated PODTA and PODTA;TRE-KLF4 mice at days 7 and 14. n = 4 per group; **P < 0.01; ***P < 0.001 as compared to the respective genotype at day 0 (VEH-treated); $P < 0.05 as compared to day 7 NTS-treated PODTA mice; two-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc test. (B) Representative images of PAS stain from PODTA and PODTA;TRE-KLF4 mice after NTS treatment (arrows indicate sclerotic glomeruli with extracapillary proliferation and crescent formation; arrowheads indicate interstitial and periglomerular proliferation; asterisks indicate tubular dilatation and proteinaceous casts). Representative images and quantification of immunostaining for (C and D) Nephrin, (E and F) CD44, (G) Ki67*CD44* cells per glomeruli, (H and I) FN1, and (J and K) ITGB6 expression in NTS-treated PODTA and PODTA;TRE-KLF4 mice. n = 4 per group; 20 glomeruli per mouse; **P < 0.01; ***P < 0.001 as compared to VEH-treated respective genotypes; $P < 0.05 as compared to NTS-treated PODTA mice; one-way ANOVA with Tukey's multiple comparisons post hoc test (D). *P < 0.05, **P < 0.01, and ***P < 0.001 as compared to NTS-treated PODTA mice; unpaired t test (F, G, I, and K). Dotted white lines indicate glomerular area; solid white lines indicate area of extracapillary proliferation; asterisks indicate tubular staining for ITGB6. Scale bars, 40 μm (B), 20 μm (C), and 50 μm (E, H, and J).
likely secreted from podocytes in the setting of KLF4 knockdown (22, 35). Similar to our previous study (13), we detected interleukin-6 (IL-6) in the conditioned medium, but with insufficient signal and spectral counts for accurate quantitation. While our protocol for the derivation of conditioned medium in this manuscript was identical to that used in these previous studies, an immunoassay such as an enzyme-linked immunosorbent assay (ELISA) is a more sensitive method and allows for the detection of femtomolar quantities of protein in its native state as compared to the reduction, alkylation, and trypsin digestion necessary for peptide detection via tandem mass spectrometry. Therefore, the potential for signal loss (peptide count) of any single protein is significantly higher than that exists with an antibody-targeted native protein ELISA, which might explain the insufficient IL-6 signal and spectral counts from our proteomics.

To the best of our knowledge, this is the first study to report a murine single-nucleus atlas of proliferative glomerulopathy, with PEC activation and proliferation mediated specifically by loss of podocytes as defining features. While recent single-cell/single-nucleus studies have identified bona fide PEC populations, these are in the absence of pathologic PEC activation and proliferation (37, 38). Efforts to map the transcriptomes of PECs have been hampered by their low abundance in adult kidney cortex, as well as glomerular isolation techniques that shear away significant portions of Bowman’s capsule. Our expanded subclustering analysis enabled the identification of aPECs as compared with proliferating PT and pseudotime analysis exposed the transcriptional changes required to transition from a state of quiescent PEC to aPEC. In addition, snRNA-seq demonstrated that the increase in mesenchymal and inflammatory markers from the glomerular RNA-seq was largely present in aPECs, suggesting a secondary event from podocyte loss. Furthermore, snRNA-seq offers several advantages when compared with single-cell sequencing, including the improved detection of rare cell types (23). Protocols for the isolation of nuclei do not require incubations at high temperature, and samples display minimal expression of stress genes. However, snRNA-seq carries some limitations including lower sequencing depth compared with other methods. As a result, we may have failed to capture low abundance transcripts. It is also noteworthy that we failed to observe a difference in podocyte number between Klf4−/− and Klf4+/+ mice, which could be due to the low overall abundance of podocytes relative to the whole kidney cortex as previously reported by other laboratories (23).

To ascertain the putative mediators of podocyte-PEC cross-talk in proliferative glomerulopathies, we integrated our glomerular RNA-seq dataset (providing increased depth of sequencing coverage but no ability to discern cell types as with snRNA-seq) with proteomics and snRNA-seq. We subsequently used known ligand-receptor interactions (28) in combination with KLF4 and STAT3 ChIP-seq data to determine which of these ligand-receptor pairs might trigger PEC proliferation in the setting of podocyte-specific Klf4 knockdown. While our unbiased approach previously described the candidate ligand-receptor interaction of MIF-CD74 in proliferative glomerulonephritis (7), snRNA-seq subclustering analysis did not reveal a significant increase in CD74 in the aPEC subclusters. Potential reasons might be limitations with depth of transcriptome coverage with snRNA-seq as compared to bulk RNA-seq.

FN1 is an ECM molecule that binds to several partner receptors including numerous integrin family members: αVβ6, αVβ3, CD44, and the urokinase receptor. Integrin subunits αV and β6 belong to the family of heterodimeric integral membrane proteins that have established functions in cell surface adhesion and signaling. The αV subunit heterodimerizes with β6 to create a functional multimeric protein capable of binding FN1 (along with other ligands with R-G-D sequence) (33, 34). As compared to other integrin receptors (i.e., αVβ3) that are localized to podocytes, glomerular basement

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Fig. 9. Schematic of proposed podocyte-PEC cross-talk in setting of podocyte-specific loss of Klf4. In this Klf4−/− murine model of proliferative glomerulopathy, podocyte-specific knockdown of Klf4 induces podocyte loss and triggers PEC activation and proliferation, which is postulated to be mediated by paracrine signaling between injured podocytes and quiescent PECs poised for activation. Receptors, such as αVβ6, expressed specifically in quiescent PECs may respond to secreted proteins from injured podocytes, serving as the initial signals for proliferation, migration, and activation, leading to de novo expression of receptors, including CD44, which further potentiate PEC activation and proliferation. Potential noteworthy ligand-receptor interactions are provided (not all-inclusive).
membrane, and mesangial cells, recent studies report expression of αvβ6 primarily in quiescent PECs (by immunostaining of normal kidney biopsies), with a concomitant increase in PECs and tubular cells in proliferative glomerulonephritis (39). Our snRNA-seq and subclustering analysis revealed that CD44 is primarily expressed de novo in aPECs as compared to ITGB6 that is abundantly expressed in both quiescent PECs and aPECs. Data from the human protein atlas validated that ITGB6 is expressed in quiescent PECs under basal conditions as compared with CD44. In addition, knockdown of Itgb6 in aPECs significantly attenuated markers of PEC activation, regardless of FN1 treatment. Collectively, these data support a putative role for FN1-αvβ6 in triggering the activation of PECs.

Because of activation of Janus kinase/STAT signaling in various human kidney diseases and murine injury models, STAT3 has emerged as a potential therapeutic target (40). Because we previously demonstrated that the loss of KLF4 triggers STAT3 activation and that Klf4ΔPod mice demonstrated improvement in albuminuria following treatment with S3I-201, a small-molecule inhibitor of STAT3, we further investigated the effects of S3I-201 treatment on podocyte differentiation and PEC activation. Compared to DMSO-treated controls, in Klf4ΔPod mice, podocyte loss and PEC activation were attenuated following treatment with S3I-201, leading to an overall reduction in kidney injury. These results are consistent with previous reports that establish podocyte-specific deletion of STAT3 as protective in NTS-induced nephritis, a murine model of RPGN (8). In addition to the therapeutic role of selective STAT3 inhibition in this model of proliferative glomerulopathy, we provide additional evidence for the effectiveness in targeting KLF4-STAT3 signaling by demonstrating the salutary role of podocyte-specific KLF4 induction in NTS-induced nephritis. Because STAT3 inhibition has been associated with systemic toxicity (41), small-molecule agonists that induce KLF4 expression could be used in combination with selective STAT3 inhibitors, to harness their synergistic effects, while simultaneously reducing systemic toxicities associated with pharmacological STAT3 inhibition.

Together, these studies demonstrate that disruption of podocyte-specific KLF4 serves as a robust model of proliferative glomerulopathy and investigate the potential ligand–receptor interactions that can be targeted for therapy. Furthermore, we identify a novel subclustering of aPECs as well as the potential transcriptional changes required to transition from the quiescent to activated state using snRNA-seq. While inhibition of STAT3 activation and/or induction of KLF4 expression may serve as potential approaches in mitigating podocyte loss and PEC activation, we provide evidence for previously unidentified downstream ligand–receptor interactions between injured podocytes and aPECs that might also be targeted for future therapy.

These PEC-rTA mice were bred with TRE-GFP mice [Tg(tetO-HIST1H2B/GFP)47Efu/J; the Jackson laboratory] where GFP expression is dependent on the engagement of a TRE, to produce DOX-inducible PEC reporter mice (PEC-rTA:TRE-GFP). To induce transgene expression, mice were fed DOX grain-based rodent diet (no. S3888, Bio-Serv).

To generate PODTA:TRE-KLF4 mice, Nphs2-rTa mice were bred with TRE-KLF4 mice [FVB.Cg-Tg(tetO-KLF4)32831Rup/Mmjax; the Jackson laboratory]. Experimental mice received DOX food starting at 8 weeks of age and continued until mice were euthanized.

Cell culture
Conditionally immortalized human podocytes were gifts from P. Mundel (Massachusetts General Hospital, Boston, MA). Methods for podocyte cultivation, immortalization, and differentiation were based on a previously described protocol (43). These cells proliferate under permissive conditions (IFN-γ at 33°C) but differentiate under non-permissive conditions (37°C).

Primary mPECs were isolated from PEC-rTA:TRE-GFP mice that were fed DOX for 1 week before isolation. To specifically isolate the PECs, mouse glomeruli were initially retrieved from whole kidney cortex using magnetic Dynabeads as previously described (44). Following digestion of glomeruli into single cells with collagenase, GFP+ cells were sorted with the BD FACSAria III cell sorter and plated in collagen-coated plates before harvesting cells for RNA. Isolated aPECs from Klf4ΔPod mice were treated with FN1 (250 ng/ml) or phosphate-buffered saline (PBS) for 24 hours before harvesting the cells for RNA.

Glomerular RNA-seq
Mouse glomeruli were isolated as described (45). Briefly, mice were perfused with Hanks’ balanced salt solution containing iron oxide (2.5 mg/ml) and 0.1% bovine serum albumin (BSA). At the end of perfusion, kidneys were removed, decapsulated, minced into 1-mm3 pieces, and digested in PBS containing collagenase A (1 mg/ml) and deoxyribonuclease I (100 U/ml). Digested tissue was then passed through a 100-μm cell strainer and collected by centrifugation. The pellet was resuspended in 2 ml of PBS, and glomeruli were collected using a magnet. The purity of glomeruli was verified under microscopy. Total RNA was isolated from mouse kidney glomeruli with the RNAeasy kit (Qiagen).

Glomerular RNA-seq data were processed as previously described (46). Briefly, sequencing reads were aligned to the mouse genome (mm10) using Spliced Transcripts Alignment to a Reference (2.4.1c) (47). Aligned read pileups were quantified (GRCM38/mm10 assembly) at the transcript level using featureCounts (v1.4.6) (48). Read counts were normalized to counts per million, and differentially expressed genes were identified using BioJupies (49). Enrichment analysis was performed using the online tool Enrichr (16, 17, 50). Specific databases used include WikiPathways (18), KEGG (51), GO (52), Jensen Compartment (22), Protein-Protein Interaction (PPI) Hub (53), and Reactome (54).

**METHODS**

**Generation of transgenic mice**

Generation of Klf4ΔPod (FVB/N) mice was previously reported (13). Klf4fl/fl;TdT and Klf4ΔPod;TdT mice were generated by breeding Klf4fl/fl and Klf4ΔPod;TdT mice carrying the TdTomato reporter cassette [Gt(ROSA)26Sor mm14(CAG-tdTomato)Hze; the Jackson laboratory] and backcrossing to the FVB/N background for nine generations. Genotyping was performed as previously reported (13).

Mice expressing GFP+ PECs were generated using the previously reported PEC-rTA mice, with a hybrid promoter expressed in PECs used to drive expression of an enhanced reverse tetracycline transactivator (42).
of lysis buffer containing 20 mM tris-HCl (pH 8), 320 mM sucrose, 5 mM CaCl₂, 3 mM MgAc₂, 0.1 mM EDTA, and 0.1% Triton X-100. The tissue was initially dissociated by pipetting up and down 10× with a p-1000 tip and then being passed through a 25G syringe 10×. The tissue was incubated on ice for 10 min and then passed through a 30-μm CellTrics filter. The nuclei were pelleted by centrifugation (5 min, 500g, 4°C) and washed with PBS after removal of supernatant. The nuclei were pelleted again and resuspended in 1 ml of PBS containing 0.04% BSA and ribonuclease inhibitor (0.2 U/μl) before generating counts with hemocytometer. The nuclei were then diluted and prepared for snRNA-seq with the 10X Chromium System according to the manufacturer’s instructions (10X Genomics). Sequencing was performed using an Illumina NextSeq 550 platform.

Raw sequencing data were demultiplexed and aligned to a mouse pre-mRNA reference genome using Cell Ranger on SeaWulf, the high performance cluster (HPC) at the Stony Brook University. Quality control, dimensionality reduction, and clustering were performed using the R package Seurat (56). Genes expressed in a minimum of three cells were retained. Cells expressing less than 200 or more than 5000 genes were excluded. Cells expressing more than 10% mitochondrial genes were also excluded. The R package Monocle (v2) was used for pseudotime trajectory analysis of the PEC/Prolif.PT cluster (26, 27).

Proteomics
Tandem mass spectrometry was performed on the supernatant from human podocytes with and without KLF4 knockdown (Scr-shRNA and Klf4-shRNA) using both label-free and isobaric tag labeling methods. Cells were seeded at 600,000 per 15-cm plate and placed in 37°C to differentiate. After 24 hours, medium was removed, and cells were washed five times with 1× PBS. Phenol-red, insulin-transferrin-selenium (ITS), and serum-free RPMI were replaced. After 48 additional hours, supernatant was harvested and centrifuged for 5 min at 1500g. Samples were reduced (dithiothreitol), alkylated (iodoacetamide), digested with trypsin, and cleaned up using hydrophobic-lyophilic-balanced (HLB) pack C-18. Samples (4 μl) were injected onto a 20-cm-long ReproSil C-18 (3 μM particle) column and run on the Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) or TripleTOF 5600+ (Sciex). Analysis was carried out using Thermo Fisher Proteome Discoverer and Scaffold.

S3I-201 treatment in mice
Klf4ΔΔNod (FVB/N) mice, starting at 7 weeks of age, were administered either S3I-201 (10 mg/kg; Santa Cruz Biotechnology Inc.) or DMSO, intraperitoneally, three times weekly until 11 weeks of age as previously reported (13).

NTS treatment in mice
In the NTS model, 10-week-old PODTA;TRE-Klf4 and PODTA littermates were first sensitized with an intraperitoneal injection of 0.5 mg of sheep IgG (Jackson Immunoresearch) with complete Freund’s adjuvant (Millipore Sigma) or PBS. Five days later, mice were administered 100 μl of NTS, intraperitoneally as previously described (13). Urine was collected before IgG injection and after 7 and 14 days. Mice were euthanized on day 14 after the first NTS injection, and kidneys were harvested for histology, protein, RNA, and glomerular isolation.

Real-time PCR
Total RNA was extracted by using TRIzol (Life Technologies). First-strand complementary DNA (cDNA) was prepared from total RNA (1.5 μg) using the SuperScript IV VILO Master Mix (Life Technologies), and diluted cDNA (1 μl) was amplified in triplicate using PowerUp SYBR qPCR Master Mix on an ABI QuantStudio 3 (Applied Biosystems). All primers used in the studies were designed using National Center for Biotechnology Information PrimerBLAST, and they were validated for efficacy before application. All quantitative PCR primer sequences are listed in table S1. Light cycle analysis software was used to determine crossing points using the second derivative method. Data were normalized to housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase) and presented as an FC increase compared with RNA isolated from the control group using the 2-∆ΔCT method.

Measurement of urine albumin and creatinine
Urine albumin was quantified by ELISA using a kit from Bethyl Laboratory Inc. Urine creatinine levels were measured in the same samples using the Creatinine (Urine) Colorimetric Assay Kit (500701; Cayman) according to the manufacturer’s instructions. The urine albumin excretion rate was expressed as the ratio of albumin to creatinine.

Light microscopy
Mice were perfused with PBS, and the kidney was fixed in 10% phosphate-buffered formalin overnight and switched to 70% ethanol before processing for histology. Kidney tissue was embedded in paraffin by American Histolabs, and 4-μm-thick sections were processed with PAS stain (Sigma-Aldrich).

Immunofluorescence and immunohistochemistry
Specimens were initially baked for 60 min in a 55° to 60°C oven and then processed as previously described (13). Briefly, formalin-fixed and paraffin-embedded sections were deparaffinized, and endogenous peroxidase was inactivated with H₂O₂. All kidney sections from mice were prepared in identical fashion. Immunofluorescence for rabbit anti-RFP (600-401-379; Rockland), goat anti-human KLF4 antibody (AF3640, R&D Systems), rabbit anti-pSTAT3 antibody (D3A7; Cell Signaling Technology), rabbit anti-Nephrin (NB1-30130; Novus), mouse anti-WT1 (sc-7385, Santa Cruz Biotechnology Inc.), rat anti-CD44 (103001, BioLegend), mouse anti-ITGB6 (AF2389, R&D Systems), and rabbit anti-FN (AB2033, Millipore) were performed as recently reported (13, 59, 60). Slides were photographed using a Nikon Eclipse 990 microscope with a digital camera. Quantification of percent area stained was completed using ImageJ 1.26 t software [National Institutes of Health (NIH), rsb.info.nih.gov/ij] on at least 20 glomeruli per sample.
Immunofluorescence staining in cultured aPECs was performed as previously described (13). In brief, aPECs were fixed in 3.7% formaldehyde and permeabilized with 0.25% Triton X-100. Cells were subsequently blocked in 10% normal horse serum and incubated with the specified primary antibodies followed by the corresponding secondary antibodies and Hoechst nuclear counterstain. Slides were photographed using a Nikon Eclipse i90 microscope with a digital camera.

Study approval
Stony Brook University Animal Institute Committee approved all animal studies, and the NIH Guide for the Care and Use of Laboratory Animals was followed strictly.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abb6600

View/request a protocol for this paper from Bio-protocol.

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