Spatiotemporal landscape of kidney tubular responses to glomerular proteinuria

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Significance statement

Glomerular proteinuria induces major changes in kidney function and cardiovascular risk profile, but the precise effects of increased protein exposure on tubular function and gene expression were not well delineated. This paper describes in detail the spatiotemporal landscape of tubular responses to elevated glomerular protein filtration in mice, using single nuclei sequencing, functional imaging and antibody staining. It demonstrates that proteinuria is a potent stimulus for signaling in tubular epithelial cells and causes extensive remodeling and injury, in a segment specific manner, leading to large shifts in the balance of reabsorptive and secretory processes. This helps to explain the outsized effect of proteinuria on body homeostasis and suggests that protein filtration is an important link between glomerular and tubular function *in vivo*.

Abstract

Background

Large increases in glomerular protein filtration induce major changes in body homeostasis and increase risk of kidney functional decline and cardiovascular disease. We investigated how elevated protein exposure modifies the landscape of tubular function along the entire nephron, to understand the cellular changes that mediate these important clinical phenomena.

Methods

We conducted single nuclei RNA sequencing, functional intravital imaging, and antibody staining to spatially map transport processes along the mouse kidney tubule. We then delineated how these were altered in a transgenic mouse model of inducible glomerular proteinuria (POD-ATTAC) at 7 and 28 days.

Results

Glomerular proteinuria activated large-scale and pleotropic changes in gene expression in all major nephron sections. Extension of protein uptake from early (S1) to later (S2) parts of the proximal tubule initially triggered dramatic expansion of a hybrid S1/2 population, followed by injury and failed repair, with the cumulative effect of loss of canonical S2 functions. Proteinuria also induced acute injury in S3. Meanwhile, overflow of luminal proteins to the distal tubule caused transcriptional convergence between specialized regions and generalized dedifferentiation.

Conclusion

Proteinuria modulated cell signaling in tubular epithelia and causes distinct patterns of remodeling and injury in a segment specific manner.

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Introduction

Large increases in glomerular protein filtration induce major changes in kidney function and body homeostasis, including salt and water retention[1], diuretic resistance[2], and hyperlipidemia[3] (the nephrotic syndrome[4]). Meanwhile, proteinuria is a strong and independent risk factor for decline in kidn function[5] and cardiovascular disease[6]. These observations imply that increased luminal exposure to proteins exerts complex effects on the behavior of tubular epithelial cells[7], including both adaptive and injurious responses, but the nature of these was not well understood[8].

Increased protein exposure could influence cell signaling in tubules by several different mechanisms. First, uptake and metabolism of proteins within lysosomes can activate pathways like mTOR[9], which are known to be important in the kidney[10]. Second, when intracellular protein degradation capacity is exceeded, endoplasmic reticulum (ER) stress can result, triggering unfolded protein responses[11–17]. Third, many plasma proteins are highly bioactive, and thus capable of extrinsic activation of cell signaling pathways[18–20]. Fourth, the osmotic effects of proteins might induce luminal stretch, which could stimulate mechanosensitive pathways[21]. Finally, proteinuria in glomerulopathies is often accompanied by decreases in glomerular filtration rate (GFR), which may also impact on the differentiation state of tubules[22].

The proximal tubule (PT) plays a critical role in renal protein handling by reabsorbing and degrading filtered proteins, to reclaim important nutrients and cargo[23]. Uptake across the apical membrane occurs via receptor mediated endocytosis (megalin and cubilin system)[24], and proteins are rapidly trafficked to cathepsin rich lysosomes to undergo catabolism. The high capacity and efficiency of this retrieval system means that almost all plasma proteins are removed by the time the filtrate reaches the distal tubular segments, which are therefore not adapted to protein uptake and metabolism. Hence, an increase in protein load may exert heterogenous effects along the nephron, depending on the intrinsic capacity of cells within each region to respond to and process this.

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The PT also secretes non-filtered organic anions and uremic toxins to eliminate them from the body. To execute this function, PT cells express organic anion transporters (OATs) in the basolateral membrane[25], and contain extensive machinery (peroxisomes and cytochrome P450s) to metabolize and detoxify organic solutes. In addition, OATs can transport lipids, including fatty acids, and thus influence systemic lipid homeostasis[26]. Previous studies have suggested that transport processes along the PT are to some extent spatially organized, with reabsorption predominating in the early part (Segment 1 – S1)[27], and organic solute secretion in the later S2[28,29]. Adaptation to these tasks is reflected in differences between S1 and S2 cells in ultrastructure[23], autofluorescence[30], metabolism[31] and gene expression[32,33]. Yet the morphological distinction between S1 and S2 occurs late in development[34], and PT-derived cells *in vitro* exhibit an intermediate S1/2 transcriptome[35], suggesting that unknown environmental factors shape PT cell phenotype in the mature kidney *in vivo*.

Using labeled ligands and intravital kidney imaging in mice, we have shown recently that reabsorption of filtered proteins occurs mainly in S1 cells, which have a highly developed apical endo-lysosomal system[36,37]. However, S2 cells also contain substantial endocytotic machinery, and extension of uptake to this region can occur when glomerular protein filtration increases[38,39]. The repercussions of this for other specialized S2 functions were unclear, but loss of organic anion secretion is an independent risk factor for kidney disease progression[40], and the development of associated metabolic[41] and cognitive complications[42].

Here, we have used single nuclei RNA sequencing, antibody staining and functional intravital imaging, to precisely map the spatial profiles of protein uptake and organic anion secretion along the PT. Moreover, by utilizing an established mouse model (POD-ATTAC) of inducible podocytopathy resembling focal segmental glomerulosclerosis (FSGS, a glomerulopathy often leading to nephrotic syndrome) and following events over time, we assess the spatiotemporal effects of proteinuria on tubular function.

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Methods

Reagents

Unless stated otherwise, all reagents were purchased from Sigma Aldrich.

Dyes

The following dyes were purchased from the manufacturer: Dextran Alexa 647 (D22914, Thermo Fisher Scientific) and fluorescein (F6377, Sigma Aldrich). Fluorescein was dissolved in 0.9% NaCl. Lysozyme and β-lactoglobulin were labeled with Atto-565 as described previously[36].

Animals

Experiments were performed in accordance with the regulations of the Zurich or Geneva cantonal veterinary offices, under the animal authorization GE-70/19, GE-181/19 and TI-04/2017. 7 to 12-week-old male C57BL/6J mice (supplied by Janvier) were housed at 20°C with free access to food and water. For the POD-ATTAC model[43], 8 week-old males on an FVB background were used. Mice were randomly assigned to control (n=3), 7 days (n=3) or 28 days (n=4) groups. The dimerizer (Clontech Laboratories, Inc.) was prepared in accordance with product datasheet and injected intraperiteonally 5 times with 0.2µg/g of dimerizer. Mice were euthanized after 7 days or 28 days.

Intravital imaging

Animals were anesthetized with isoflurane (1.5 – 5%) and oxygen (600ml/min) and the left kidney was externalized for imaging as described previously[44]. The internal jugular vein was cannulated to allow intravenous injections of dyes and reagents. Animals were placed on a custom-built temperature-controlled stage and body temperature was monitored throughout experiments. Imaging was performed using a custom-built multiphoton microscope operating in an inverted mode[45], and powered by a broadband tunable laser (InSight DeepSee Dual Ultrafast Ti:Sapphire, Spectraphysics). Intravital imaging was performed with an XLPlan N ×25/1.05 water immersion objective (Olympus) and emitted light was

collected through four gallium-arsenide-phosphide photomultiplier tubes (Hamamatsu) in a non-descanned epifluorescence detection mode. The following excitation wavelengths were used: Dextran Alexa 647 (2mg/kg) 1120nm, β-lactoglobulin-565 (25ug) 850nm and fluorescein (2mg/kg) 950nm. Image acquisition of dextran uptake was every 2.5seconds. Probenecid (P36400, Thermo Fisher Scientific, 100mg/kg) was dissolved in 0.9% NaCl and injected 5 minutes before fluorescein.

Histological and immunofluorescence staining

PFA-fixed, paraffin-embedded blocks were sectioned at 5µm. For immunofluorescence staining, antigen retrieval was performed at 110°C in citrate buffer at pH 6 or in Tris EDTA buffer at pH 9 for 12 minutes. After permeabilization with 0.1% TritonX-100 (Surfact-Amps detergent solution, Thermo Fisher Scientific) for 5 minutes, tissue sections were blocked with 1% BSA and 10% donkey or goat serum before incubation overnight at 4°C with following primary antibodies: goat anti-Cathepsin L (R&D Systems, AF1515-SP, 1:100), rabbit anti-OAT1 (Alpha Diagnostic International, OAT11-A, 1:100), rabbit anti-SGLT2 (Proteintech, 24654-1-AP, 1:25), rabbit anti-PMP70 (PA1-650, Thermo Fisher, 1:100), rat anti-LAMP1 (ab25245, Abcam, 1:100), mouse anti-Lrp2 (CD7D5) (Novus Biologicals, NB110-96417, 1:50), sheep anti-Cubilin (R&D Systems, AF3700, 1:50), rabbit anti-NCC Nterminal (#1869, Loffing lab, 1:1000) or rabbit anti-NKCC2 (#817, Loffing lab, 1:4000), mouse anti-STAT3 (Santa Cruz Biotech, sc-8019, 1:100), goat anti-Kim1 (R&D Systems, AF1817, 1:100). After washing with PBS, sections were incubated at room temperature for 2 hours with the following secondary antibodies: donkey anti-goat-AF647 (705-606-147, Jackson ImmunoResearch Europe Ltd, 1:500), donkey anti-rabbit-AF488 (711-546-152, Jackson ImmunoResearch Europe Ltd, 1:500), donkey anti-rabbit-AF647 (711-606-152, Jackson ImmunoResearch Europe Ltd, 1:300), goat anti-rat-Cy5 antibody (112-607-003, Jackson ImmunoResearch Europe Ltd, 1:500), goat anti-mouse-AF568 (A-11004, Thermo Fisher Scientific, 1:500), donkey anti-goat-AF555 (Abcam, ab150130, 1:500) and donkey anti-sheep-AF647 (A-21448, Thermo Fisher Scientific, 1:500). Tissue was subsequently washed with PBS, and cellular DNA was stained with 5µg/ml Hoechst 33342 (H1399, Molecular Probes) for 10 minutes at room

temperature. Slices were mounted with Dako mounting medium (Agilent, Santa Clara). Visualization of the staining was performed with an Axio Scan.Z1 slidescanner using a Plan Apochromat 40x/0.95 air immersion objective and a confocal Leica SP8 inverse STED 3X microscope using an HC PL APO CS2 20x/0.75 oil immersion objective.

For immunohistochemistry experiments, endogenous peroxidase activity was blocked using 3% H_2O_2 for 5 minutes and permeabilization performed with 0.1% TritonX-100 (Surfact-Amps detergent solution, Thermo Fisher Scientific) for 5 minutes. Sections were then blocked in PBS goat serum 5% for 1 hour at room temperature and the following primary antibodies incubated overnight at 4°C: rabbit anti-VCAM1 (ab134047, Abcam 1:50), rabbit anti-SGLT2 (Proteintech, 24654-1-AP, 1:25), rabbit anti-NCAM1 (ab220360, Abcam 1:200). After washing with PBS, sections were incubated at room temperature for 1 hour with the biotinylated goat anti-rabbit (BA-1000, Vectorlabs, 1:200) and signal detection was performed using the Vectastain Elite ABC kit peroxidase (PK-6101, Vectorlabs) then the DAB substrate kit peroxidase (SK-4100, Vectorlabs), following the manufacturer recommendations. Sections were progressively dehydrated in increasing concentrations of EtOH then cleared in Histochoice (H2779, Sigma-Aldrich) and mounted using Pertex xylene-based medium. Visualization was performed with an Axio Scan.Z1 slidescanner using a Plan Apochromat 20x/0.8 air immersion objective.

Image analysis

All images for comparison were captured using the same imaging settings. All image processing was done in FIJI[46]. Regions of interest were drawn manually to obtain fluorescent signals from the endo-lysosomal system or the cytosol. The nuclear signal was removed by applying a lower threshold. For better visualization of the images, contrast and brightness were modified and applied to all parts of the figures equally. Fluorescent signals from each marker were normalized from min (=0) to max (=1) for each animal to plot tubule ROIs depending on the level of each marker expression. For histochemical images, signals were thresholded to remove background fluorescence and count all pixels above the threshold as a

positive staining for the marker. For the identification of VCAM1+ structures, serial sections were aligned using ZEN software alignment tool.

Single-nuclei isolation

Single nuclei isolation from tissue was performed as previously described[47]. Briefly, mice were perfused with ice-cold PBS and kidney halves collected for snRNA-seq analysis. The half kidney was cut into small pieces (>25) and transferred into a 2-mL Dounce homogenizer (Sigma, Cat#D8938) loaded with 1 mL of NEZ Lysis Buffer (Sigma, Cat#N3408) with RNase inhibitor (NEB, Cat#M0314) at final concentration of 0.4 U/µl) on ice. Samples were then Dounce homogenized on ice with five strokes of the looser pestle every 2 minutes for 8 minutes (25 strokes in total). Samples were then slowly Dounce homogenized 25 times with the tighter pestle on ice. The homogenized sample was filtered through a 40-µm Falcon Nylon Cell Strainer, then the filter was washed with 8 mL of 1% BSA PBS, and the nuclear suspension spun in a precooled (4°C) centrifuge at 650g for 8 minutes. Supernatant was removed and the pellet resuspended in 1 % BSA PBS (plus RNase inhibitor) and moved to a low-bind Eppendorf tube. Nuclear quality and number were assessed with trypan blue staining and a hemocytometer. Nuclei were sorted as DAPI positive on a BD Aria into 2% BSA solution with RNA inhibitor.

Single nuclei RNAseq library generation

Single-cell transcriptomes was performed using 10X Chromium single cell platform (10X Genomics). After sorting, nuclei were counted and immediately processed according to the 10X Chromium protocol. The recommended volume of single cell suspension was loaded on a Chromium Single Cell Controller (10x Genomics) targeting 8,000-10,000 cells per sample. Barcoded single-cell gel beads in emulsion (GEMs) were created by 10x Genomics Chromium TM and then reverse transcribed to generate single-cell RNA-seq libraries using Chromium Single Cell 3' Library and Gel Bead Kit v3 (10X Genomics) according to manufacturer's instructions. Resulting short fragment libraries were checked for quality and quantity using an Agilent 2100 Bioanalyzer and Invitrogen Qubit Fluorometer. Sequencing Unique molecular

identifiers (UMIs), which were incorporated into the 5' end of cDNA during reverse transcription, were used to quantify the exact number of transcripts in a cell. Paired-end sequencing was carried out on Illumina NextSeq500platform using 150-cycle High Output. FASTQ files originating from GSE151167 and GSE139107, along with the newly generated GSO218376 were homogenously processed by CellRanger (version 5.0) and reads were aligned to mouse reference genome (mm10). The Cell Ranger count function output filtered gene–cell expression matrices, removing cell barcodes not represented in cells. Finally, a UMI count table was generated for downstream analysis.

Single nuclei RNAseq data analysis

Downstream analyses were performed in R (version 4.3.0) using Seurat package (v5). To filter only high-quality cells using the same quality control criteria among the different databases, we applied filters on unique molecular identifier (UMI), excluding nuclei with less or more than three times the median of the nFeature_RNA detected for each sample and nuclei with a percentage of UMIs mapped to mitochondrial genes >5 and ribosomal genes >5. Potential doublets were removed using DoubletFinder_v3 with PCs = 1:30, pN = 0.25, pK = 0.09 and nExp = nExp_poi.adj. Ambient RNA removal was performed using SoupX with standard parameters. Each dataset was further normalized separately using the Log normalize function from Seurat, before being integrated with the Seurat Canonical Correlation Analysis method (CCA). We integrated samples originating from GSE151167 and GSE139107, along with the newly generated GSO218376 dataset using Seurat CCA workflow split by datasets. Finally, we ran the RunPCA, RunUMAP, FindNeighbors functions with default parameters on the integrated dataset. FindCluster was performed at sequential resolutions and the adequate resolution chose using Clustree and DimPlots. Cluster annotation was performed manually, based on lineage-specific marker expression. FeaturePlot, DotPlot and DoHeatMap functions from Seurat were used for data visualization, as well as dittoDimPlot from dittoSeq package. FindAllMarkers function was used to identify top markers among clusters with a min.pct = 0.25, and a logfc.threshold = 0.25, and FindMarkers when comparing two clusters. Gene Set

Enrichment Analyses were performed using gseGO function from ClusterProfiler package version 3.0.4. Trajectory inference was carried out using the slingshot function[48] setting the control samples as starting point and using UMAP coordinates. The genes differentially expressed along the trajectories were calculated using the tradeSeq algorithm (10.18129/B9.bioc.tradeSeq) including only variable features (n=2000).

Results

Spatial arrangement of solute transport function along the mouse proximal tubule

To investigate the spatial relationship between protein reabsorption and other transport functions along the mouse PT, we began by performing an unbiased analysis of gene expression data in control C57BL/6J mice, generated by single nuclei RNA sequencing in a previous study on the effects of ischemia-reperfusion injury (IRI)[49,50] (Supplementary table 1). We present gene expression analysis in C57BL/6J mice as it was the strain used for intravital imaging experiments, but similar results were obtained in the FVB background of POD-ATTAC mice (Supplementary Figure 1). In this dataset, S1 and S2 cells could be distinguished as two distinct, but overlapping clusters, whereas the S3 cluster was more segregated (Figure 1A). Among the highly expressed genes in the S1 cluster were the apical membrane reabsorptive transporters Slc6a19 (sodium-amino acid transporter), Slc5a2 (sodium-glucose transporter 2 [SGLT2]), and SLC5a12 (sodium-monocarboxylate transporter, responsible for lactate reabsorption) (Figure 1B and H). Gene set enrichment analysis (GSEA) revealed high mitochondrial activity in S1, which is known to be critical for solute reabsorption (Figure 1C). Pathways enriched in S2 included organic anion transport, lipid transport/metabolism and transferase activity, and organic anion transport score was higher in S2 (Figure 1D-E). Megalin and the sodium-phosphate transporter NaPi2a were expressed in both S1 and S2 (Figure 1F-G), whereas Slc22a6 (OAT1), Slc13a3 (basolateral sodium-dependent dicarboxylate transporter, required for OAT1 function), Abcd3 (Pmp-70, a peroxisomal marker), and Cd36 (fatty acid transporter) all localized predominantly to S2 (Figure 1B, F-K).

These data indicate that S1 cells are more adapted to perform apical reabsorption of filtered substances, whereas S2 cells are more specialized to transport and metabolize organic anions and lipids. To provide further validation of this concept, we injected control healthy mice intravenously with a fluorescently labeled small protein (lysozyme), which we have previously shown is rapidly filtered and reabsorbed in the PT[36]. Thirty minutes post injection mice were sacrificed, and kidney tissue was fixed and stained with an antibody to OAT1. As expected, OAT1 abundance was low in early, lysozyme positive PT segments, but was much higher in later regions devoid of protein (Figure 2A). Moreover, axial quantification of signal intensities revealed that OAT1 abundance increased dramatically in the region where protein uptake ceased. Since filtered endogenous proteins are degraded by lysosomal cathepsins, we also performed staining for cathepsin L, and again observed an inverse relationship with OAT1 abundance (Figure 2B).

Finally, to perform functional assessments *in vivo* we conducted intravital imaging experiments using multiphoton microscopy in mice injected intravenously with a fluorescently labeled protein (lactoglobulin, to ensure results were not unique for lysozyme) and fluorescein, a fluorescent substrate for OAT1[51]. The luminal appearance of a small dextran tracer was used to gauge the displacement of PT segments from the glomerulus, and the specificity of fluorescein was demonstrated by blockade of OAT1 with probenecid (Figure 2C-E). These experiments revealed that transport of fluorescein was low in the initial part of the PT closest to the protein filtering glomerulus, but increased markedly in later regions, beyond the protein uptake zone, thus closely matching the aforementioned expression profile of OAT1. Moreover, we did not observe cells displaying a high uptake of both protein *and* fluorescein.

In summary, these results suggest that cells in the PT of healthy mice are adapted *either* towards protein reabsorption *or* secretion of organic solutes. Moreover, the striking inverse relationship between protein uptake and OAT1 expression/activity also suggests that the former might contribute to functional demarcation of S1 and S2 segments.

Proteinuria induces widespread changes in tubular gene expression

To investigate the effects of increasing glomerular protein filtration on tubules we used the established POD-ATTAC mouse model (bred on an FVB background), where induction of caspase-8-mediated apoptosis specifically in podocytes causes abrupt loss of the normal filtration barrier and substantial proteinuria from day 2[43]. Tissue was obtained at 7 and 28 days post induction to track the temporal evolution of events (Supplementary Figure 2). These mice display progressive proteinuria and GFR decline, thus modeling glomerular disease in humans (Supplementary Figure 2). Single nuclei RNA sequencing was performed to assess changes in gene expression along the entire tubule.

In response to proteinuria, large-scale changes in gene expression occurred at both time points in POD-ATTAC mice, showing that luminal protein exposure may be a potent modulator of gene transcription in tubular epithelia. Multiple new cell clusters were identified in proteinuric mice, arising from both proximal and distal tubular segments (Figure 3A, Supplementary figure 3, Supplementary Table 2). Histological analysis of fixed kidney tissue from mice revealed that tubular lumens were dilated and filled with eosinophilic material, confirming a massive increase in luminal protein delivery (Figure 3B).

Emergence of hybrid S1/2 proximal tubular cells in proteinuria

Detailed sub-clustering of PT cells revealed normal S1, S2, and S3 groups in control mice, as assigned by established markers (Figure 4A-B and Supplementary figure 4). In proteinuric mice we observed the presence of PT cell types expressing injury markers such as Havcr1 (KIM-1), Cdh6 or Vcam1[52,53], and the appearance of a new cell population displaying an overlap of S1 and S2 marker genes (Trans_S1-S2 in Figure 4A-B and Supplementary figure 4). Temporal profiling of cell abundance in each cluster revealed a striking loss of S2 and S3 cells after 7 and 28 days in proteinuric mice, the persistence of S1/2 transition cells and progressive expansion of injured cells (Figure 4C). Conversely, the S1 cell population was much better preserved. These results indicated that proteinuria induced remodeling of later (S2/3) PT segments.

The highest expressed genes in the S1/2 transition cluster were indicative of a hybrid of both S1 and S2 segments (Figure 4D-G). Moreover, since the S1 cell population did not change to the same extent over time, it is highly likely that this new cluster arose predominantly from S2. Unsupervised comparison of the new S1/2 hybrid cluster with normal S2 cells in control animals revealed downregulation of pathways normally highly active in the latter, including organic acid metabolic processes and catalytic activity, but activation of other processes, such as receptor ligand activity, protein macromolecule adaptor activity, signal transduction, and regulation of growth (Figure 4H and Supplementary Table 4). Furthermore, differentially expressed genes included transcription/signaling factors involved in cell growth and development (e.g. Map3k1, Bmp6, Ndrg1), endo-lysosomal and actin cytoskeleton genes (e.g. Atp6v0a4, Myo9a), and genes involved in neuronal/synapse development and axon guidance (e.g. Tanc1, Sema3c) (Supplementary figure 5).

Taken together, these results suggest that urinary proteins induce a cell plasticity response leading to the extension of the protein reabsorptive segment of the PT through the transition of S2 cells into a hybrid S1/2 cell state. To provide further validation for this concept, we performed antibody staining for proteins normally highly abundant in S1 (Lamp1 and cathepsin L)[36] and S2 (OAT1 and pmp70, a peroxisomal protein[30]), respectively. Compared to control mice, after 7 days proteinuric mice displayed a large increase in the number of PT cells displaying high abundance of both S1 and S2 markers (Figure 5A-C). Moreover, among the differentially expressed genes in the expanded S1/2 transition cluster was the transcription factor/signaling molecule Stat3 (Supplementary figure 5), and we confirmed that its abundance was increased at protein level in cells of S2 origin (Figure 5D). Interestingly, Stat3 has been previously identified as important in several kidney diseases[54].

After 28 days of proteinuria, we observed that the abundance of OAT1 in proteinuric kidneys was markedly reduced (Figure 5E), denoting a loss of canonical S2 secretory function. Conversely, the expression of megalin/cubilin in cortical PTs was similar in both proteinuric and control mice

(Supplementary Figure 6). After 28 days, cubilin staining was more patchy, but large clusters of highly expressing tubules still remained. Moreover, residual tubules stained strongly for SGLT2 (Supplementary Figure 6), thus verifying relative preservation of S1, even after 4 weeks of heavy proteinuria.

Lineage analysis if injury responses in the proximal tubule

To better understand the origins of PT cells expressing injury markers in proteinuric kidneys, we performed a lineage analysis using cells belonging to the control group as the starting point. The lineage control – 7 days – 28 days was then calculated using a Slingshot algorithm[48]. The experimental time was used as the ground truth to corroborate the suitability of the calculated lineage. From this, we derived a pathway from S2 cells to S1/2 transition and S1, and then to late injury/failed repair (Figure 6A-B). In addition, a distinct injury trajectory arising from S3 was also identified (Figure 6C-D). To further validate these concepts, we conducted antibody staining for established markers of acute injury (Havcr1/KIM-1) and failed repair (VCAM-1)[52]. This revealed increased abundance of KIM-1 in S3s in proteinuric kidneys (and to a lesser extent in S2) (Figure 6E-F). VCAM-1 staining mainly localized to cortical convoluted PTs, which were mostly distinct from SGLT2 positive (S1) segments in serial sections (Figure 6G-H).

Taken together, these results further support the concept that extension of protein uptake along the PT initially induces remodeling of S2 cells towards an S1 phenotype, but that with time features of late injury/failed repair also develop, which may exacerbate the loss of canonical S2 functions.

Comparison of injury profiles between proteinuria and ischemia-reperfusion injury

To further assess to what extent gene expression profiles in proteinuric states are specific to this condition, we compared results with published data from a previous study of IRI[49,50]. For the IRI mice, data were taken at two time points: early (4h, 12h, 64h, 96h) and late (14d, 28d, 6weeks) (Supplementary figure 7). Comparison of the datasets revealed an acute dedifferentiation and subsequent partial recovery

of all three segments PT (S1-3) in IRI, in contrast to the selective and sustained loss of S2/3 cells observed in proteinuria.

Proteinuria triggers genetic reprogramming and transcriptomic convergence in the distal tubule

New cell clusters were identified in POD-ATTAC mice emerging from the thick ascending limb of Henle (TAL) (Supplementary Figure 8), the distal convoluted tubule (DCT) and the principal cells (PCs) of the connecting tubule (CNT) (Figure 7A-B and E). Among the processes altered in new clusters were metabolic pathways (glycolysis, NAD), inflammasome assembly, mTOR and TGF-B signaling, autophagy, cellular proteostasis and development (Figure 7C), confirming that protein exposure has broad effects on distal tubular gene expression. Interestingly, these new clusters displayed partial transcriptomic convergence (Figure 7D), implying some commonality in response to proteinuria.

Comparison of the TAL-new cluster to normal TAL cells revealed activation and suppression of numerous genes (Supplementary Figure 8). GSEA identified upregulation of developmental processes and response to stimulus in new clusters, but downregulation of solute transport processes (Supplementary Figure 8). Interestingly, among the suppressed genes in the TAL-new cluster was uromodulin (Supplementary Table 5), which has been strongly linked to risk of kidney disease in genetic studies[55]. Meanwhile, comparison of DCT-new cells to normal DCT cells revealed activation of multiple pathways, related to processes/structures such as morphogenesis, cell projection, and the cytoskeleton (Supplementary Figure 9). Conversely, among the downregulated processes were cation and inorganic ion homeostasis, implying a loss of solute transport, which was further supported by suppression of the sodium-chloride co-transporter Slc12a3 (NCC, Supplementary Table 6).

Taken together, these data suggest that in the context of massively increased glomerular protein filtration, overflow of proteins to the distal tubule causes extensive reprogramming, with activation of developmental pathways and partial transcriptomic convergence between the segments. This results in a

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generalized dedifferentiation of the epithelia and concomitant loss of specialized transport processes. To provide further evidence of this, we performed antibody staining for Ncam1 - a marker of early tubular development that is normally not expressed in mature kidney epithelia[56] - and found that its abundance is markedly increased in proteinuric mice (Supplementary Figure 9). Conversely, proteinuric kidneys displayed decreased expression of the apical solute transporters NKCC2 and NCC, which normally demarcate the TAL and DCT, respectively (Supplementary Figure 9). Since these transporters are the major targets of medically used diuretic drugs, their disappearance might contribute to the development of diuretic resistance, which often arises in patients in the setting of heavy proteinuria.

Discussion

By performing a detailed spatiotemporal analysis of responses along the entire nephron, we show that induction of proteinuric glomerulopathy causes large-scale changes in tubular cell gene expression profiles. These include the marked expansion of a hybrid S1/2 cell population, appearance of injury and failed repair in specific PT segments, and loss of differentiation status in the distal tubule. Thus, proteinuria causes major shifts in the normal balance of reabsorptive and secretory functions. These findings provide several new insights into renal epithelial cell biology and could also explain some of the dramatic changes in body homeostasis that occur in humans with proteinuric kidney diseases.

First, they suggest that luminal protein filtration plays a role in shaping the axial topography of the PT. Further studies will be required to identify the precise intracellular signaling mechanisms that mediate this phenomenon, but we note the intimate relationship between protein endocytosis, lysosomal function and mTOR activity[9], and the fact that depleting mTOR *in vivo* alters transporter expression in PTs[10]. Moreover, a recent *in vitro* study highlighted a possible role for the phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) pathway[57]. Meanwhile, our data might also explain why the expression of apical sodium coupled transporters like NaPiIIa and NHE increases in proteinuric rodent models[58,59], and why PT phosphate transport is higher in children with nephrotic syndrome[58]. Importantly, if proteinuria

induces an early switch in PT function towards solute reabsorption, this could represent a target for intervention to reduce fluid retention in proteinuric diseases (e.g. with SGLT2 inhibitors).

Second, encroachment of protein uptake into S2 is associated with loss of canonical functions in this region, probably due to a combination of partial remodeling towards an S1 phenotype and subsequent development of a late injury/failed repair response. The relative resistance of S1 to proteinuria is most likely explained by its high lysosomal degradation capacity. A deficit of PT secretory function could help to explain why patients with nephrotic syndrome often become partially resistant to drugs like loop diuretics[60], and why uremic solute excretion is disproportionately affected in chronic kidney disease[61]. Another important finding from our study was that transport and metabolism of lipids is normally more active in S2. Therefore, loss of S2 function might contribute to the well-recognized association between proteinuria and dyslipidemia[26].

Third, the activation of developmental pathways suggests that protein filtration may be an important environmental modulator of gene expression during organogenesis. Of note, previous studies in the rat have shown that it is only possible to morphologically identify distinct S1 and S2 segments post birth, when the glomerular filtration barrier matures and the leakage of proteins is markedly reduced. A distinct S1 segment then emerges in a retrograde direction[34]. Thus, changes occurring in a proteinuric state could be conceptualized as a reversal of tubular maturation in normal development.

Fourth, we also identified a distinct injury trajectory arising from S3 in proteinuric mice. Of note, we recently discovered that protein degradation fragments generated by S1 lysosomes are released back into the tubular lumen, and that S3 avidly reabsorbs small peptides[37]. Thus, we speculate that increased lysosomal catabolism of proteins in S1 and S1/2 transition in proteinuric states might result in peptide overload and injury in downstream S3 cells.

Fifth, the segment specific effects of proteinuria in the PT stand in contrast to those of IRI and a tubular toxic (aristolochic acid) form of nephropathy, where a generalized dedifferentiation of all PT segments occurs[33]. This highlights the heterogeneity of kidney diseases and underlines the need to identify spatial patterns of injury in an organ with such a complex three dimensional structure.

Sixth, we observed that protein exposure causes extensive reprogramming in distal tubular segments, with activation of developmental pathways and downregulation of specialized transport processes. This phenomenon might further exacerbate diuretic resistance in nephrotic patients. We cannot identify from our study how exactly proteinuria drives changes in gene expression, but GSEA analysis revealed activation of non-renal pathways (e.g. neuronal), which could be consistent with extrinsic signaling from filtered bioactive peptides[19]. Meanwhile, it has very recently been shown that ureteric obstruction also causes de-differentiation of distal tubular segments[21], suggesting some similarity in responses.

There are several potential limitations to our study. Experiments were performed in mice, and we therefore cannot be certain that findings are translatable to other species. However, older studies suggested that lysosomal cathepsin staining is also concentrated in S1 in rats[62], and that activity in S2 segments dramatically increases in response to proteinuria[63]. The relatively abrupt onset of increased protein filtration in POD-ATTAC mice may not fully recapitulate the typical temporal profile in humans. Moreover, factors other than proteinuria might also contribute to loss of organic solute transporters in kidney disease, such as the uremic milieu[64]. Finally, the effects of albumin and other serum components cannot be separated in animal models of glomerular disease, and a recent *in vitro* study suggested that these have different actions in tubular cells[20].

In conclusion, we show that proteinuria exerts complex and pleiotropic effects on the renal tubule; understanding the nature of these is likely to be critical in developing new treatments to slow the progression of kidney disease and manage its associated systemic complications.

Disclosures

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Author contributions

A.F., A.R., M.B., T.V., I.B.S., D.D., S.C. and M.K. performed experiments. A.F., M.B., A.R., I.B.S., D.L., and D.M. analyzed the data. P.E.C., S.d.S. and A.M.H. conceptualized and supervised the project. A.M.H. wrote the manuscript with input from all authors.

Data sharing

Single nuclei RNA sequencing data that support the findings of this study have been deposited in GSEO with the accession number GSE218376 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE218376) and the login token ejgxugguzfkrxql. The corresponding quality control plots and the scripts necessary to reproduce the analysis are available for download:
https://zenodo.org/records/10722341?token=eyJhbGciOiJIUzUxMiIsImlhdCI6MTcwOTEzNjQwMywiZXhwIjoxNzExNjcwMzk5fQ.eyJpZCI6ImJjMGY1NjQ3LWZjNWUtNDA5NS05MWYzLWU2MzExMDA4ODZlMiIsImRhdGEiOnt9LCJyYW5kb20iOiJiNjZkYzU4MmU4NTUyMjRjZDA5MWY5YzFlZDE5NWUwYiJ9.W0M_9HFgQ7xxp2v7rgxqJzeVY1HA-ppluAauoJxWyWkH6Xlf_bfOl2yDL4spL6zK--9Rh_yTnW6DHpu0k8SvUA

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Figure legends

Figure 1. Gene expression patterns in S1 and S2 segments of the proximal tubule. (A) UMAP of single-nuclei RNAseq data showing an overview of clusters annotated according to classical marker genes and new (N) clusters. (PT = proximal tubule, TAL = Thick ascending limb, DCT = distal convoluted tubule, PC = principal cells, IC = intercalated cells, podo = podocytes, endo = endothelial cells, uro = urothelial cells). **(B)** FeaturePlot showing marker gene expression in different cell clusters. **(C)** Gene Set Ontology Analysis showing top differentially regulated pathways using gseGO function from clusterprofile, in S1 versus S2 clusters. **(D)** UMAP showing S1 and S2 clusters. **(E)** Pathway score of organic anion (OA) transport

(GO_MGI_0015711) calculated using Addmodule score function on MAGIC_RNA assay. **(F-G)** FeaturePlot showing Megalin (Lrp2) and NaPi2a (Slc34a1) are expressed in both S1 and S2. **(H)** SGLT2 (Slc5a2) is predominantly expressed in S1. **(I-K)** Cd36 (lipid transporter), OAT1 (Slc22a6) and pmp70 (peroxisomal marker) are predominantly expressed in S2.

Figure 2. Spatial arrangement of solute transport along the proximal tubule. (A) Fixed kidney cortex tissue from mice injected intravenously with fluorescently labeled lysozyme, showing high protein uptake in early (S1) segments of the proximal tubule (PT), and high OAT1 staining in S2 (G = glomerulus). Regions of interest (ROI) were drawn around individual sections of PT, and the mean fluorescence value within each is depicted in the histogram. Values were ordered according to lysozyme signal, sorted from highest to lowest (n=3). **(B)** Lysosomal cathepsin L abundance also displayed an inverse relationship with OAT1. ROIs were drawn around individual sections of PT, and the mean fluorescence value within each is depicted. Values were ordered according to cathepsin L signal, sorted from highest to lowest (n=3). **(C)** Intravital imaging with multiphoton microscopy, showing reabsorption of labeled lactoglobulin in S1, and a high uptake of the fluorescent OAT1 substrate fluorescein in S2. ROIs were drawn around individual sections of PT, and the mean fluorescence value within each is depicted. Values were ordered according to the arrival time of a small injected dextran in the tubular lumen, to denote displacement from the glomerulus (n=3). **(D)** The uptake of fluorescein was inhibited by the OAT1 blocker probenecid. **(E)** Schematic diagram showing that PT cells are adapted either for protein reabsorption (S1) or organic anion secretion (S2). Scale bars = 20um.

Figure 3. Overview of single cell sequencing data showing all tubular segments and new clusters in controls and proteinuric kidney disease. (A) UMAP of single-nuclei RNAseq data of mice (n=3 for control and 7 days, n=4 for 28 days), showing manually annotated clusters: S1 segment from proximal tubule (PT-S1), S2 segment (PT-S2), S3 segment (PT-S3), decreased PT cells (PT-D1) in proteinuria, increased PT cells (PT-N1, PT-N2), thick ascending limb (TAL), increased TAL cells (TAL-N1, TAL N2), distal convoluted tubule

(DCT), increased DCT cells (DCT-N1), principal cells (CNT-PC and CNT-PC2), intercalated cells type A (ICA), intercalated cells type B (ICB), endothelial cells (Endo), parietal epithelial cells (Parietal epithelial), immune cells (Immune), unknown proliferating cells (Proliferating), unknown decreased cells (Decreased), urothelial cells (Uro), and podocytes (Podo). **(B)** Hematoxylin and eosin staining in kidneys of transgenic (TG) mice after 7 days revealed dilated tubules filled with eosinophilic material (arrows), confirming a high luminal protein concentration. These changes were more severe after 28 days. Scale bars = 500um.

Commented [A8]: Change to appear as no quantification is provided

Figure 4. Gene expression changes in the proximal tubule in response to proteinuria. (A-B) UMAP plot of single-nuclei RNAseq data of POD-ATTAC mice (Control, 7 days and 28 days) depicting proximal tubule cell states (S1, S2, S3, Trans_S1-S2, Dedifferentiated_S1-S2, Injured_S3, Failed Repair, Unknown) manually annotated according to markers of differentiation, injury and failed repair (n=66426 nuclei). **(B)** Dot plot showing the gene expression of the differentiation, injury and failed repair makers by the identified cell states. **(C)** Cell proportion (%) of total cell count across control, 7 days and 28 days in different sub-clusters. **(D-E)** Heatmap adapted from Ransick et al., Dev Cell, 2019, showing top10 genes from S1 and S2 in the normal nephron according to the Kidney Explorer Tool. **(F)** Heatmap showing top10 genes from the Transition-S1/S2 hybrid cluster in POD-ATTAC mice. (**G**) Corresponding legend of nephron segments. **(H)** Gene Set Ontology Analysis showing top differentially regulated pathways in the Transition-S1S2 cluster versus normal S2 cells.

Figure 5. Identification of S1/2 transition cells in proteinuric kidneys. (A-C) Fixed tissue sections were stained with antibodies for S1 (Lamp1 and Cathepsin L) and S2 (OAT1 or PMP70) markers. After 7 days, the number of double positive segments (upper right quadrants denoted by red lines) was markedly increased in transgenic (TG) mice compared to wild-type (WT), indicating expansion of S1/2 transition cells. Scale bars= 50 um. Plots display normalized (0 to 1) signal intensity in individual tubular segments (n=3). **(D)** Antibody staining for STAT3 showing increased abundance in TG animals in segments of S2 origin (OAT1 positive). Scale bars= 50 um. Data represent mean intensity values, error bars SEM. Groups

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were compared using parametric t test (n=3). **(E)** Abundance of OAT1 expression was significantly lower in TG mice than WT after 28 days. Scale bars= 500 um. Data represent mean intensity values, error bars SEM. Groups were compared using parametric t test (n=3).

Figure 6. Lineage analysis of injury trajectories in the proximal tubule. (A) UMAP of the proximal tubular cell states associated to the S1-S2 injury trajectory classified according to the dedifferentiated, injury and failed repair markers or the experimental time point (Control, day7, day28). Black arrow indicates the trajectory of injury of S1 and S2 proximal tubular cells calculated using Slingshot defining "Control" as starting point. **(B)** Heatmap showing the expression of the reference markers of S1, S2, injury, failed repair and the new Trans_S1-S2 markers along the pseudotime of the trajectory identified in A. **(C)** UMAP of the proximal tubular cell states associated to the S3 injury trajectory classified according to the dedifferentiated, injury and failed repair markers or the experimental time point (Control, day7, day28). Black arrow indicates the trajectory of injury of S3 proximal tubular cells calculated using Slingshot defining "Control" as starting point. **(D)** Heatmap showing the expression of the reference markers of S3, injury and failed repair markers along the pseudotime of the trajectory identified in C. **(E)** Feature plot showing the gene expression of Slc5a2 (S1 marker), Havcr1 (KIM-1) and Vcam1 by proximal tubular cells at day 7. **(F)** Antibody staining for KIM-1 showing increased expression in transgenic (TG) mice at day 7, compared to wild-type (WT). Staining was most prominent in S3 in the outer stripe of the outer medulla (OSOM) and was also observed in some cortical OAT1 S2 positive segments (arrowhead). Scale bar= 200 um overview images and 50 um in zoomed-in images. Data represent mean values, error bars SEM. Groups were compared using parametric t test (n=3). **(G)** Antibody staining for VCAM1, confirming increased expression in cortical convoluted proximal tubules in TG mice after 7 days compared to WT. Data represent mean values, error bars SEM. Groups were compared using parametric t test (n=3). **(H)** Antibody staining for the S1 marker SGLT2 in serial sections displayed minimal overlap with VCAM1 staining.

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Figure 7. Gene expression changes in the distal tubule in response to proteinuria. (A) HeatMap of single-nuclei RNAseq data of POD-ATTAC mice (Control, 7 days and 28 days) in principal cells (PC), connecting tubule (CNT), and distal convoluted tubule (DCT), **(B)** Distal-new clusters showing top 10 differentially expressed genes between Distal and Distal-new clusters. The upper panel corresponds to genes increased in Distal-new and the bottom panel to genes downregulated in Distal-new. **(C)** Supervised pathway analysis showing selected pathways differentially expressed between and PC, CNT, DCT and new clusters. **(D)** UMAPs of single-nuclei RNAseq data of POD-ATTAC mice (Control, 7 days and 28 days), subset to distal nephron clusters, manually annotated according to marker genes. **(E)** Cell proportion (%) of total cell count across control, 7 days and 28 days in different sub-clusters as described in (D).

Supplementary Table 1. Overview of the single nuclei datasets used in this study.

Supplementary Table 2. FindAllMarkers output for cell clusters in the POD-ATTAC dataset.

Supplementary Table 3. FindAllMarkers output for the proximal tubule subset in the POD-ATTAC dataset.

Supplementary Table 4: Significantly altered genes between S1/2 transition PT cluster in POD-ATTAC mice and normal S2 PT cluster.

Supplementary Table 5: Significantly altered genes between normal TAL cluster and TAL-new cluster in POD-ATTAC mice.

Supplementary Table 6: Significantly altered genes between normal DCT cluster and DCT-new cluster in POD-ATTAC mice.