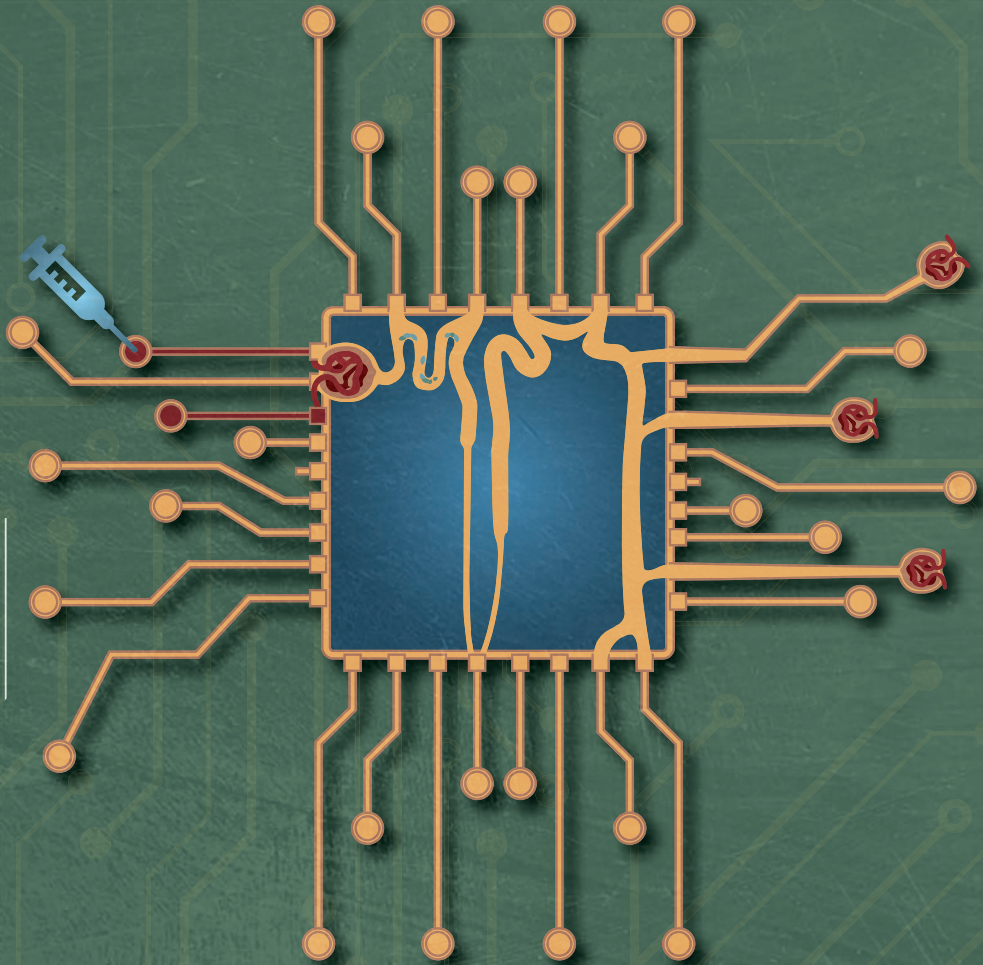


DECODING RECOVERY

OMICS INSIGHTS INTO PODOCYTE AND TUBULAR CELL REPAIR



MARTIJN VAN DEN BROEK

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Decoding Recovery

Omics Insights into Podocyte and Tubular Cell Repair

Martijn van den Broek



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Martijn van den Broek

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Decoding Recovery

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aan de Radboud Universiteit Nijmegen
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in het openbaar te verdedigen op

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from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.M. Sanders,
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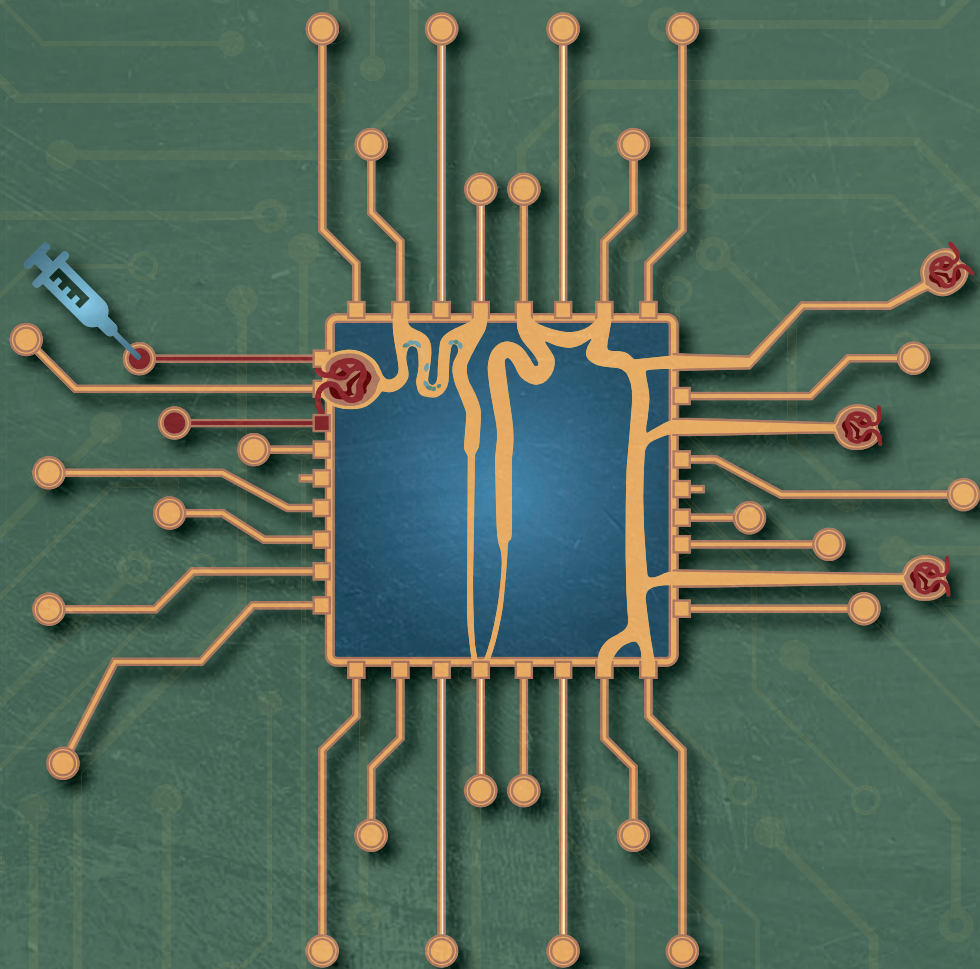
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Chapter 1.

Introduction and outline of the thesis

1. Introduction to the anatomy and function of the kidneys

1.1 The kidneys

I like to drink my coffee hot, so it takes me 5 minutes to finish a cup of coffee. It is fascinating to realize that in the 5 minutes that I spend drinking the 150 mL of coffee, all the blood in my body (+/- 5 litres) has passed my kidneys once, producing roughly 500 mL of pre-urine¹. If this 500 mL of pre-urine would leave my body, it would have been impossible for me to stay hydrated. Luckily, the kidneys concentrate this 500 mL of pre-urine into approximately 4 mL of actual urine that is transported out of the body. Evidently, the filtration of our blood is one of the important tasks of our kidneys, and this takes place in the filtering units of the kidneys: the nephron (Figure 1). Each kidney contains about one million nephrons, through which blood is filtered via the glomerulus to produce pre-urine. The pre-urine is processed and concentrated in the tubules, consisting of the proximal tubule, the loop of Henle, the distal tubule and eventually the collecting duct (Figure 1). Each part of the nephron has its own function, biology, and physiology. Since this thesis will focus on the glomerulus and the proximal tubule, a more elaborate introduction will be given for those two segments of the nephron.

1.2 The glomerulus

The glomerulus consists of a network of small capillaries surrounded by Bowman's capsule (Figure 2). In the small capillaries of the glomerulus, blood is pushed from the inside of the capillary to the outside³. This capillary wall comprises several filtering cell and protein layers, called the glomerular filtration barrier, that all contribute to the retention of essential components of the blood within the capillary, such as cells and large proteins like albumin, but also ensures the passage of smaller waste molecules to the outside of the capillary. All the capillaries of the glomerulus are structurally supported by the mesangial cells⁴. The mesangial cells play a key role in the production of extracellular matrix proteins to provide support to the glomerulus, but also in directing and supporting glomerular development. The glomerular endothelial cells (GEnCs) form the first layer in the glomerular filtration barrier and are covered by a negatively charged glycocalyx on the inside of the capillary. Together, the glycocalyx and GEnCs form a size-selective barrier for the filtrate. Afterwards, the glomerular basement membrane is crossed, which consists of a mesh of extracellular proteins. These proteins are produced by GEnCs and also, for a large part, by the podocytes. The podocytes are the final layer of filtration and have a large cell body with many long extensions that are called

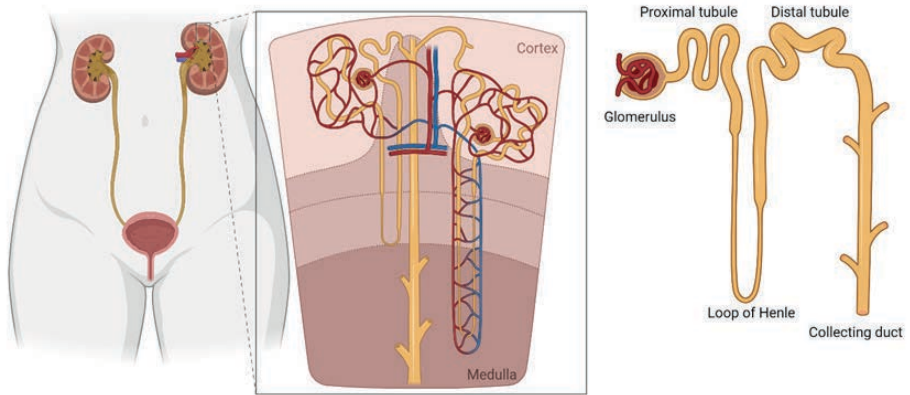


Figure 1. Overview of the kidneys. Human body with two kidneys, ureters, and bladder. A zoom-in of the kidney reveals the anatomy of the nephrons, which consist of a network of small capillaries (the glomerulus) where "unfiltered" blood enters the nephron. In the glomerulus, the blood is filtered, and the filtrate is transported to the collecting duct via the proximal tubule, the loop of Henle and the distal tubule. Image created with Biorender.

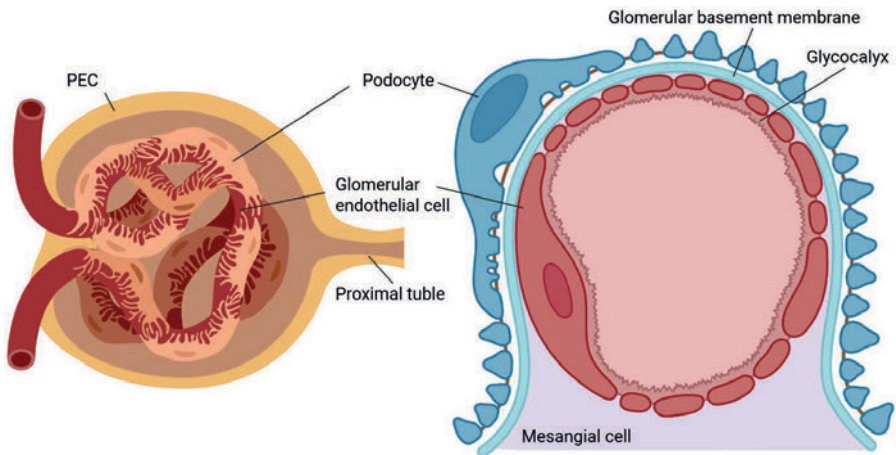


Figure 2. Zoom in on the glomerulus and the glomerular filtration barrier. On the left, a 3D schematic of the glomerulus, with glomerular endothelial cells, wrapped by podocytes and surrounded by PECs that form Bowman's capsule. The only way out of the capsule is through the proximal tubule. On the right, a schematic, more detailed drawing of the glomerular filtration barrier is depicted. Mesangial cells provide support for the capillaries, which are covered by the glycocalyx on the inside of the capillary. The glomerular filtration barrier, consisting of the glycocalyx, the fenestrated glomerular endothelial cells, the glomerular basement membrane and the podocyte foot processes with slit diaphragm, makes sure that larger proteins and cells are kept within the capillary, whereas smaller toxins and waste products are filtered to the outside of the capillary. Image created with Biorender.

foot processes. Between the foot processes, the slit diaphragm is formed by extracellular proteins, such as nephrin and neph1³. In this process, pre-urine is produced and contained in the glomerulus by Bowman's capsule. Bowman's capsule is populated by parietal epithelial cells (PECs) and forms a leak-tight barrier with only one way out: the proximal tubule. The proximal tubule begins the tubular system, which comprises the proximal tubule, the loop of Henle, the distal tubule, and concludes in the collecting duct. In the tubular system, the pre-urine produced in the glomerulus is further processed. Some nutrients like glucose, salts and water are reabsorbed from the pre-urine, while toxic metabolic waste products are excreted in the pre-urine⁵.

1.3 The proximal tubule

More than half of the entire kidney mass is accounted for by the proximal tubule, and more than half of the filtered load of salt and water is absorbed in the proximal tubule⁵. The proximal tubule is the main metabolically active part of the kidney, which is needed for the active transport that occurs to reabsorb solutes from the pre-urine and excrete waste products into the urine. Gluconeogenesis, an energy-demanding process to generate glucose, also takes place in the proximal tubular epithelial cells (PTECs) to produce glucose for other segments of the nephron. The PTECs contain a brush border, which maximizes surface area for active absorption and excretion. PTECs also contain a high number of mitochondria that supply the energy needed for all the active transport and gluconeogenesis. Another extraordinary feature of the proximal tubule is that it can regenerate after injury⁶⁻⁸. The cell type responsible for regeneration has been heavily debated. In recent years, a morphologically different cell type identified in the proximal tubules has been proposed as a candidate cell type to aid in regeneration after injury. This cell type can be found in a scattered distribution, which has provided its name: scattered tubular cells (STCs)⁶⁻⁸. They not only express different proteins, the STCs also do not have a brush border or a high amount of mitochondria compared to the proximal tubule cells.

Because the glomerulus and proximal tubule have distinct but essential roles, damage to either compartment can cause different but serious kidney diseases. This thesis will focus on two of these very different diseases: nephrotic syndrome, which is caused by podocyte injury and acute kidney injury caused by injury to the proximal tubules with the aim to contribute to better treatment options. In the following section, this introduction will focus on podocyte injury and current treatment regimens to treat glomerular

disorders caused by podocyte injury. In the third section, the reader will find more on the background of STCs, injury in the proximal tubule and the potential regenerative role of STCs after proximal tubular injury.

2. Podocytes

2.1 Nephrotic syndrome

Nephrotic syndrome (NS) is used to describe a combination of clinical symptoms: proteinuria, hypoalbuminemia and oedema⁹. NS is caused by injury to the podocytes, leading to leakage of proteins to the urine that should normally be retained in the blood. The podocyte injury can be primary (idiopathic), secondary to other systemic diseases such as diabetes, or the podocytes may be abnormal due to genetic variations that usually presents as congenital NS within the first months of life¹⁰. The cause of idiopathic NS (iNS) can be classified based on the histology in a kidney biopsy. The most common causes of iNS are membranous nephropathy (MN), focal segmental glomerulosclerosis (FSGS), and minimal change disease (MCD). In children below 10 years of age, MCD is the most common cause of NS (>70%), MN is rare (<5%), and FSGS accounts for approximately 10% of cases^{9,11}. Interestingly, in adults, these numbers are almost reversed: the most common cause of NS is MN, whereas MCD and FSGS account for approximately 10-20% each. The histology of MCD is characterized by the absence of abnormalities on light microscopy (Figure 3). Although not visible on light microscopy, electron microscopy reveals podocyte foot process effacement, a characteristic of podocyte injury. In FSGS, podocyte foot process effacement is also visible, however, FSGS is also detectable on light microscopy. In FSGS, segments of some glomeruli are sclerotic, caused by podocyte loss and subsequent extracellular matrix deposition. MCD and FSGS are hypothesized to represent a continuum of the same disease, where MCD represents the earlier stage with a better prognosis and FSGS represents the later stage in which the initial foot process effacement has led to podocyte loss and the formation of scar tissue¹². The causes of both MCD and FSGS are still under debate but seem to originate from factors in the blood, possibly with an immune origin¹²⁻¹⁵.

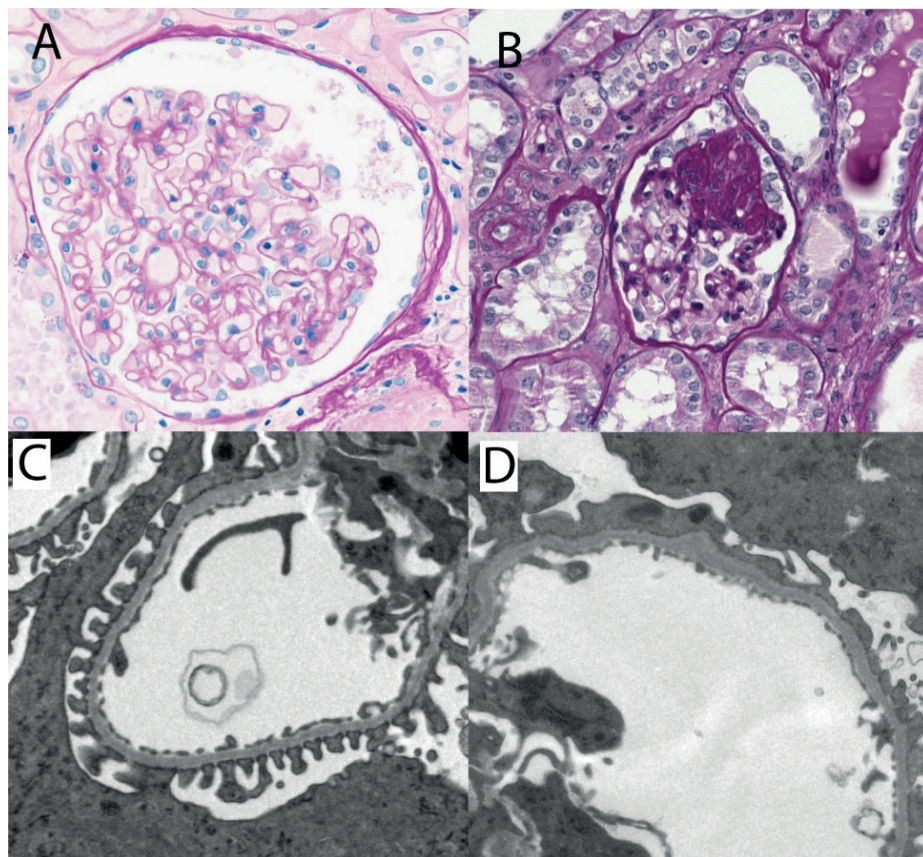


Figure 3. Kidney histology in health and disease. Histology of (A,B) the human kidneys by light microscopy or (C,D) rat kidneys by electron microscopy in (A,C) healthy kidneys, (B) a kidney with focal segmental glomerulosclerosis or (D) a kidney with minimal change disease. Histological images of human tissue were made using nephrectomies available at the Smeets lab, or rat kidneys available from the study described in Chapter 4.

2.2 Treatment of MCD and FSGS

Glucocorticoids have been the first-line treatment for both MCD and FSGS for over 60 years¹⁶⁻¹⁸. After the initial use of the glucocorticoid prednisone for MCD, at that time termed lipid nephrosis, evidence was presented that MCD was caused by a disorder of T-cell function, providing a rationale for the use of the immunosuppressive prednisone¹⁹. However, to date, the exact molecular mechanism of action by which prednisolone, the commonly used active form of prednisone, reduces proteinuria remains elusive. Besides immunosuppressive effects, prednisolone has also been shown to have direct effects on the podocytes²⁰. Since podocyte injury is a main feature of iNS, it

remains unclear whether prednisolone induces remission primarily through its immunosuppressive effects, through direct protective actions on podocytes, or through a combination of both. It is even possible that other kidney cell types are involved in the beneficial effects, since both PECs and PTECs also express the glucocorticoid receptor²¹. Interestingly, both stimulation and inhibition of the glucocorticoid receptor in PECs have been demonstrated to lower proteinuria in an experimental mouse model²². In PTECs, the glucocorticoid receptor expression was found to be lower in human proteinuric kidney disease compared to healthy kidneys²³. The ability of all these cell types to respond to glucocorticoids complicates studies looking into the molecular mechanism, since there might not be one single cell type solely responsive and responsible for recovery. Also, due to the widespread expression of the glucocorticoid receptor in our body, prednisolone treatment is associated with several, sometimes severe, side effects. These side effects include growth impairment in children, hypertension, glaucoma, weight gain or obesity, and psychological/behavioural abnormalities^{16,24}. Evidently, to improve treatment and reduce side effects for patients with iNS, we need to unravel the mechanism of action of prednisolone.

3. Proximal tubule

3.1 Acute kidney injury

Acute kidney injury (AKI) is a life-threatening complication and is observed in approximately 30% of patients admitted to the intensive care unit^{25,26}. It can be defined by an abrupt decrease in glomerular filtration, usually due to ischemic or toxic injury to the PTECs. To restore function and recover from AKI, the proximal tubule must completely regenerate. Incomplete regeneration is associated with progression to chronic kidney disease (CKD), and this frequently leads to end-stage kidney disease in which patients require dialysis or a kidney transplantation²⁶⁻²⁸. The process of incomplete regeneration is also called maladaptive repair. In maladaptive repair, injured PTECs are not cleared and the environment surrounding the proximal tubule cells is transformed into a pro-inflammatory and pro-fibrotic environment, stimulating interstitial fibrosis and irreversible tubular damage. Hence, stimulating effective regeneration or inhibiting maladaptive repair would both be promising therapeutic targets in the treatment of AKI and avoiding the progression to CKD. To further study these mechanisms, the origin and biology of the regenerating cells in the proximal tubule need to be unravelled.

3.2 Scattered tubular cells

There is still debate on the origin and exact function of the regenerating cells in the kidney, but STCs are a well-known candidate to fulfil this role^{6,28}. STCs are found in a scattered distribution throughout the proximal tubule in healthy human kidneys. They can be recognized by their different phenotype with typical expression of markers like CD44, vimentin, CD24 and CD133 that are not expressed in PTECs (Figure 4A)⁶. Another characteristic of these cells is observed using electron microscopy: STCs have a lower number of mitochondria and do not show a well-defined brush border (Figure 4B). There are still multiple questions regarding STCs and one of them is their origin. Two main theories have been investigated, one of them proposing that STCs are a fixed intratubular population and the other one proposing that every PTEC can dedifferentiate upon injury and become a STC^{7,8,29,30}. A second question regards the characteristics and function of STCs. One possibility is that they repopulate the proximal tubule and thereby lead to effective repopulation. However, STC-like cells in rodent models have also been found to promote maladaptive repair, characterized by a prolonged pro-inflammatory and pro-fibrotic gene expression profile³¹⁻³⁴. Overall, if STCs are capable of both successful and maladaptive repair, the molecular mechanism underlying this repair should be studied in human tissue to provide insights for patients with AKI.

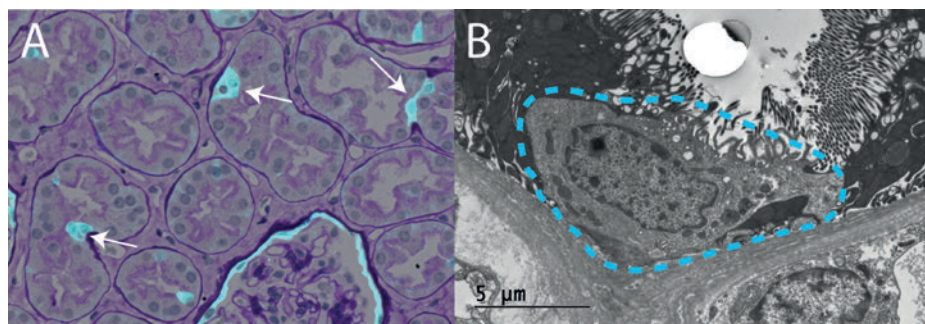


Figure 4. Scattered tubular cell histology. On the left, periodic-acid Schiff (PAS) stain of the kidney with immunofluorescence overlay for a marker of the STC (annexin A3, cyan). White arrows are showing some scattered cells in the proximal tubules. On the right, an electron microscopy image shows a STC (circled in blue by a dotted line) that has a smaller brush border and less mitochondria, compared to its neighbouring proximal tubule cells. Images are made with material available in the Smeets lab.

4. New and advanced *in vitro* model systems in kidney research

1

In the last decade, more effort has been directed towards improving the *in vitro* human model systems to better recapitulate the *in vivo* physiology for various reasons^{35,36}. The translation gap between animal models and patients has significantly contributed to the search for better model systems. One of the simplest options to study human biology *in vitro*, is to collect primary human cells for cell culture. However, the accessibility of primary cells and the limited lifespan in culture are complicating the extensive use of primary cells. Collecting primary human kidney cells from kidney tissue is only rarely possible, and although primary cells have been isolated from the urine, firstly it must be noted that the quality of these cells is questionable since they have survived for an unknown period of time in the urine and have been subjected to osmotic stress, pH changes, hypoxia, and to cytotoxic waste products present in the urine. Secondly, donor variation can lead to results that are not reproducible or need a lot of biological replicates to reveal an effect, leading back to the first issue: the material is scarce. Thirdly, some cell types, like the podocyte, are terminally differentiated *in vivo* and therefore will lose all their *in vivo* characteristics when they start dividing in culture³⁷. To overcome the issue of accessibility and the problem of maintaining viable podocytes in culture, conditionally immortalized podocyte (ciPOD) cell lines have been established³⁸. These primary human cells have been harvested once, overcoming the accessibility issue, and replicate under permissive conditions, while stopping replication and starting podocyte differentiation under non-permissive conditions. However, these human two-dimensional (2D) conventional cell models lack key protein markers, multiple other cell types and communication, blood flow with the accompanying oxygen and nutrient gradient, and a three-dimensional (3D) microenvironment. Compared to the traditionally used ciPOD, induced pluripotent stem cell (iPSC)-derived podocytes show improved podocyte characteristics, such as expression of key podocyte proteins like synaptopodin, Wilms tumour 1 (WT1) and vinculin³⁹⁻⁴¹. Since iPSCs are also readily available, these podocytes offer an improved 2D research model to study podocyte biology, however, they still lack communication with other kidney cell types and blood flow.

To increase human relevance of the model, there is a need for the addition of blood flow, a 3D environment and the inclusion of other cell types, such as

mesangial cells, glomerular endothelial cells and proximal tubular cells^{42,43}. Two main advancements in the field focus on glomerulus-on-a-chip models and human iPSC-derived kidney organoids. Glomerulus-on-a-chip models can incorporate both flow and other cell types, making it possible to investigate cellular communication in a physiologically more relevant model. The human iPSC-derived kidney organoid on the other hand, incorporates multiple other cell types and a 3D environment, which allows for studying the communication network between multiple cell types and produces morphologically more mature cell types. Despite these advances, the organoids do not yet contain a fully mature filtration barrier. However, recent advances that combine kidney organoids with flow have shown improved maturation in the kidney organoids⁴⁴.

5. Scope and outline of the thesis

The first aim of my thesis was to investigate the mechanism of podocyte recovery by prednisolone to ultimately find specific treatment options for iNS, where podocyte injury is a key feature. Secondly, we aimed to characterize the human STC population, because STCs may be a promising therapeutic cell source in patients with AKI to stimulate regeneration.

In **chapter 2**, we characterized podocytes in human iPSC-derived 3D kidney organoids. These kidney organoids are increasingly used as an improved model to study podocyte biology instead of previously used and less complex 2D cell culture models, which contain only a single cell type. We characterized the maturity of the podocytes in the kidney organoids, the presence and functionality of other kidney cell types and demonstrated NS disease modelling in these organoids.

In **chapter 3**, an overview is provided of the already described effects of glucocorticoids on the podocyte, including a discussion on the different model systems used to investigate this. After reviewing the information in the literature, **chapter 4** describes our endeavour to unravel the molecular mechanism of prednisolone on podocytes. The chapter describes the establishment of a rat model for iNS which is partially rescued by prednisolone. The rat podocytes are analysed using single-nucleus RNA and ATAC sequencing to discover genes that are crucially involved in the recovery of podocytes. Next, these results are functionally validated *in vitro*, using inhibitors in the ciPOD model and the more

complex 3D kidney organoids, to investigate whether inhibition of those targets might be beneficial for podocytes to recover post injury.

Finally, in **chapter 5** we investigated the STC population within the proximal tubule. Using human kidney tissue, the human STC population is characterized. We reveal their preferred location within the kidney, the expression of markers related to maladaptive repair, their transcriptomic profile, and explore the heterogeneity within the STC population.

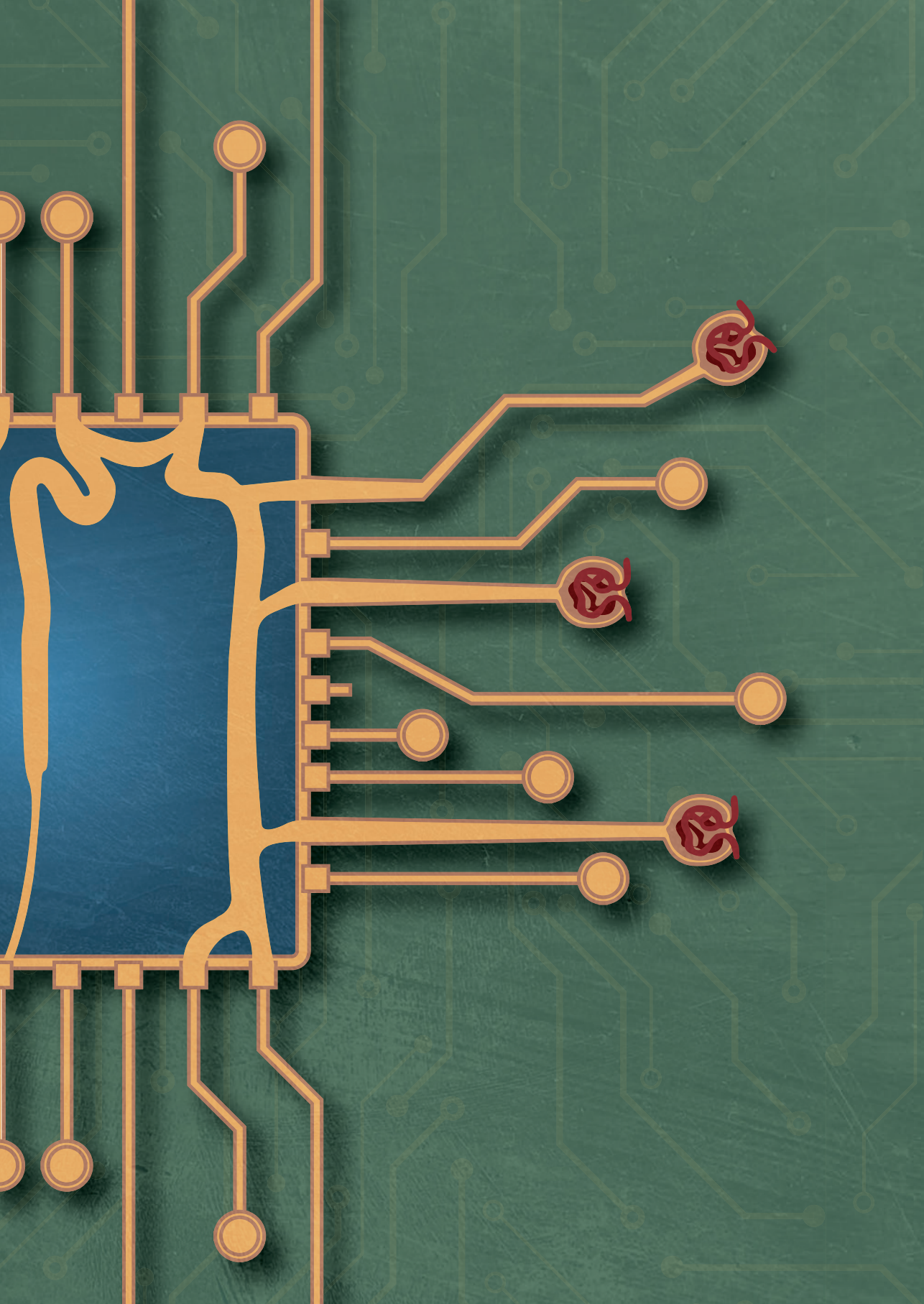
Chapter 6 discusses the main results and conclusions of all prior chapters, including their limitations and how they relate to the literature.

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Human pluripotent stem cell-derived kidney organoids for personalized congenital and idiopathic nephrotic syndrome modelling

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Abstract

Nephrotic syndrome (NS) is characterized by severe proteinuria as a consequence of kidney glomerular injury due to podocyte damage. *In vitro* models mimicking *in vivo* podocyte characteristics are a prerequisite to resolve NS pathogenesis. The detailed characterization of organoid podocytes resulting from a hybrid culture protocol showed a podocyte population that resembles adult podocytes and was superior compared with 2D counterparts, based on single-cell RNA sequencing, super-resolution imaging and electron microscopy. In this study, these next-generation podocytes in kidney organoids enabled personalized idiopathic NS modelling, as shown by activated slit diaphragm signalling and podocyte injury following protamine sulfate, puromycin aminonucleoside treatment and exposure to NS plasma containing pathogenic permeability factors. Organoids cultured from cells of a patient with heterozygous *NPHS2* mutations showed poor *NPHS2* expression and aberrant *NPHS1* localization, which was reversible after genetic correction. Repaired organoids displayed increased VEGFA pathway activity and transcription factor activity known to be essential for podocyte physiology, as shown by RNA sequencing. This study shows that organoids are the preferred model of choice to study idiopathic and congenital podocytopathies.

Keywords: Nephrotic syndrome, Human iPSC-derived kidney organoids, 2D iPSC-derived podocytes

Introduction

Podocytes play an important role in the glomerular filtration barrier. Podocytes possess interdigitating foot processes that are bridged by a protein complex called the slit diaphragm, which contains proteins such as nephrin (NPHS1) and podocin (NPHS2). Malfunctioning of podocytes (podocytopathy) causes massive proteinuria, resulting in nephrotic syndrome (NS)^{1,2}. In patients with idiopathic NS (iNS) a kidney biopsy shows no abnormalities in light microscopy. Complete foot process effacement, as determined by electron microscopy, defines iNS as a podocytopathy. The identification of mutations in congenital NS in important podocyte genes (NPHS1 and NPHS2)^{3,4} also defines iNS as a podocytopathy.

The pathophysiology of iNS has not yet been clarified. Within the spectrum of NS, a unique subset of patients have presumed pathogenic circulating permeability factors (CPFs) that result in podocyte injury and eventually recurrence of the disease after kidney transplantation. Clinical evidence suggests CPFs involve the immune system (i.e. T and B cells)⁵⁻⁷. However, there is still no consensus about the nature of the CPFs⁸⁻¹⁰.

In order to gain more insight into podocytopathies, animal models and basic *in vitro* models have been explored, but these have failed to provide full mechanistic insight. Hence, there is an unmet need for models that recapitulate glomerular physiology and pathology to resolve the molecular mechanism of underlying glomerular diseases. Previously, podocyte cell lines, human induced pluripotent stem cell (iPSC)-derived 2D podocytes, and 3D kidney organoid models have been established¹¹⁻¹⁷. The advantages of 2D podocyte models are the straightforward and standardized culture conditions, although the presence and localization of slit diaphragm proteins are not convincing^{18,19}. Kidney organoid models are more complex and require challenging culture protocols, but podocyte characteristics, including slit diaphragm proteins, have been demonstrated^{12,18,20,21}. Despite the substantial progress that has been made in the kidney organoid field, current organoids resemble immature tissue, show limited vascularization, and do not have a functional glomerular filtration barrier^{17,21,22}. Recently, Tanigawa and colleagues successfully modelled Finnish-type congenital NS (NPHS1 mutant) in kidney organoids, identified slit diaphragm abnormalities in these podocytes and could correct the mutation genetically²³. The study by Tanigawa et al. emphasized the potential of kidney organoids for personalized regenerative medicine and for the accurate investigation of NS *in vitro*.

The aim of our study was to investigate the potency of current kidney organoids to model podocytopathies, as observed in NS, accurately. In this study, we established a hybrid differentiation protocol that resulted in kidney organoids containing a podocyte population that approaches adult podocyte expression levels. We succeeded in modelling podocyte pathophysiology that leads to congenital (NPHS2 mutant) NS as well as iNS in patients. Our data indicate that iPSC-derived kidney organoids are the preferred *in vitro* system to model NS, since cellular signalling cascades are induced upon injury that are absent in 2D iPSC-derived podocytes and a podocyte cell line.

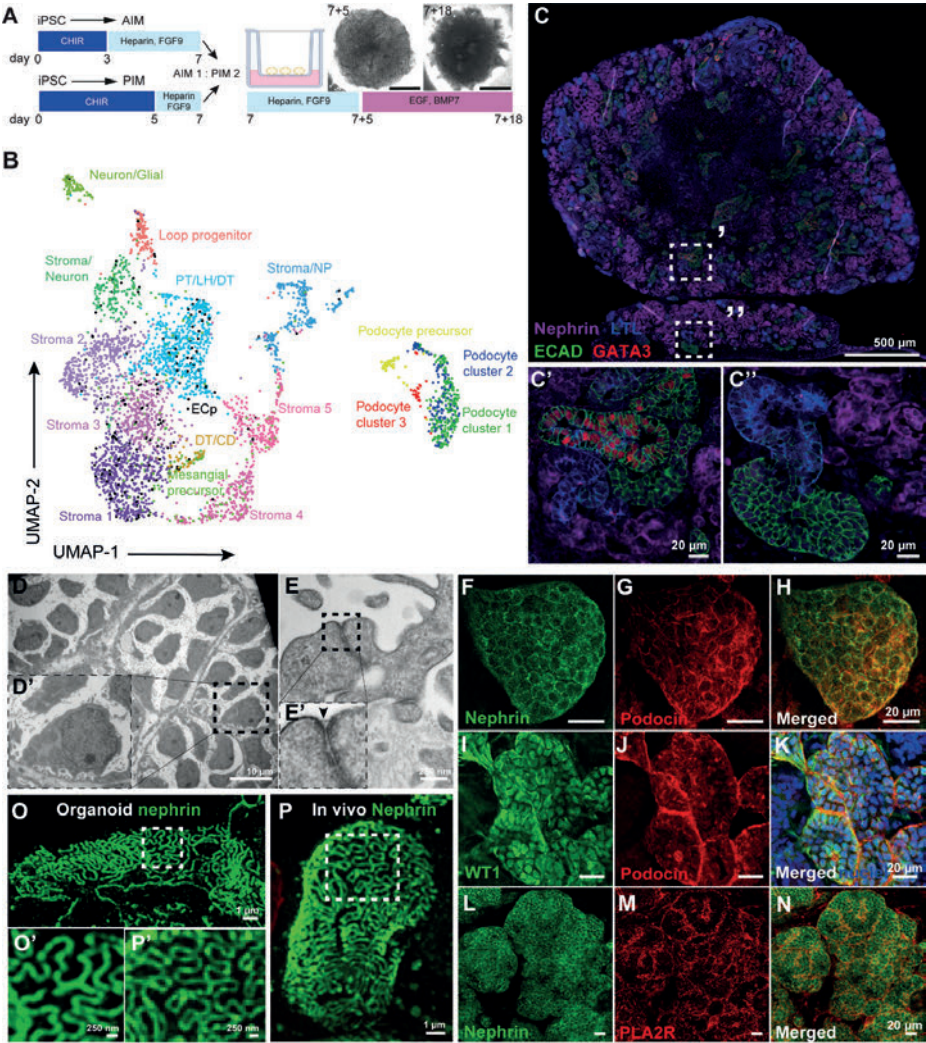
Results

Podocytes in kidney organoids show superior characteristics compared to 2D podocytes

Human iPSCs were successfully cultured into kidney organoids using a hybrid directed differentiation protocol based on that described by Uchimura and Takasato et al.^{17,22}. Briefly, iPSCs were differentiated towards metanephric mesenchyme (MM) by exposure to the WNT signalling agonist CHIR 99021 for 5 days and into GATA3⁺ epithelium by CHIR 99021 exposure for 3 days (Takasato et al., 2015). On day 7, GATA3⁺ and MM cells were mixed in a 1:2 ratio, to simulate signalling cues between both populations that are essential for nephrogenesis (Figure 1A) (Uchimura et al., 2020). Using this approach, segmented patterning was induced and organoids consisted of kidney cells, including podocytes, proximal tubule, loop of Henle, distal tubule, and collecting duct-like cells, as well as endothelial and mesangial cell precursors, stromal cells, and off-target neuronal progenitors, as shown by single-cell RNA sequencing (scRNA-seq) and immunofluorescence staining (Figure 1B – C", Supplemental figure 1, Table S1).

Podocytes that mirror *in vivo* characteristics, e.g. the presence of slit diaphragm proteins, as closely as possible are required to study NS *in vitro*. Hence, the podocytes in the kidney organoids were thoroughly characterized. The presence of podocyte cell bodies and foot processes as well as a slit diaphragm could be observed at the ultrastructural level (Figure 1D-E'). Podocyte-specific proteins, such as podocin, nephrin, Wilms' tumor-1 (WT-1), and synaptopodin were also expressed (Figure 1F-K, Supplemental figure 2). Phospholipase A2 receptor (PLA2R; also known as PLA2R1) was also detected in organoid podocytes (Figure 1L-N). PLA2R is the main target

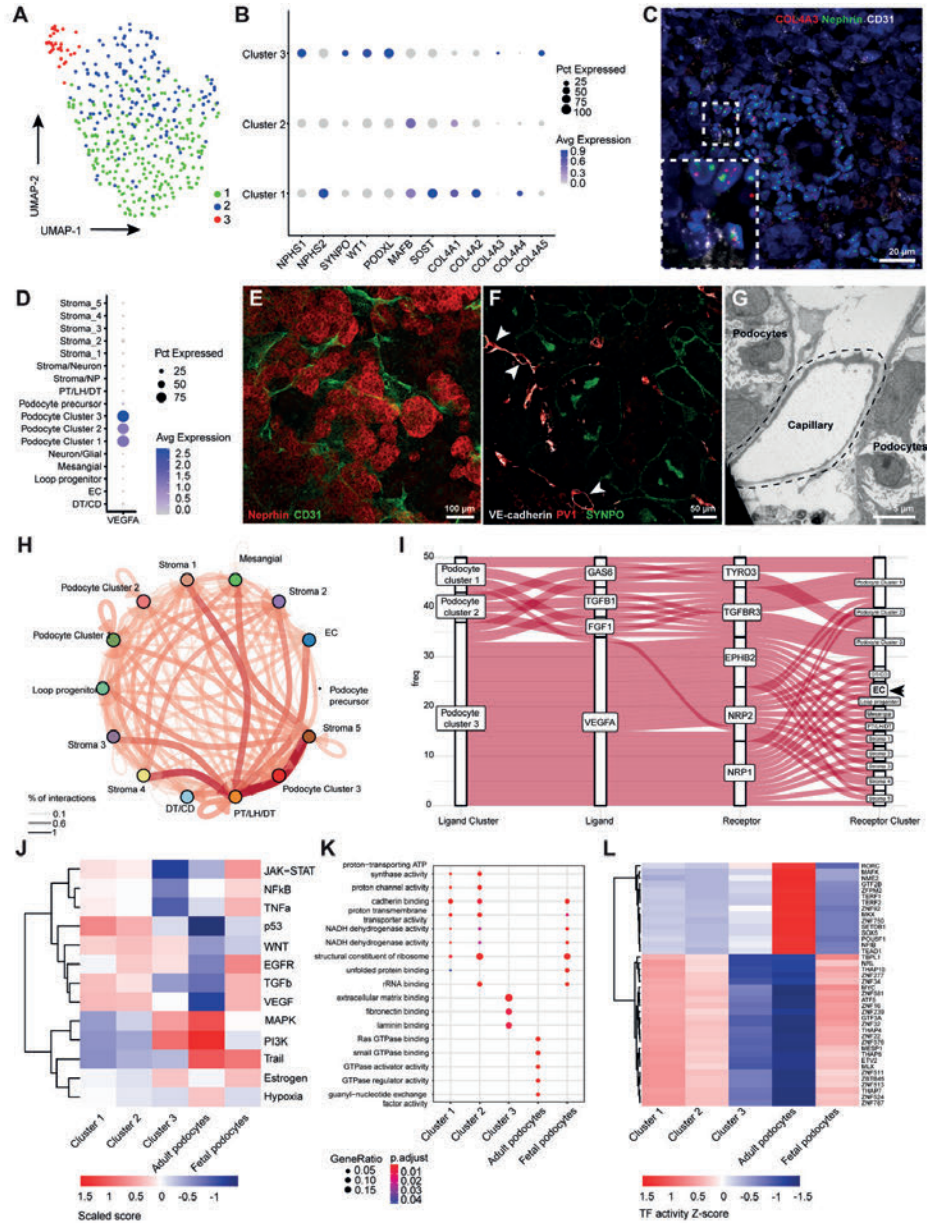
receptor of autoantibodies present in the majority of NS patients with primary membranous nephropathy²⁴. Using super-resolution imaging, slit diaphragms that showed nephrin expression could be detected between podocyte foot processes in organoids, showing similar morphological characteristics to *in vivo* adult slit diaphragms (Figure 10-P').



< Figure 1. Human iPSC-derived kidney organoids contain 17 distinct cell clusters, including well-developed podocytes showing filtration slits. (A) Schematic of the iPSC-derived kidney organoid culture protocol. iPSCs were differentiated in 2D either towards anterior intermediate mesoderm (AIM) or posterior intermediate mesoderm (PIM) and aggregates (300,000 cells) were made using a 1:2 AIM/PIM ratio on day 7. Organoids were cultured on the air-liquid interface of Transwell™ filters for an additional 18 days, using a mixture of growth factors as indicated. BMP7, bone morphogenic protein 7; CHIR, CHIR99021; EGF, epidermal growth factor; F9, fibroblast growth factor 9. (B) Uniform manifold approximation and projection (UMAP) integration of 3,900 single cells from human iPSC-derived kidney organoids results in 17 different cell clusters. CD, collecting duct precursors; DT, distal tubular cells; ECp, endothelial cell precursors; LH, Loop of Henle cells; NP, neuronal progenitors; PT, proximal tubular cells. (C-C'') Segmented patterning in human kidney organoids shows the presence of podocytes (NPHS1, magenta), proximal tubules [lotus tetragonolobus lectin (LTL, blue], distal-like [E-cadherin (ECAD), green] and collecting duct-like [ECAD (green) and GATA binding protein 3 (GATA3) (red)] cells. (D-E') TEM micrographs of organoid-derived podocytes showing cell bodies and interdigitating foot processes including a filtration slit, indicated by an arrowhead in E'. (F-H) Filtration slit proteins podocin (red) and nephrin (green) are expressed at the podocyte cell membranes. (I-K) The podocyte-specific transcription factor Wilms' Tumor-1 (WT1, green) is expressed in podocyte nuclei (podocin, red; nuclei, bleu). (L-N) Phospholipase A2 receptor (PLA2R, red) expression at the podocyte cell membranes (nephrin, green). (O-P') Super resolution filtration slit imaging of nephrin in (O-O') organoid-podocytes and in a morphologically healthy appearing glomerulus of a (P-P') Munich Wistar Frömter rat.

Analysis of scRNA-seq data revealed three podocyte subclusters, each expressing a distinct profile of podocyte markers, independent of cell cycle phase (Figure 2A-B and Supplemental figure 3A). Notably, cluster 3 showed expression of the collagen 4 alpha 3, 4 and 5 (COL4A3, COL4A4, and COL4A5) genes (Figure 2B and S3B). The developmental expression of the aforementioned collagen chains is initiated at the capillary loop stage in fetal podocytes, whereas expression is exclusive for mature podocytes²⁵ (Supplemental figure 3B). The COL4A3 mRNA expression was confirmed in organoid podocytes (NPHS1⁺) using *in situ* hybridization (Figure 2C). During nephrogenesis, at the S-shaped body phase, podocytes start to secrete vascular endothelial growth factor A (VEGFA), which is essential for podocyte differentiation and attraction of endothelial cell precursors, as a first step towards the formation of a glomerular filtration barrier^{26,27}. All organoid podocyte subclusters expressed VEGFA, with cluster 3 showing the most prominent VEGFA expression (Figure 2D). In fact, VEGFA expression in cluster 3 was 1.5- to 2-fold higher than the expression observed in fetal or adult control podocytes (Supplemental figure 3C). The interaction between podocytes and endothelial cells dictates glomerular filtration barrier formation and is essential for glomerular maturation^{28,29}, which, notably, was not observed in our kidney organoids. Cluster of differentiation 31 (CD31; PECAM1), vascular endothelial (VE)-cadherin (CDH5), plasmalemmal vesicle associated protein-1 (PV-1; PLVAP) and Eps15 homology domain-containing 3 (EHD3) were expressed by

endothelial capillary structures (Figure 2C, E-G, Supplemental figure 4), including luminal spaces in the organoids. However, organoid vasculature was interspaced between podocytes rather than invading as normally occurs at the capillary loop stage during glomerulogenesis *in vivo*.



< Figure 2. Kidney organoid-derived podocyte subcluster 3 partially resembles adult podocytes. (A, B) UMAP integration of 401 podocytes (A) identified three subclusters based on differentially expressed genes (B). Avg, average; Pct, percent. (C) RNAscope analysis showing nephrin (NPHS1), collagen IV alpha three (COL4A3), and endothelial marker cluster of differentiation 31 (CD31) expression. Inset shows an enlarged view of the boxed area. (D) Vascular endothelial growth factor A (VEGFA) expression among the podocyte subclusters. (E,F) CD31-, VE-cadherin- and plasmalemmal vesicle associated protein-1 (PV-1)-stained endothelial capillaries are interspaced between podocytes expressing (E) NPHS1 and (F) synaptopodin (SYNPO) in the organoids. Arrowheads indicate endothelial capillaries with lumen. (G) Ultrastructural analysis showing the luminal space of a capillary adjacent to organoid podocytes. (H) Cell-cell interactions between all cells present in kidney organoids using CellPhoneDB and CrossTalker packages. Thickness and depth of colour of the arrows correspond to the percentage of interactions. (I) Autocrine and paracrine podocyte ligand-receptor interactions showing a dominant role for VEGFA signalling originating from cluster 3 to VEGF-related receptors in, amongst others, endothelial cell precursors (EC). Analysis was performed using CellPhoneDB and CrossTalker packages. (J-L) Comparative (J) PROGENy, (K) GO terms and (L) DoRothEA analysis showing (J) pathway activity, (K) biological processes and (L) transcription factor activity between the three podocyte subclusters, adult and fetal podocytes [adult and fetal podocytes sequencing data extracted from Gene Expression Omnibus (GSE118184 and GSE112570)]. Abbreviations as in Figure 1.

To understand the observed maturation differences within the three clusters of podocytes, we further investigated the cell-cell communication between podocytes and endothelial cells by performing ligand-receptor analysis using CellPhoneDB and CrossTalker packages (Figure 2H)^{30,31}. Podocyte cluster 3 showed enriched VEGFA interactions with endothelial cell precursors receptors ephrin type-B receptor 2 (EPHB2), neuropilin 1 and neuropilin 2 (NRP1 and -2), which could not be identified in the other podocyte clusters (Figure 2I). Concomitantly, using PROGENy³², podocyte cluster 3 showed an increased phosphoinositide 3-kinase (PI3K) pathway activity (Figure 2J) which, in concert with VEGF signalling, is associated with COL4A3 gene regulation^{33,34}. Podocyte cluster 3 mimics adult podocytes most closely when compared to fetal and adult pathway activities that were extracted from the Kidney Interactive Transcriptome database (Figure 2J) (The Humphreys Laboratory; <https://humphreyslab.com/SingleCell/>)³⁵. Gene ontology (GO) terms showed distinct biological processes related to extracellular matrix binding in podocyte cluster 3, which were not significantly enriched in the other podocyte clusters or in adult podocytes (Figure 2K). GO terms of podocyte cluster 1 and 2 showed overlap with biological processes active in fetal podocytes (Figure 2K). To infer transcription factor (TF) activity differences between the podocyte clusters that may aid in resolving molecular cues involved in podocyte maturation, we used the DoRothEA computational package³⁶. We found that podocyte cluster 1 and 2 resemble a fetal podocyte TF profile, whereas the transcriptional circuit in podocyte cluster 3 showed

partial overlap with adult podocytes (Figure 2L). However, some emerging TFs, like TEA domain transcription factor 1 (TEAD1) and nuclear factor I B (NFIB), involved in cellular differentiation and tissue homeostasis, were less active in podocyte cluster 3 compared to adult podocytes (Figure 2L)³⁷⁻³⁹. Ofnote, zincfingerprotein 750 (ZNF750) waslessenriched incluster3 podocytes compared to adult podocytes. Normally, ZNF750 represses kruppel like factor 4 (KLF4), a known epigenetic modulator involved in podocyte differentiation⁴⁰. Also, SRY-Box transcription factor 5 (SOX5) known to co-activate WT1⁴¹, shows limited activity in cluster 3. Altogether, we show that essential podocyte markers are expressed in the entire kidney organoid podocyte population, but we identified a subpopulation of podocytes that resemble adult podocytes at the transcriptional level.

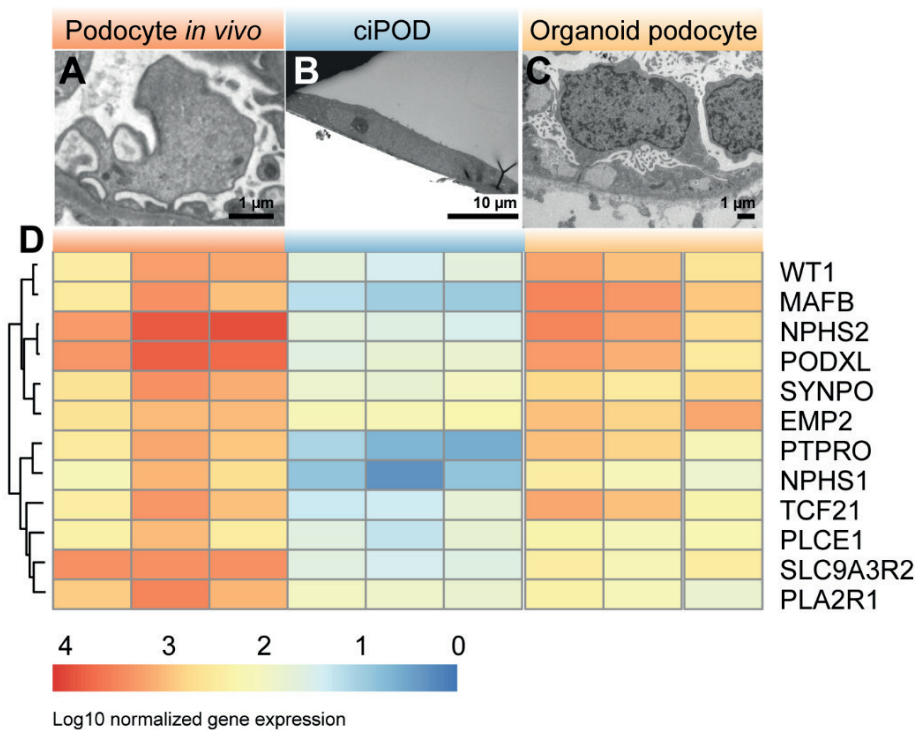


Figure 3. Organoid podocytes show superior morphology and transcriptome compared with 2D podocytes in vitro. (A-C) TEM micrographs showing the ultrastructural analysis of (A) normal human podocytes in situ, (B) conditionally immortalized podocytes (ciPOD) and (C) organoid podocytes. (D) Heatmap of bulk RNA sequencing data showing the expression signature of podocyte-specific markers in normal human glomeruli, ciPODs and organoid podocytes.

Next, we compared the performance of the current organoid model compared with a frequently used human 2D *in vitro* podocyte cell line, i.e. a widely used conditionally immortalized podocytes cell line (ciPOD)¹⁴. Morphologically, ciPODs do not show the typical appearance of a "floating" cell body with primary and secondary (foot process) structures (Figure 3B). Notably, within the organoids, podocytes strongly resembled human podocytes (Figure 1E' and 3A-C). Transcriptome analysis revealed that organoid podocytes resemble human podocytes much more than ciPODs do (Figure 3D), in particular when evaluating typical podocyte markers such as *NPHS1*, *NPHS2*, *WT1*, *PODXL*, and *SYNPO*. Altogether, the organoid podocytes in part resemble the transcriptome profile of human adult kidney podocytes, which includes the expression of typical podocyte markers that are essential for the study of NS pathology *in vitro*.

Impaired protein processing towards the cell membrane in kidney organoids of a patient with *NPHS2* mutations is abrogated after genetic correction

Podocin is known to play an important role in slit diaphragm organization. Intact podocin is required for nephrin membrane trafficking, ultimately leading to oligomerization of podocin clusters and nephrin, which is required for development of filtration slits. NS patients with mutated *NPHS2* exhibit aberrant nephrin expression, thereby emphasizing the crucial role of podocin in podocyte physiology^{42,43}. Here, we used kidney organoids to model congenital nephrotic syndrome caused by *NPHS2* mutation.

Compound heterozygous mutations p.Arg138Gln (exon 3) and p.Asp160Tyr (exon 4) in the podocin (*NPHS2*) gene were identified in a patient with congenital nephrotic syndrome. Erythroblasts from this patient were successfully reprogrammed into iPSC and cultured into organoids. Podocin protein expression was almost absent on the cell membrane and, although nephrin expression was present, membrane expression was patchy or in some podocytes a weak cytoplasmic stain was detected (Figure 4A-C), which is in line with observations in NS patients⁴². Correction of the exon 3 mutation by CRISPR/Cas9 resulted in restored podocin protein expression colocalizing with nephrin expression at the cell membrane in organoids (Figure 4D-F). Bulk transcriptomics of organoid *NPHS1*⁺ podocytes showed a distinct podocyte mRNA expression profile for both mutant and repaired organoid podocytes, for example *SYNPO*, *MAFB*, *WT1*, *NPHS1* and *NPHS2* expression (Figure 4G), resembling the transcriptome of isolated human glomeruli (Supplemental figure 5A). *NPHS2* gene expression was not affected by the *NPHS2* mutations.

Both *NPHS2* mutations result in protein retention in the endoplasmic reticulum and do not interfere with mRNA expression^{44,45}.

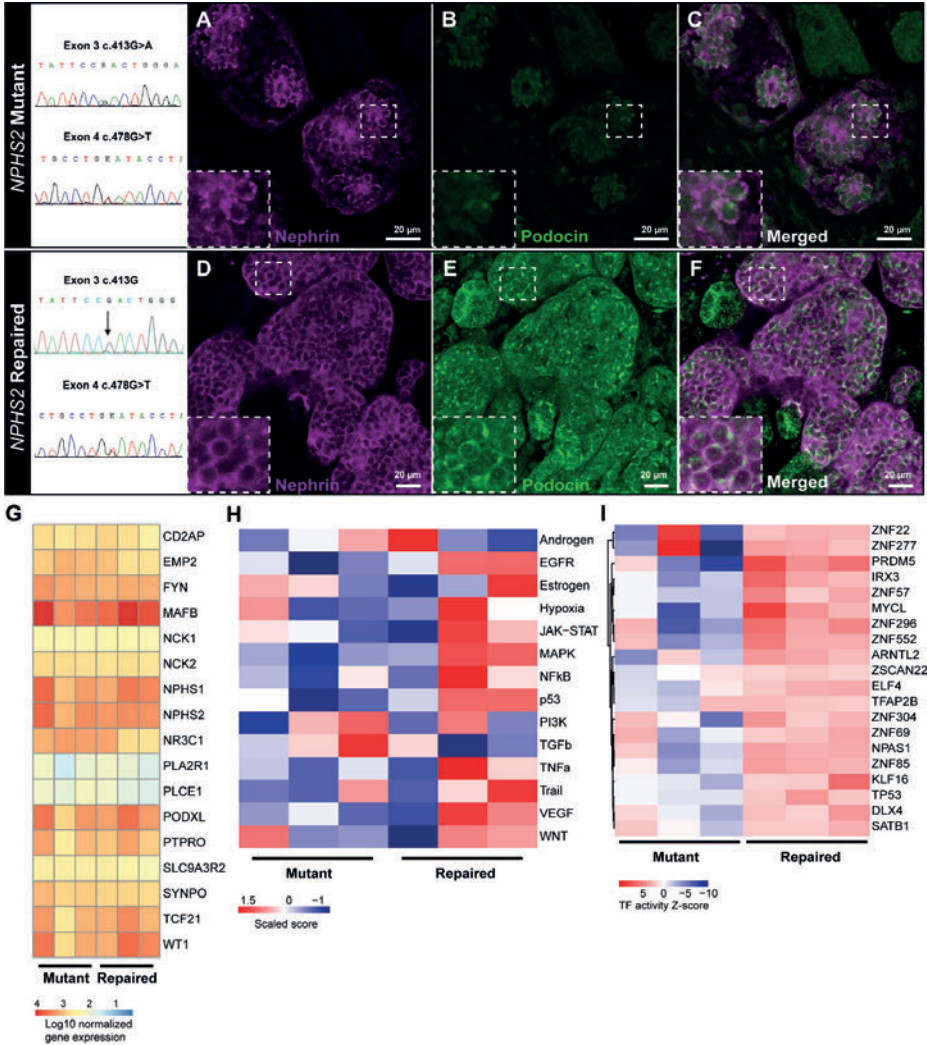


Figure 4. Congenital nephrotic syndrome modelling of patient-derived *NPHS2* mutant and genetically repaired iPSC kidney organoids. (A-F) Nephlin (magenta) and podocin (green) expression in (A-C) *NPHS2* mutant and (D-F) genetically repaired kidney organoids. Insets show enlarged views of the boxed areas. (G) Comparative bulk transcriptomics of podocyte-specific genes in mutant versus repaired organoid podocytes. (H,I) Comparative (H) PROGENy pathway activity analysis and (I) DoRothEA transcription factor activity of mutant versus repaired organoid podocytes. (G-I) Bulk transcriptomics was performed from RNA isolated from three independent batches of *NPHS1*⁺ sorted podocytes from mutant and repaired organoids. Per batch, ten mutant or repaired organoids were pooled and used for sorting podocytes by FACS.

In vivo, podocytes are the major VEGF producers in the glomerular compartment, which act via autocrine and paracrine signalling to maintain the glomerular filtration barrier³³. Autocrine VEGFA signalling regulates slit diaphragm proteins, including podocin⁴⁶. In line with this, we showed, using PROGENy, that repaired organoid podocytes displayed enriched VEGF pathway activity compared with mutant organoid podocytes (Figure 4H and Supplemental figure 5B). Repaired organoid podocytes closely resembled fetal podocytes with respect to VEGF pathway activity, whereas mutant organoid podocytes differed from fetal podocytes (Supplemental figure 5B). We identified that fibroblast growth factor (FGF) receptor 4 (FGFR4) signalling is enhanced in repaired organoid podocytes compared with mutant organoid podocytes (Supplemental figure 5C – 6; adjusted p-value <0.05⁴⁷). FGF signalling is likely to be involved in podocyte differentiation and plays a role in podocyte recovery following injury⁴⁸. All up- and downregulated genes in mutant versus repaired organoids are shown in Supplemental figure 5C,D.

The transcription factor activity of repaired organoid podocytes showed a distinct signature compared with mutant organoid podocytes (Figure 4I). The top 20 enriched TFs included, amongst others, PR domain zinc finger protein 5 (PRDM5), E74-like ETS transcription factor 4 (ELF4) and zinc finger protein 69 (ZNF69), which are involved in the regulation of genes involved in podocyte physiology^{12,13}. Altogether, we have successfully modelled congenital NS in organoids. The repaired *NPHS2* mutation resulted in restored podocin expression and nephrin localization, as well as the rescue of pathway and TF activities essential in podocyte physiology.

Idiopathic NS modelling using kidney organoids shows downstream slit diaphragm signalling events and granule formation in podocytes

The protamine sulfate model is a known *in vivo* model of specific podocyte injury induction⁴⁹. Protamine sulfate abolishes the negative charge on podocytes, thereby inducing foot process effacement accompanied by changes in downstream slit diaphragm signalling, cytoskeleton rearrangements, and activation of injury-associated mechanisms. The protamine sulfate model is reversible by heparin treatment, which restores podocyte charge, the cytoskeleton and normal foot process formation⁴⁹.

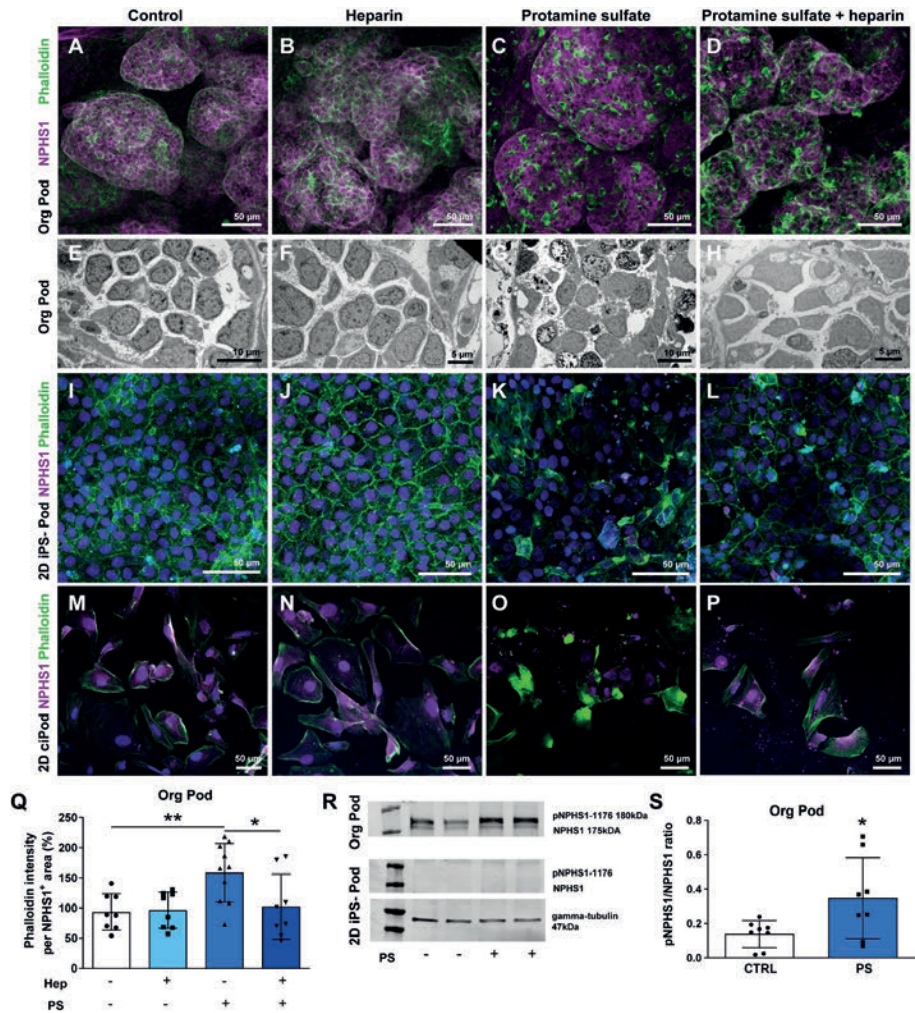


Figure 5. Kidney organoids are the preferred model of choice to model idiopathic nephrotic syndrome. (A-D) Podocyte cytoskeleton (phalloidin (green), NPHS1 (magenta)) and (E-H) ultrastructure analysis of protamine sulfate-treated and heparin-rescued kidney organoid podocytes. (I-L) Podocyte cytoskeleton analysis (phalloidin (green), NPHS1 (magenta)) of protamine sulfate-treated and heparin-rescued 2D iPSC-derived podocytes. (M-P) Podocyte cytoskeleton analysis (phalloidin (green), NPHS1 (magenta)) of protamine sulfate-treated and heparin-rescued 2D ciPODs. (Q) Quantification of phalloidin intensity in protamine sulfate-treated and heparin-rescued kidney organoid podocytes. $*=p<0.05$, $**=p<0.01$ (one-way ANOVA analysis followed by Tukey post-test). Data are presented as mean $\pm$ s.d. from three independent experiments using at least two biological replicates per condition. (R) Western blotting analysis of pNPHS1 and NPHS1 protein expression in protamine sulfate (PS)-treated kidney organoids and 2D iPSC-derived podocytes. Gamma tubulin was used as a reference protein. (S) Semi-quantification of the pNPHS1/NPHS1 ratio of protamine sulfate-treated kidney organoids. $*=p<0.05$ (unpaired t-test). Data are presented as mean $\pm$ s.d. from three independent experiments using at least two biological replicates per condition.

Organoids treated with protamine sulfate showed clear podocyte cytoskeleton rearrangements (Figure 5C, 5Q) and podocyte injury, as shown by vacuole formation and cell degeneration at the ultrastructural level (Figure 5G) compared with controls (Figure 5A,B,E,F). The protamine sulfate-induced injury could largely be reversed by heparin treatment (Figure 5D,H,Q). iPSC differentiated into 2D podocytes, from the same iPSC line as used for organoids, as well as ciPODs also showed protamine sulfate-induced cytoskeletal rearrangements (Figure 5K and Supplemental figure 7), which also showed partial recovery after heparin treatment (Figure 5L, Supplemental figure 7). Interestingly, only the organoids showed nephrin and protamine sulfate-induced phospho-nephrin expression upon western blotting (Figure 5R-S), which is indicative of slit diaphragm signalling following injury, whereas nephrin expression could not be detected in 2D iPSC-derived podocytes or in ciPODs (Figure 5R, Supplemental figure 7).

To investigate further the preferred NS model of choice, i.e. 2D iPSC-derived podocytes versus 3D iPSC-derived organoids, the podocin mutant and repaired iPSC lines were evaluated in both models. NPHS2 mutant and repaired organoids were exposed to protamine sulfate and/or treated with heparin. In line with our previous results, these organoids showed cytoskeleton rearrangements as a result of protamine sulfate exposure (Supplemental figure 8A,F), which was only significantly reversed upon heparin treatment in podocin-repaired organoids (Supplemental figure 8F).

Moreover, pNPHS1 expression in both organoid lines was not significantly induced upon protamine sulfate treatment. Nevertheless, a slight trend in pNPHS1 expression in the repaired line could be observed (Supplemental figure 8B,C,G,H). Both mutant and repaired iPSCs, differentiated in 2D culture towards podocytes, responded to protamine sulfate, resulting in injury, which recovered upon heparin treatment, as shown by actin cytoskeleton expression (Supplemental figure 8E,J). However, no nephrin and pNPHS1 expression was observed in 2D iPSC-derived podocytes (Supplemental figure 8D,I), which agrees with our earlier results. These results support the suggestion that 3D organoids are the preferred model of choice to study podocytes accurately *in vitro*.

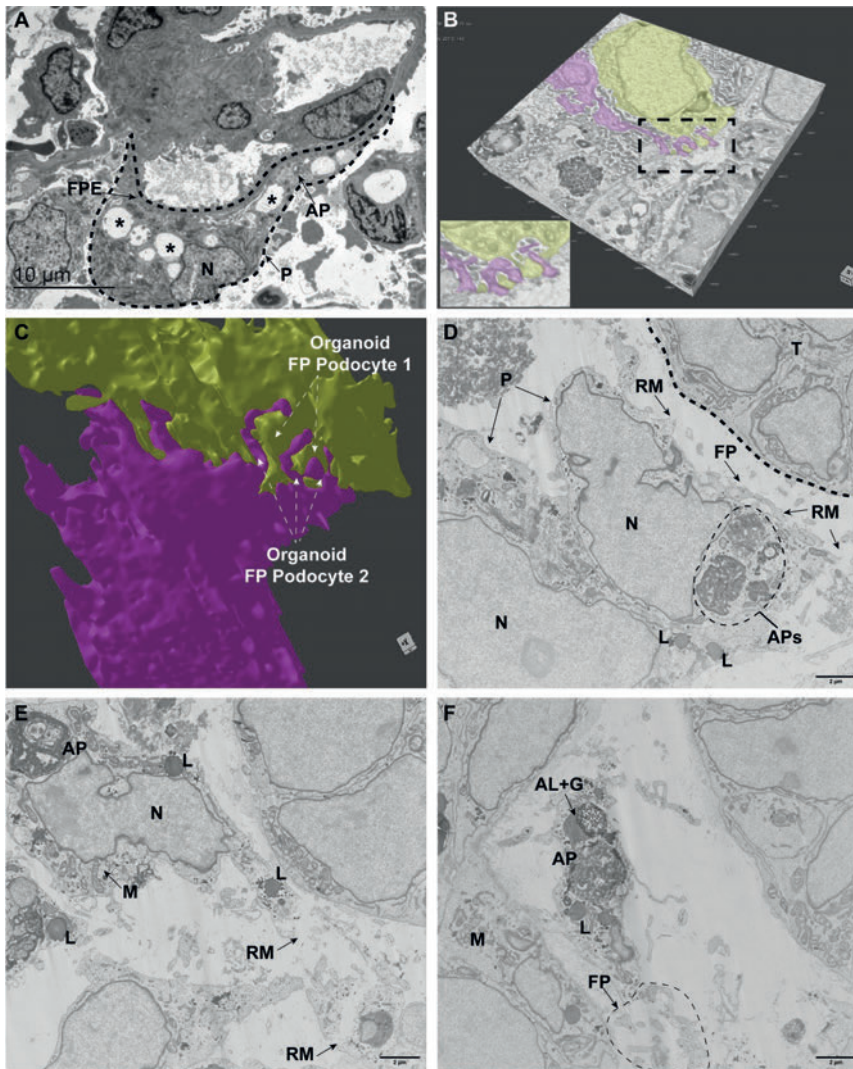


Figure 6. Puromycin aminonucleoside-induced injury in kidney organoids results in autophagic activity comparable to human minimal change disease NS. (A) Ultrastructure analysis of a human MCD kidney biopsy specimen as observed by TEM. The podocyte (dashed line, P) showed vacuole formation (some are highlighted with asterisks), foot process effacement (FPE) and autophagosomes (AP). N, nucleus. (B) 3D volume rendering of vehicle-treated kidney organoid, imaged by FIB-SEM, showing podocytes (yellow, magenta) and foot processes. The complete z-stack, including annotations, is shown in Supplemental movie S1. (C) 3D rendering of the segmented podocytes shows interdigitating podocyte foot processes (arrows). See Supplemental movie S2 for 3D rendering following segmentation. (D-F) FIB-SEM volume imaging (stack slide number shown in D: 327, E: 735, F: 1456) from PAN-injured podocytes (P) showing autophagosomes (APs), lysosomes (L), foot processes (FP) with ruptured membranes (RM). AL+G, autolysosome with glycogen; M, mitochondria; N, nucleus; T, tubular cells. Scale bars 2 μ m. The complete z-stack, including annotations, is shown in Supplemental movie S3.

Using protamine sulfate, the early events affecting podocyte injury through cytoskeleton remodelling and affected slit diaphragm signalling can be modelled *in vivo* (Verma et al 2018) and in kidney organoids (this study). The protein sulfate model is, however, an acute podocyte injury model. Therefore, we investigated the puromycin aminonucleoside (PAN) model and compared PAN-induced organoid podocyte injury to human minimal change disease (MCD) NS pathology.

Using focus ion beam scanning electron microscopy (FIB-SEM) volume imaging and transmission electron microscopy (TEM) we analysed vehicle and PAN-treated (25 µg/ml, 24h exposure) organoids and compared the results with kidney biopsy tissue from a patient suffering from MCD. In human MCD, vacuoles, autophagosomes and foot processes effacement were observed (Figure 6A). FIB-SEM volume imaging of the vehicle-treated organoid podocytes showed polarized cell bodies, foot processes and filtration slits (Figure 6B and Supplemental movie S1). Segmentation and 3D-rendering analysis confirmed interdigitating foot processes between podocytes (Figure 6C and Supplemental video S2). FIB-SEM volume imaging of the PAN-injured organoid podocytes showed autophagic activity in multiple podocytes, as indicated by autophagosomes and autolysosomes (Figure 6D-F and Supplemental video S3). Moreover, parts of the podocyte cell membrane were ruptured and podocyte foot processes were affected. These findings in organoids are in line with previous human studies showing that podocyte autophagy is correlated with podocyte foot process effacement and proteinuria in MCD NS^{50,51}. These results demonstrate that organoid podocytes show similar pathological characteristics compared with podocyte damage in patients suffering from MCD, thereby emphasizing the potency of organoids for accurately modelling podocyte changes that lead to NS in patients.

Next, as a proof of concept, we showed that organoid podocytes respond to CPFs present in plasma from patients with NS recurrence after kidney transplantation (Supplemental figure 9). We treated organoids for 4h with patient plasma collected during plasmapheresis in the active course of the disease and plasma obtained after remission. Control plasmapheresis plasma was collected from an iNS patient after kidney transplantation, with no disease recurrence (plasma presumably free of CPFs). By flow cytometry analysis, we could show that NPHS1⁺ organoid podocytes exhibited increased cytoplasmic granule formation when exposed to active plasma compared with control and remission plasma (Supplemental figure 9A). Podocyte granule formation is an

acknowledged marker for podocyte injury⁵²⁻⁵⁴. Podocyte morphology and nephrin protein expression were not affected after 4h plasma exposure (Supplemental figure 9B-C). In addition, we exposed organoids to plasma for 36h and observed the initiation of cytoskeleton remodelling by actin condensation at the podocyte basement membrane (Supplemental figure 9D). Our data suggest that organoid podocytes might be a useful model for investigating podocyte pathophysiology activated by CPFs and, ultimately, may contribute to the identification of CPFs involved in iNS.

Discussion

In the present study, we successfully modelled congenital (*NPHS2* mutant) and idiopathic NS using kidney organoids. Using iPSCs derived from a patient with two heterozygous podocin mutations, we could show the absence of podocin expression and aberrant nephrin localization in kidney organoids, which was rescued by CRISPR/Cas9 stem cell gene editing prior to differentiation into kidney organoids. As a result, VEGFA pathway and transcription factors activity, essential in podocyte physiology, were restored. iNS was modelled in organoids using plasma from a patient with post-transplant disease recurrence that resulted in podocyte injury. Moreover, we induced iNS-associated podocyte injury using protamine sulfate and PAN. We report that kidney organoids show superior morphology and expression of podocyte markers and activate downstream signalling cascades upon injury, which are absent in 2D iPSC-derived podocytes and human ciPOD, thereby making kidney organoids the preferred *in vitro* model to study podocyte injury that results in NS in patients.

NS pathophysiology is complex and the molecular mechanisms underlying the disease are still poorly understood, mainly owing to a lack of accurate human models. From previous studies, it is known that the podocyte is the main culprit in NS, consequently affecting glomerular filtration barrier integrity and leading to endothelial cell injury⁵⁵⁻⁵⁸. For decades, murine and human ciPODs have been used to unravel NS pathophysiology. Although these models have expanded our knowledge regarding podocytopathies in NS, we and others showed that *in vitro* 2D conditionally immortalized podocyte models do not fully exhibit *in vivo* podocyte characteristics^{18,19,55,59,60}. Here, we showed that kidney organoids are a promising tool to study both congenital and idiopathic NS because organoid podocytes express essential proteins, have a morphology

closely resembling *in vivo* podocytes, and respond similarly to injury triggers. The 3D organoids show a superior expression profile compared with the 2D iPSC-derived podocytes. In 2D culture, nephrin is absent or the localization is still intracellular rather than between the interdigitating foot processes. Also, downstream filtration slit diaphragm signalling is absent in injured 2D iPSC-derived podocytes, emphasizing the limited differentiation properties of podocytes in 2D culture. It is known that 3D glomerulus-like structures isolated from organoids maintain marker expression for 96h, whereas 2D podocytes migrating from these organoid glomeruli showed a clear alteration in their transcriptome¹⁸. There is an unmet need for better *in vitro* models to recapitulate the *in vivo* glomerular filtration barrier, with emphasis on the podocyte. The 3D configurations and the role of mechanical as well as extracellular matrix (ECM) cues that influence cellular (de)differentiation in the development of accurate organoid models need to be addressed in order to establish improved glomerular models *in vitro*. The effect of ECM cues and material stiffness on organoid differentiation was nicely shown by Garetta et al. and also by Geuens and co-workers^{11,61}. Organoids encapsulated in soft biomaterials showed accelerated differentiation, less fibrosis upon aging, and reduced off-target cell populations, all aspects that will aid the signalling required to enhance the maturation of podocytes, but also other kidney segments like the proximal tubule and the collecting duct.

Kidney organoids hold great promise as the next step in NS modelling, nevertheless, tissue maturity and the presence of off-target cells are often being criticized. To overcome maturation challenges, organoid transplantation *in vivo* or onto chicken chorioallantoic membranes can be performed^{11,13,21,62}. Organoid maturation can be achieved by the use of renal subcapsular transplantation of human kidney organoids in immunodeficient mice for the adaptive immune system, which results in size-selective filtration across the organoid glomerular filtration barrier⁶². This transplantation approach emphasizes the potential of organoids to develop a mature glomerulus, which would be useful for studying a wide range of glomerular disorders; however, animals would still be required, which hampers high-throughput screens and is in conflict with the current attention for animal welfare. Altogether, when focusing on podocytopathies such as NS, the organoids we present in this study represent a useful tool to model idiopathic and congenital injury responses.

Despite the fact that podocytes are one of the best differentiated cell types in kidney organoids, the human adult podocyte signature is not yet fully

accomplished in kidney organoids^{18,60,63}. Our computational analysis revealed heterogeneity throughout the organoid podocyte populations based on collagen IV alpha chain expression. It is known that at the comma, S-shaped, and early capillary loop stage of glomerulogenesis, podocyte progenitors express collagen IV alpha chains 1 and 2. This collagen IV network is replaced by collagen IV alpha chains 3, 4, and 5 at the beginning of capillary loop stages and, ultimately, these chains are solely expressed by mature podocytes²⁵. The exact mechanism of the collagen IV isoform switch remains elusive, although it has been described to aid in podocyte differentiation and is likely under the control of VEGF, which in turn is regulated by, amongst others, PI3K signaling^{29,33,34}. In line with this, we showed that podocyte subcluster 3 had increased collagen IV alpha 3 and VEGF expression as well as PI3K signalling compared with the other two podocyte subclusters. Moreover, solely cluster three shows ligand-receptor interactions with endothelial cell precursor receptors, as a potential first step towards glomerular filtration barrier formation, thereby confirming that cluster 3 is more mature compared with the other two podocyte clusters. Cluster 3 resembled in part adult podocytes in terms of collagen IV alpha 3 expression. The data we collected in this study could aid in establishing the culture conditions required to achieve a fully mature podocyte population in organoids.

The PI3K signalling pathway, which regulates VEGF signalling, can be induced by tyrosine kinases such as epidermal growth factor receptor (EGFR)⁶⁴, which is achieved in our kidney organoid culture protocol by EGF supplementation. Despite the EGFR stimulation, the consequent PI3K activation did not result in homogenous VEGF expression levels as VEGF expression varied between the podocyte clusters we identified in our organoids. Also, the TF signature in podocyte cluster 3 showed closest, but still incomplete, resemblance with adult podocytes. To accelerate the differentiation of podocytes, VEGF signalling is likely one of the major actors²⁷.

To stimulate VEGF signalling, dynamic modulation of WNT signalling has been shown to be a promising approach to enhance podocyte differentiation in kidney organoids and corresponding VEGFA levels. Consequently, enhanced VEGFA levels promoted a defined vascular network, which ultimately is essential for glomerular filtration barrier maturation⁶⁵. When focusing on VEGF signalling regulation, a magnitude of factors are involved. The promoter region of VEGF contains multiple trans-activating factors and responsive elements⁶⁴, such as hypoxia inducible factor-1 (HIF1), that might open avenues to

transcriptional stimulation of VEGF expression throughout all podocytes present in the organoids. Notably, organoids are cultured under normoxic conditions, in contrast to glomerulogenesis *in vivo*, which occurs under hypoxic conditions⁶⁶. A hypoxic environment will activate HIF transcription factors, regulate VEGF signalling, and consequently stimulate podocyte differentiation⁶⁶. Mimicking the delicate balance of VEGF signalling during the different stages of nephrogenesis will be a challenge *in vitro*²⁷. Obviously, podocyte maturation not only depends on VEGF signalling but relies on a plethora of factors. For instance, podocyte maturation also relies on the concerted actions with other cell types, such as podocyte-macrophage crosstalk⁶⁷, fluid flow including nutrient exchange, as well as microenvironmental ECM cues^{11,61,62,66,68}.

In conclusion, we report successful patient-based idiopathic and congenital podocytopathy modelling in iPSC-derived human kidney organoids. We showed that 3D kidney organoids are the model of choice for investigating podocyte changes that result in NS in patients, as confirmed in three iPSC lines, compared with 2D cultured podocytes, and in particular that a specific podocyte cluster in part resembles adult podocytes. Kidney organoids represent a powerful tool for studying underlying NS disease mechanisms, may have the potential to identify pathogenic CPFs in patients' plasma, and allow for the study of functional genomics. Altogether, kidney organoids will help to improve our understanding of podocytopathies and may be used for developing novel effective therapies to treat NS. Once a mature glomerular filtration barrier is developed *in vitro*, this high-end platform could be used beyond studying podocytopathies and may have the potential to become an accurate model for resolving the pathophysiology of a wide range of glomerular disorders.

Materials and Methods

Ethical statement

Human adult skin fibroblasts derived from a healthy volunteer and erythroblasts from a paediatric patient suffering from congenital NS, after giving informed consent, were used to generate iPSCs. This study was conducted in accordance with the Helsinki Declaration as revised in 2013. Permission for the creation and use of iPSCs in this study was obtained from the local ethical commission for human-related research of the Radboud university medical center, Nijmegen (approval numbers: 2015-1543 and 2006-048). Human kidney tissue was used for Immunostainings and EM analysis. Ethical permission for the use of archived human kidney material was given by the local commission for human-related research of the Radboud university medical center, the Netherlands (approval number: 2018-4086).

Patient and healthy control plasma

This study was approved by the regional medical-ethical committee (Arnhem-Nijmegen), under file number 09/073, and has been carried out in compliance with the Declaration of Helsinki as revised in 2013. All human participants signed informed consent prior to the study, as specified in the International Committee of Medical Journal Editors recommendations.

Reagents and resources

Reagents and resources (e.g. antibodies, chemicals, commercial assays, software and algorithms) are listed in Table S5.

Cell culture

iPSC generation

Healthy control iPSC were generated using fibroblasts from a healthy volunteer at the Radboudumc Stem Cell Technology Center through the use of lentiviral vectors, previously described by Warlich et al.⁶⁹. In short, *Escherichia coli* were used to clone lentiviral vectors containing the transcription factors Oct4, Klf4, Sox2 and cMyc together with the fluorescent marker dTomato (pRRL.PPT.SF.hOKSMco-idTom.pre.FRT). HEK293T cells were then used for viral production using packaging (pCMV-VSV-G) and envelope (pCMV-dR8.91.) vectors, respectively. Fibroblasts were transduced with viral supernatants and reseeded on plates with inactivated mouse embryonic fibroblast (MEF) feeder cells to support the growth and pluripotency of iPSCs. Emerging iPSC colonies

were picked, expanded and assessed for activation of stem cell markers to confirm pluripotency.

Mutant iPSC, containing 2 mutations in the podocin encoding gene (*NPHS2*; Chr1(GRCh37); NM_014625.3:c.413G>A (p.(Arg138Gln); heterozygote) and c.478G>T (p.(Asp160Tyr); heterozygote) were generated from erythroblasts from a paediatric patient using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher, Breda, The Netherlands) according to the manufacturer's protocol. In short, erythroblasts were transduced using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit and reseeded on plates with inactivated MEFs to support the growth and pluripotency of iPSCs. Emerging iPSC colonies were picked, expanded and assessed for activation of stem cell markers to confirm pluripotency.

iPSC maintenance culture

iPSCs were cultured using Essential™ 8 (E8) medium (Gibco, Thermo Fisher) supplemented with E8 supplement (50X, Gibco) and 0.5% (v/v) antibiotic-antimycotic (Gibco) on Geltrex-coated (Thermo Fisher) cell culture plates (Greiner, Alphen aan den Rijn, the Netherlands). Upon 70-90% confluency iPSCs were washed 3 times with PBS and subsequently passaged in colonies using 0.5 mM EDTA (Thermo Fisher) in PBS for 5 minutes at room temperature. For cell seeding, iPSCs were washed 3 times with PBS and subsequently disassociated into single cells using TrypLE Select Enzyme (Thermo Fisher) for 2 minutes at 37°C.

ciPOD culture

The ciPOD used in this study was kindly provided by Prof. Saleem (Bristol, UK)¹⁴. ciPODs were cultured using uncoated 75 cm² culture flasks (Greiner) at 33°C with 5% (v/v) CO₂ in DMEM/F12 (Gibco) supplemented with 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium (Sigma-Aldrich, Zwijndrecht, the Netherlands), 10% heat inactivated FCS (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco). Passaging and seeding of cells was performed by washing cells with PBS and subsequently detaching them using Accutase® (Sigma-Aldrich), according to the manufacturer's protocol. ciPODs were seeded at 62,500/cm² in black flat bottom 96-wells plates (Corning, Corning, New York) for immunofluorescent imaging or 6-wells plates (Greiner) for protein and RNA isolation and were incubated at 37°C with 5% (v/v) CO₂, which initiated differentiation of the ciPODs.

All cell lines used were tested negative for contamination, including *Mycoplasma*.

Differentiation protocols

2D podocyte differentiation

An adapted protocol based on Rauch et al. and Musah et al.^{15,16} was used to differentiate iPSC into podocytes. iPSCs were seeded at 15,000 cells/cm² on Geltrex-coated cell culture plates in E8 medium supplemented with E8 supplement, 0.5% (v/v) antibiotic-antimycotic and 1X RevitaCell (Gibco). One day later, defined as day 1, differentiation of iPSCs into metanephric mesenchyme was started by adding Essential™ 6 (E6) medium (Gibco), supplemented with 15 ng/mL human recombinant bone morphogenetic protein 7 (RD system, Minneapolis, Minnesota), 10 ng/mL human recombinant activin A (RD systems), 100 nM retinoic acid (Sigma-Aldrich) and 1X non-essential amino acids (Gibco). At day 11, podocyte differentiation was started by culturing cells in E6 medium supplemented with 1X non-essential amino acids and 10 ng/mL vascular endothelial growth factor subtype A (RD systems). At day 20, iPSC-derived podocytes were mature and used for experiments.

3D organoid differentiation

iPSCs were seeded on Geltrex-coated cell culture plates using E8 medium supplemented with E8 supplement, 0.5% (v/v) antibiotic-antimycotic and 1X RevitaCell at a density between 18,750 - 22,900 cells/cm². Twenty-four hours after seeding, culture medium was replaced by E6 medium supplemented with 6 µM CHIR (Tocris, Bio-Techne, Abingdon, United Kingdom) to initiate differentiation (defined as day 0). From day 3 onwards, half of the wells were cultured in E6 medium supplemented with 200 ng/mL FGF9 (RD systems) and 1 µg/ml heparin (Sigma-Aldrich) until day 7 (anterior intermediate mesoderm (AM)), whereas the other wells remained on E6 medium with CHIR until day 5 (posterior intermediate mesoderm (PM)). From day 5 onward, all cells were cultured in E6 medium with FGF9 and heparin. At day 7, cells were harvested using trypsin-EDTA and a cell suspension was made consisting of AM and PM cells in a 1:2 ratio. Cells aggregates, consisting of 300K cells, were transferred to Transwell™ plates (Corning) and incubated for at least 1 hour at 37°C, 5% (v/v) CO₂, with E6 medium supplemented with 5 µM CHIR to induce self-organizing nephrogenesis. After 1 hour, medium was replaced for E6 medium supplemented with FGF9 and heparin. At day 7+5 (i.e. 5 days later), medium was changed to E6 supplemented with EGF (10 ng/ml, RD systems) and BMP7 (50 ng/ml, RD systems). Organoids were used for experiments at day 7+17 or 7+18.

Protamine sulfate exposure and heparin rescue

Organoids, ciPODs and 2D iPSC-derived podocytes were exposed to protamine sulfate treatment and rescued with heparin. Briefly, protamine sulfate (2 mg/ml, Sigma-Aldrich) was dissolved in E6 medium and organoids and 2D cell cultures were exposed at 37°C, 5% (v/v) CO₂ for 2 hours. After 2 hours, cells (organoids and 2D cells) exposed to protamine sulfate were processed for protein isolation and immunofluorescent staining, as described in a following sections. Sequentially, protamine sulfate injured organoids and 2D cell cultures were exposed to heparin (800 µg/ml in E6, heparin sodium salt from porcine intestinal mucosa, Sigma) at 37°C, 5% (v/v) CO₂ for 2 hours. Ultimately after 2 hours, these organoids and cell cultures (groups: control, heparin control, protamine sulfate plus heparin treatment) were also processed for protein isolation and immunofluorescence staining.

Puromycin aminonucleoside treatment of organoids and sample preparation for electron microscopy and FIB-SEM volume imaging

Kidney organoids were exposed to vehicle or puromycin aminonucleoside (25 µg/ml, MedChemExpress, Monmouth Junction, NJ) at 37°C, 5% (v/v) CO₂ for 24h. Next, organoids were washed in PBS and fixed using 2.5% (w/v) paraformaldehyde (PFA) (Sigma-Aldrich) + 0.1% (v/v) glutaraldehyde (GA) (Electron Microscopy Sciences (EMS), Hatfield, PA) in 1 mM phosphate buffer, at pH 7.4. After one hour the samples were transferred to 4% (v/v) PFA in 1mM phosphate buffer, pH 7.4 for 48 hours. After fixation, the samples were stored in 0.1M cacodylate buffer (EMS) at 4°C until further processing for EM. To enhance the sample contrast for electron microscopy, an OTO based protocol was used^{70,71}. Briefly, the samples were incubated in 2% (w/vl) osmium tetroxide (Structure Probe, Inc. (SPI), West Chester, PA / 1.5% (w/v) potassium ferrocyanide (Merck)) in 0.1 M cacodylate buffer with 2 mM CaCl₂ (Merck) for one hour at room temperature, thereafter washed and incubated in 0.5% (w/v) thiocarbohydrazide solution (Sigma-Aldrich) for 30 min at room temperature (RT). After rinsing, the samples were incubated again in 2% (w/v) osmium tetroxide for 30 min at RT, and then rinsed and incubated in 2% (w/v) aqueous uranyl acetate (EMS) at 4°C overnight. After washing, the samples were incubated in lead aspartate (Merck) solution (pH 5.5) for 30 min at 60°C, rinsed and dehydrated in ascending ethanol series. The dehydrated samples were then transferred into a mixture of acetone and Durcupan (Sigma-Aldrich) in mixtures of 3:1, 1:1, each step for 1.5 hours, followed by a 1:3 mixture for overnight. Subsequently, the samples were incubated in 100% Durcupan for 4 hours before embedding and polymerization with fresh Durcupan.

After polymerization, semi-thin sections (500 nm) were cut with a Reichert Ultracut S ultramicrotome (Leica, Rijswijk) and stained with toluidine blue (Sigma-Aldrich). The region of interest was cut out of the resin block and glued on an SEM stub with carbon tape, using conductive silver paint. The samples were coated with a gold sputter coater (Edwards, Stockholm, Sweden) before introduction into the FIB/SEM, a Zeiss Crossbeam 550 FIB/SEM (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

Multiple coarse trenches were milled (using the 30 kV@30nA probe) to choose the regions of interest for further 3D volume imaging. Parameters for serial sectioning imaging of large regions were set using the Atlas 3D software (Atlas Engine v5.3.3). The large trenches were first smoothened using the 30 kV@1.5nA FIB probe, thereafter, a 30 kV@700pA probe current was used for serial FIB milling. InLens secondary and backscattered EM images were simultaneously collected at an acceleration voltage of 2.0 kV with a 300 pA probe current. The backscattered grid was set to -876 V. For noise reduction images were acquired using line average ($N=4$) and a dwell time of 0.7 ms. The milling and imaging processes were continuously repeated and long series of images were acquired, creating original stacks of 30x24x12 μm for both the control and PAN-injured samples. The imaging voxel size in both cases was set to 5x5x10 nm.

Image post processing and segmentation

The FIB-SEM images were processed using MATLAB (The MathWorks Inc., R2018b, Natick, MA) and ImageJ (US National Institutes of Health, Bethesda, MD) to correct for uneven illumination, misalignment, de-stripping and contrast enhancement. A summary of each processing step is as follows:

Alignment: The consecutive slices were aligned using normalized cross correlation. Briefly, the first image in the stack was chosen as reference and the second image was translated pixel by pixel across the reference and a normalized cross correlation matrix was obtained using the "normxcorr2" function. The location of the highest peak in the cross-correlation matrix (representing the best correlation) was then used to calculate the translation required to align the two images. Once the moving image was aligned with the reference image, it served as the reference for alignment of the subsequent slice.

Uneven illumination: In order to obtain a homogeneous background, the uneven illumination between the upper and lower half of the images was corrected using the "rolling ball background subtraction" feature in ImageJ. No smoothing was performed during subtraction.

Histogram matching: In order to avoid losing the colour depth and the original contrast and brightness of the images after background correction, the histogram of each image was matched with its corresponding original image using "imhistmatch" function in MATLAB with "polynomial" mapping method. The number of bins was chosen equal to the number of bins in the original image.

Vertical stripes: Removing the vertical stripes in the stacks was done following a wavelet-FFT filtering approach described by Münch et al.⁷². Briefly, the high frequency information corresponding to the vertical stripes was successively condensed into a single coefficient map using decomposition by "coif" wavelet family. Subsequently, a 2D-fourier transform was performed to further tighten the stripe information into narrow bands. Finally, the condensed stipe information was eliminated by multiplication with a gaussian damping function and the de-striped image was reconstructed by inverse wavelet transform.

3D segmentation: Dragonfly™ image analysis and deep-learning software (Objects Research Systems, version 2020.1, Montreal, QC, Canada) was used to segment all image data. For efficient interpolation during segmentation, the images were first binned by a factor of 2 in the X and Y direction (image plane) resulting in a pixel size of 10x10 nm. Subsequently, the stack was down-sampled by a factor of 2 in all directions, resulting in a cubic voxel size of 20x20x20 nm.

Transmission electron microscopy (TEM)

Organoids were fixed overnight using 2.5% (w/v) glutaraldehyde dissolved in 0.1 M sodium cacodylate. Tissue fragments were then fixed for 1 hour using 2% OsO₄ buffered in Palade's buffer (0.23 % (w/v) CH₃COONa, 0.58% (w/v) C₈H₁₁N₂NaO₃ dissolved in MilliQ (pH 7.3)). Tissue was dehydrated through an increasing series of alcohol and embedded in Epon 812 (Sigma-Aldrich). Using a Leica Ultracut, 90 nm ultra-thin sections were cut, mounted on copper grids and contrasted at room temperature with uranyl acetate and lead citrate. Sections were assessed using a Jeol JEM 1400 electron microscope at 60kV. For image collection, a digital camera was used (Gatan Inc. Pleasanton, CA, USA).

Preparing paraffine sections of human iPSC-derived kidney organoids

iPSC-derived kidney organoids were cut from the Transwell™ filter and fixated in 4% (v/v) formalin on ice for 20 minutes. Fixed iPSC-derived kidney organoids were stripped of the filter membrane using a scalpel. Multiple organoids from similar conditions were stacked on top of each other and embedded in a cryomold (Tissue-Tek, Sakura Finetek Europe B.V., Alphen aan de Rijn, the Netherlands) using 2.25% (w/v) agarose gel (ThermoFisher). After embedding for 5 minutes at 4°C, the iPSC-derived kidney organoids were transferred to embedding cassettes and paraffinized. After paraffinization, iPSC-derived kidney organoids were cut at a thickness of 4 µm using a cryo-microtome and mounted on FLEX IHC Microscope Slides (DAKO, Agilent Technologies, Amstelveen, Netherlands).

Immunofluorescence staining

Paraffine tissue

Using a series of xylol (2x) and 100% (v/v) ethanol (3x), paraffine slides were deparaffinized. Antigen retrieval was performed by boiling slides in Tris-buffered EDTA (VWR Chemicals, Amsterdam, the Netherlands) for 10 minutes in a microwave. Primary (1:100) and secondary (1:200) antibodies were diluted in PBS containing 1% (v/v) BSA (Sigma-Aldrich). Primary antibodies were incubated overnight at 4°C, while secondary antibodies were incubated at room temperature for 2 hours. After each antibody incubation, slides were washed 3x in PBS for 5 minutes. Slides were mounted in Fluoromount-G® (Southern Biotech, Sanbio, Uden, Netherlands). Primary and matching secondary antibodies are listed in Table S2. Images were captured using the Zeiss LSM 880 confocal microscope or Leica DMI6000B high-content microscope.

Whole organoid staining

Whole-mount staining was performed according to Takasato et al.⁷³. Briefly, organoids were fixed using 2% (w/v) PFA at 4°C for 20 minutes. After PBS wash, organoids were blocked in blocking buffer containing 10% (v/v) donkey serum (GeneTex, Irvine, California) and 0.6% (v/v) Triton-X in PBS at room temperature for 2h. Primary antibodies (Table S2) were diluted 1:300 and incubated at 4°C overnight. Next, organoids were washed using PBS-Triton-X (0.3% (v/v), PBTX). Secondary antibodies (Table S2) were diluted 1:400 in PBTX and incubated at room temperature for 2h. Organoids were washed using

PBS and mounted using Fluoromount-G®. Images were captured using the Zeiss LSM 880 confocal microscope.

Organoid filtration slit super-resolution imaging

Sample processing and subsequent imaging were performed as described before⁷⁴. In brief, 4 µm paraffin sections were directly mounted on coverslips (VWR). To correct for PFA-induced autofluorescence, samples were incubated with 100 mM glycine in PBS for 10 minutes. Samples were blocked with 1% (v/v) fetal bovine serum, 1% (v/v) goat serum, 1% (v/v) bovine albumin and 0.1% (v/v) cold fish gelatin in PBS at RT for 1 hour. Primary antibody against nephrin (guinea pig, PROGEN, GP-N2, 1:100) was diluted in blocking solution and detected by a secondary anti-guinea pig Cy3-antibody (Jackson ImmunoResearch Laboratories Inc., 1:800) that was also diluted in blocking solution. 3D-SIM images were acquired using a Zeiss Elyra PS.1 system. Using Zeiss ZEN black software, 3D-SIM images were reconstructed. As control *in vivo* tissue we used sections of male Munich Wistar Frömter rats, age ranging from 41-54 weeks. The animals were housed under standard conditions. Animal procedures were approved by the German government officials (LANUV NRW 50.203.2 – AC 10/06) and performed in accordance with the European Communities Council Directive (86/609/EEC).

2D podocyte staining

iPSC-derived podocytes and ciPODs cultured in 96-well plates were fixed using 2% (w/v) PFA for 10 minutes at room temperature. Cells were blocked using blocking buffer (same as used for whole organoid stain) at room temperature for 2h. Primary antibodies (Table S2) were diluted 1:100 and incubated at 4°C overnight. Next, cells were washed using PBTX and were incubated using secondary antibody diluted 1:200 in PBTX and incubated at room temperature for 2h. Cells were washed and mounted using Fluoromount-G®. Images were captured using the Leica DMI6000B high-content microscope.

Protein harvesting, gel electrophoresis and Western blot

Tissue was collected and incubated on ice for 60 minutes using RIPA buffer containing 1X RIPA containing a phosphatase inhibitor Na_3VO_4 (Cell Signaling, Leiden, the Netherlands), 1 mM phenylmethylsulfonyl fluoride (PMFS, Sigma) and 2% (w/v) sodium dodecyl sulphate (SDS, Sigma) in PBS. After incubation, tissue was centrifuged at 16,000xg for 15 minutes at 4°C. The supernatant containing protein was collected and stored at -80°C for further processing. Protein samples were mixed 1:4 with 4X Laemmli buffer (Bio-Rad, Lunteren,

Netherlands) containing 10% (v/v) β -mercaptoethanol (Merck) and incubated at 95°C for 5 minutes. Samples were spun down and loaded onto a 4-20% (w/v) polyacrylamide precast mini-PROTEAN TGX gel (Bio-Rad) for subsequent gel-electrophoresis. Gels were then transferred onto a nitrocellulose blotting membrane (Amersham, GE Healthcare, Eindhoven, the Netherlands) and blocked for 1 hour at room temperature using Odyssey blocking buffer (LI-COR Biosciences, Bio-Rad) in a 1:1 dilution with tris-buffered saline (TBS) containing 0.1% (v/v) Tween® 20 (Merck). Subsequently, membranes were washed 3 times for 5 minutes with TBS-T and incubated overnight with primary antibodies at 4°C. After washing the membranes again 3 times for 5 minutes with TBS-T, membranes were incubated with matching secondary antibodies for 1 h at room temperature. Membranes were washed using TBS and afterwards imaged and semi-quantified using a LI-COR Odyssey CLx imaging system. Primary- and corresponding secondary antibodies are listed in Table S3.

Fluorescence-activated cell sorting (FACS)

Organoids cultured on Transwell™ plates were washed twice in PBS, dissociated using Accutase® and transferred to sterile 6-wells plates (without Transwell™ inserts). Organoids from similar culture conditions were pooled and incubated for 15 minutes at 37°C. During incubation, the cell suspension was resuspended every 2 minutes for homogenization purposes. After incubation, the cell suspension was put on ice for 1 minute to inactivate the Accutase®, cells were strained using a 40 μ m cell strainer (Corning) and the cell strainer was washed repeatedly with 10% (v/v) FCS in DMEM/F12 to collect all cells. The strained cell suspension was centrifuged for at 250xg for 5 minutes at 4°C and supernatant was discarded. Cells were washed with FACS buffer (PBS- 1% (v/v) BSA) once and incubated with primary antibodies for NPHS1 (1:100, RD systems, #AF4269) in FACS buffer for 45 minutes at 4°C. After incubation, cells were centrifuged at 250xg for 5 minutes at 4°C and again washed with FACS buffer. After washing, cells were again centrifuged at 250xg for 5 minutes at 4°C and subsequently incubated with matching secondary antibodies (1:200, donkey anti-sheep Alexa Fluor™ 647, ThermoFisher, #A-21448) for 30 minutes at 4°C. Cells were centrifuged at 250xg for 5 minutes at 4°C and washed twice in FACS buffer. After washing, cells were incubated with DAPI (300 nM, ThermoFisher) in FACS buffer 10 minutes prior to FACS (for live/dead gating). Detection events were gated to select cells and doublet control was performed for both forward and side scatter. Living cells (DAPI⁻) were selected and gated on NPHS1⁺ (podocytes). Cells were sorted by immune fluorescence using a fluorescence cell sorter (BD Biosciences; FACSAria™ II SORP 18-color) and DIVA8 software. After cell

sorting, DAPI⁻/NPHS1⁺ cells were collected in RLT buffer (RNeasy, Qiagen, Germantown, Maryland) for further processing.

Plasma exposure and granule formation analysis using flow cytometry

D7+17 organoids were exposed to 10% (v/v) plasmapheresis plasma supplemented with anticoagulants heparin (100 µg/ml, Sigma Aldrich) and PPACK (10 µM, Santa Cruz Biotechnology) and incubated at 37°C, 5% (v/v) CO₂, for 4 hours and 24 hours. The plasma was collected during plasmapheresis of a patient with posttransplant recurrent NS, during the active course of the disease and after remission. After collection, plasmapheresis plasma was aliquoted and stored at -80°C. As controls we used E6 culture medium, E6 medium supplemented with anti-coagulants heparin (100 µg/ml) and PPACK (10 µM) and plasmapheresis plasma collected from an iNS patient after kidney transplantation, with no disease recurrence (plasma presumably free of CPFs) and supplemented with anticoagulants. Next, single organoids were washed with PBS and digested using Accutase® for 15 minutes at 37°C, during this step the suspension was resuspended every 5 minutes. Accutase® was inactivated by adding DMEM/F12 supplemented with 10% (v/v) FBS. The organoid suspensions were centrifuged at 250xg for 5 minutes. Cells were washed once using wash buffer (PBS containing 1% (v/v) BSA) and again centrifuged. Next, the cells were incubated using a primary antibody against NPHS1 (R&D Systems, #AF4269), diluted 1:100 in FACS buffer (PBS supplemented with 1% (v/v) BSA) for 1 hour at 4°C. After 2 washing steps, the cells were incubated using a secondary antibody donkey anti-sheep Alexa Fluor 647™ (ThermoFisher, #A21448) 1:200 in FACS buffer for 45 min at 4°C. Cells were centrifuged at 250xg for 5 minutes at 4°C and washed twice in FACS buffer. After washing, cells were incubated with DAPI (300nM, ThermoFisher) in FACS buffer 10 minutes prior to FACS (for live/dead gating). Detection events were gated to select cells and a doublet control was performed for both forward and side scatter. Living cells (DAPI⁻) were selected and gated on NPHS1⁺ (podocytes). Cells were measured with a flow cytometer (NovoCyte 3000 flow cytometer, Agilent), as previously described by den Braanker et al.⁵⁴. The granule formation in living NPHS1⁺ podocytes was quantified by sideward scatter.

Transcriptome analysis

scRNA-seq

D7+17 organoids were washed with PBS and digested using Accutase® at 37°C for 15 minutes. Accutase® was inactivated by adding DMEM/F12 with 10% (v/v) FBS. The cell suspension was filtered through a 40 µm cell strainer and spun down at 1250xg for 5 minutes. To reduce background and obtain a single cell suspension, this step was repeated once more. Cells were then resuspended in PBS containing 0.04% (v/v) BSA and counted using a Neubauer counting chamber (Carl Roth, Karlsruhe, Germany). Next, 1000 cells/ µL per sample were loaded onto a 10x Chromium Next GEM Chip G according to the manufacturer's instructions and processed in a Chromium controller (10x Genomics, Pleasanton, California). All single cell sequencing libraries were generated using 10x Chromium Next GEM v3.1 kits (10x Genomics) and sequencing was performed on a Novaseq6000 system (Illumina, San Diego, California), using an S2 v1.5 100 cycles flow cell (Illumina) with run settings 28-10-10-90 cycles. After sequencing, Cell Ranger (v.3.1.0) was used to align the reads to the human genome GRCh38, using default settings.

Standard scRNA-seq analysis workflow

The pre-processed reads were analysed using the R package "Seurat" (version 4.0.3)⁷⁵. Data was loaded using the "Read10X" function and transformed into a Seurat object. A Seurat object was created with features (genes) that appeared in at least 3 cells and cells that contained at least 200 different features as a first filtering step. Next, cells were filtered out containing <200 or > 3000 features to filter out damaged cells and doublets. Also, cells in which mitochondrial genes contributed for >20% to the total gene expression were believed to be damaged or apoptotic and therefore discarded. After these quality control checks, data was log normalized and variable features were identified using the "FindVariableFeatures" function with the vst selection method. Afterwards, the Seurat package was used to scale the data, and run a principal component analysis (PCA) dimensionality reduction. Data from the PCA was used to construct a Shared Nearest Neighbor (SNN) graph taking into account 20 principal components (PCs), which was subsequently used to identify clusters using the "FindClusters" function with the resolution set to 0.7. For visualization, the "RunUMAP" function was used. Next, clusters markers were obtained by running the "FindAllMarkers" function. Annotation of the clusters was performed manually using these cluster markers, and cluster markers described in literature and the Humphreys Kidney Interactive Transcriptomics (KIT) website (<http://humphreyslab.com/SingleCell/>).

scRNA sequencing in-depth podocyte cluster analysis

For in-depth podocyte analysis, the podocyte cluster was extracted from the total dataset and variable features were calculated again for this subset only. Next, the same workflow as described for the complete dataset after identifying variable features was performed. For comparative analysis with adult and fetal podocytes, publicly available datasets obtained from the GEO database were used (accession number GSE118184 and GSE112570, respectively). Seurat objects were created from these datasets, dotplots for NPHS1, NPHS2, podocalyxin, synaptopodin and Wilms' Tumour 1 were used to define the podocyte population within each dataset. This cluster was subsetted and merged with the subsetted podocytes from the organoids. Podocyte datasets were integrated using the "runHarmony" function from the Harmony (version 0.1.0) package⁷⁶. Subsequently, we compared the podocyte clusters for marker expression using Seurat, pathway activity using PROGENy (version 1.14.0) and transcription factor activity using DoRothEA (version 1.4.1) and visualized using the pheatmap (version 1.0.12) R-package^{32,36,77,78}. Cluster markers of podocyte clusters were determined using the "FindAllMarkers" function on a dataset with only these podocyte clusters (organoid, adult and fetal). These cluster markers were filtered to retain only significant markers (adjusted p-value < 0.05) and were divided in upregulated (log fold change > 0) or downregulated (log fold change < 0). A list of significantly upregulated cluster markers was used to perform GO and KEGG enrichment analysis, using the clusterProfiler package (version 4.0.2)⁷⁹. MSigDB enrichment analysis was done using the 'H' or 'C2' category gene set from the msigdbR package (version 7.2.1)⁴⁷.

Ligand-receptor interaction analysis

In order to assess cellular interactions between cellular cluster within the organoid we replaced the podocyte cluster that was obtained by unsupervised clustering with the three podocyte subclusters that were identified during in-depth podocyte cluster analysis. Furthermore, cells positive for *GATA3* and negative for *CDH1* were named mesangial cells and cells positive for *PECAM1* or *CD34* or *ICAM1* or *CDH5* or *PLVAP* were named endothelial cells. Ligand-receptor analysis was performed with the addition of podocyte cluster 1, 2 and 3, mesangial cells and endothelial cells added to the original clusters identified by unsupervised clustering using Seurat. scRNA sequencing matrices were log-normalized and scaled and used as input for ligand receptor inference by CellphoneDB (CellphoneDB, version 2.0.5)³⁰. Analysis was performed using the statistical_analysis mode of the CellphoneDB package. Using statistical significant interactions (p-value < 0.05) in the CellphoneDB output, ranking

and visualization of the data was performed by CrossTalker (v 1.0.0)³¹. The same clusters were used to obtain GO, KEGG and Hallmark H enrichment in a similar way as described for the podocyte clusters (organoid, adult and fetal) described above.

Bulk RNA sequencing

Sequencing was performed at Single Cell Discoveries, a (single cell) sequencing provider located in the Netherlands using an adapted version of the CEL-seq protocol. In short, total RNA was extracted from FACS-sorted podocyte cell pellets using the Ambion PureLink RNA mini kit (ThermoFisher) according to the manufacturers protocol and used for library preparation and sequencing. mRNA was processed as described previously, following an adapted version of the single-cell mRNA seq protocol of CEL-Seq^{80,81}. Briefly, samples were barcoded with CEL-seq primers during reverse transcription and pooled after second strand synthesis. The resulting cDNA was amplified with an overnight *in vitro* transcription reaction. From this amplified RNA, sequencing libraries were prepared with Illumina Truseq small RNA primers. Paired-end sequencing was performed on the Illumina Nextseq500 platform. Read 1 was used to identify the Illumina library index and CEL-Seq sample barcode. Read 2 was aligned to the hg19 human RefSeq transcriptome using BWA⁸². Reads that mapped equally well to multiple locations were discarded. Mapping and generation of count tables was done using the MapAndGo script¹. Samples were normalized using RPM normalization.

Bulk RNA sequencing bioinformatic workflow

Bulk RNA sequencing data was used to produce heatmaps of known podocyte markers, pathway analysis using progeny and differential gene expression. Differential gene expression analysis was carried out using DESeq2 version 1.32.0 with internal statistical and normalization methods (i.e. multiple testing correction with Benjamini-Hochberg). The list of up- and downregulated genes that was obtained from DESeq2 for repaired podocytes versus mutant podocytes were used as input for enrichment analysis. Enrichment analysis for GO terms, KEGG pathways and Hallmark H gene sets was performed using clusterProfiler.

RNAscope

Formalin-fixed paraffin-embedded (FFPE) kidney organoid samples were pretreated according to the protocol of ACDBio (#322452, Bio-Techne). The probes that were used in this protocol were RNASCOPE[®] probe - Hs-COL4A3 (#461861, Bio-Techne), RNASCOPE[®] probe - Hs-PECAM1-O1-C2 (#487381-C2,

Bio-Techne), RNASCOPE® probe - Hs-NPHS1-C3 (#416071-C3, Bio-Techne), and RNASCOPE® 3-Plex negative control probe (#320871, Bio-Techne). The RNAscope was performed according to the manufacturer's protocol of ACDbio for fluorescent multiplex RNA *in situ* hybridization (#320293-USM). Images were captured using a Zeiss LSM 880 confocal microscope.

CRISPR/Cas NPHS2 repair (c.413G>A to c.413G)

CRISPR/Cas repair was performed by the Radboudumc Stem Cell Technology Center. In short, 70-90% confluent *NPHS2* mutant iPSCs were disassociated into single cells using TrypLE Select Enzyme for 2 minutes at 37°C. Cells were nucleofected with an RNP complex containing Alt-R® S.p. Cas9 Nuclease V3 (#1081058, IDT, Leuven, Belgium) coupled to Alt-R® S.p. CRISPR-Cas9 sgRNA 5'AGAGTAATTATATTTCCAACCT3' (IDT) and Alt-R™ HDR donor oligo 5'GTGTTTAGAAAAAAGAGTGTTTTTTTACCAGGGCCTTTGGCTCT TCCAG-GAAGCAGATGTCCGAGTCGGAATATAATAACCCTTTCATACTCTTG 3' (PS modification, antisense; IDT) using a 4D-nucleofector System X Unit (Lonza, Basel, Switzerland) and the P3 primary Cell 4D-Nucleofector® X kit (#V4XP-3024, Lonza). Nucleofected cells were seeded in Matrigel matrix-coated (1:50, Corning) 10 cm dishes and cultured in E8 medium supplemented with Alt-R® HDR Enhancer (#1081072, IDT) and E8 supplement for 24h. On day 2, medium was refreshed to E8 medium with E8 supplement and cells were cultured until colonies were observed. Multiple clones (~25) were picked, frozen and made into a pellet for subsequent processing. After proteinase K treatment, DNA was extracted from these cell pellets and, following PCR and purification, Sanger sequencing was performed to confirm successful gene editing. Clones were screened for correct gene editing using Chromas Lite and ICE analysis software (Synthego, Menlo park, CA). For sequencing data including the repaired mutation, see Table S4.

Fiji (ImageJ) quantification

Fluorescence images obtained from protamine sulfate experiments were analysed using custom made macros in the free open-software Fiji/ImageJ (see section data availability). First, the region of interest (ROI), was automatically determined through selection of *NPHS1*⁺ area (podocytes) using a readily available Fiji threshold algorithm. The same area selection algorithm was used for all raw images. Second, mean phalloidin intensity was measured in *NPHS1*⁺ areas to automatically determine changes in cytoskeleton arrangement (cytoplasmic contraction). As an internal quality control for automatic threshold selection, images containing the respective ROI selection

were automatically created for post-processing analysis to ensure correct ROI selection in all conditions.

Statistical analysis

All data are expressed as mean $\pm$ s.d. of three independent experiments, unless stated otherwise. Statistical analysis was performed using one-way ANOVA analysis followed by Tukey post-test or, when appropriate, an unpaired t-test with GraphPad Prism version 9 (La Jolla, CA). One asterisk was used to indicate significance with $p < 0.05$, whereas two asterisks were used to indicate significance with $p < 0.01$.

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Author Contributions

J.J., M.F.S. and B.S. designed the study. J.J., B.T.B., M.B. and K.C.R cultured the iPSC-derived kidney organoids. J.J., B.T.B., M.B., GDG, F.M. Q.N., R.W and H.M. performed tissue processing, IF stainings, Western Blotting as well as RNAscope experiments. B.W. carried out electron microscopy analysis and H.D. advised on EM data analysis. D.D., M.K., R.R., A.A. performed 3D FIB-SEM analysis. E.S., T.N., R.J.M., J.F.M.W., J.V. contributed tissue specimens and plasma material. C.K. performed data acquisition experiments for single cell sequencing. C.K. and R.K. supported single cell sequencing. M.B. carried out the single cell sequencing data analyses, and was assisted by J.S.N. and I.G.C.. N.E. and V.D. performed super-resolution imaging. J.J., B.T.B. and M.B. arranged the Figures. J.J., B.T.B., M.B, M.F.S. and B.S. wrote the manuscript. J.F.M.W., N.K., R.J.M., T.N., J.V. K.C.R, C.K., A.A. and R.K. edited the manuscript and advised on data analysis and interpretation. All authors read and approved the final manuscript.

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Declaration of Interests

The authors declare no competing interests.

Data availability

The scRNAseq dataset is publicly available from Gene Expression Omnibus (GEO⁸³) with GEO Series accession number GSE181954. All scripts and codes used for data analyses are deposited on Zenodo and publicly available via <http://doi.org/10.5281/zenodo.5156000>.

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Supplementary Files and Data

The full supplementary information (including the supplemental videos) is accessible online via:

<https://journals.biologists.com/dev/article/149/9/dev200198/275303/>

Human-pluripotent-stem-cell-derived-kidney

Table S1. Top 20 differentially expressed (DE) genes based on adjusted p-value

	PT/LH/DT	Podocyte precursor	Podocyte subcluster1	Podocyte subcluster2	Podocyte subcluster3	Loop progenitor	DT/CD	EC precursor	Mesangial precursor	Stroma1	Stroma2	Stroma3	Stroma4	Stroma5	Stroma/neural progenitor	Stroma/neuron	Neuron/glat
1	IRX1	WT1	NPHS2	CLIC5	ITGA3	KIF20A	LBX1	CD34	GATA3	CTSC	CD24	ADCVAP1	ISL1	MEOX2	CD81	CLSPN	ZIC2
2	IRX2	MAFB	SOST	PTPRO	PTPRO	DLGAP5	PITX2	PLVAP	NEFM	COL3A1	ALX4	CYTL1	OSR2	DLX2	COL3A1	XRCC2	SOX2
3	MAB21L2	GADD45A	TCF21	SOST	PODXL	CENPA	POU4F1	EBF3	ANGPT1	SULT1E1	PRRX1	CNTN3	NEFM	DLX1	COL1A2	TYMS	PAX3
4	RFLNA	NPHS2	PODXL	WT1	NPHS1	CENPE	TMEM108	VCAM1	FLRT3	CRABP1	COL21A1	PRSS12	SIM2	RALYL	MT-ND1	UHRF1	REF4
5	CLDN11	MDK	PTPRO	SBSPPON	PLA2R1	KNL1	GATA3	CRABP1	VCAN	CHST2	NOVA1	TAC1	ZNF385D	PALMD	HNFB	CENPU	CACNG8
6	MECOM	MT-CO2	CLIC5	PODXL	ENPEP	CD20	GRIA4	IFITM3	FN1	NAV3	CXCL14	VEGFC	HAND2	FSTL5	LAPTM4A	DTL	POU3F2
7	CXCL12	MARCKS	WT1	NPHS2	ST3GAL6	GTSE1	MYF6	COL12A1	PDGFC	STMN2	MAB21L2	CRABP1	SLC26A7	MGP	SFRP2	FAM111B	ZIC5
8	PTN	FTH1	DDN	NPHS1	NPHS2	HMMR	LYPD1	COL6A3	PTH1R	S100A10	MAB21L1	MEIS2	CHRM2	TNMD	HSP90B1	ORC6	CHL1
9	SIX2	MT-ATP6	AQP3	AQP3	AC008264.2	TACC3	CAMK2N1	LAMA4	FHL1	NDST3	ALDH1A2	TMSB4X	TTN	IFI44L	GAS1	RAD51AP1	SMOC1
10	USH1C	KCNQ10T1	ENPEP	TCF21	CLIC5	PLK1	GATA3-AS1	CD99	AC060834.2	PENK	POSTN	FAT4	GUCY1A1	HPGD	FBLN1	MYBL2	MSX1
11	COL2A1	MT-CO3	SBSPPON	DDN	SPOCK2	NUF2	OLFML2B	PCOLCE	TNMD	MEIS2	ALX1	CDH2	CPE	TRPS1	PDGFRA	PCLAF	PHYHIP1L
12	COL9A2	SOX4	PLA2R1	VAMP8	SBSPPON	ASPM	PDGFC	HMG2	MEOX2	BCHE	CSRP2	MIR137HG	NRP2	MXK	MT-CVB	GIN52	PI15
13	EGFL6	MT-ND4	ST3GAL6	ST3GAL6	SOST	CNBB2	LIN7A	USH1C	TMSB4X	ACR3	LVRN	SPOCK3	TBX2	COL11A1	CPE	ATAD5	MAP6
14	ERG	MT-ND1	SPOCK2	ENPEP	VEGFA	CKAP2L	SNCA	DDR2	FAM110B	TPM1	FAM196A	USH1C	SORCS1	FIBIN	MT-CO3	EZF1	LMX1A
15	PLD5	MT-CO1	VAMP8	MPP5	WT1	KIF4A	FLRT3	BTBD3	BCHE	TWIST1	LUM	RPL3	CCDC141	CSMD3	MDK	MCMT10	TFAP2B
16	TSPAN13	CRABP1	CLDN5	CLDN5	PRODH2	TROAP	SHISA2	SERPINH1	MDK	COL6A3	PRRX2	RPL10	NRXN1	C3orf80	CALR	CDC45	GDF7
17	MIA	ANXA1	NPHS1	SPOCK2	MPP5	TOP2A	PTPRZ1	ROBO2	NR2F1	KLHL4	SOX11	RPL13	IGFBP3	RGCC	MT-ATP6	CENPK	RAB3C
18	CYP1B1	AIF1	MRGPRF	ARHGAP29	PTPRQ	MKI67	EGFLAM	TPM1	KIF26B	PARM1	TBX3	HGF	COL21A1	THBS4	IL11RA	CDC6	SATB2
19	SOX5	TPM2	STON2	PLA2R1	PLTP	KIF2C	CRABP1	CHST2	ADAMTS6	SLIT2	PCDH9	RPL10A	SIM1	RUNX1T1	FSTL1	MCMT4	TTYH1
20	FAT3	COL3A1	TPPP3	GADD45A	MAGI2	BIRC5	ADAMTS6	EDNRA	CREB5	MAB21L2	PCDH10	RPL30	PDZRN3	PTGFR	APP	FANCD2	WSCD2

CD, collecting duct; DT, distal tubule; EC: endothelial cells; LH, loop of Henle; PT, proximal tubule.

Table S2. Antibodies used for immunofluorescence staining.

Primary antibody	Secondary antibody
Human Nephtrin Antibody (AF4269, R&D Systems)	Donkey anti-sheep Alexa Fluor™ 647 (A21448, Thermo Fisher)
Anti-Podocin antibody (P0372, Sigma Aldrich)	Donkey anti-rabbit Alexa Fluor™ 568 (A10042, Thermo Fisher)
Anti-Human WT1 clone 6F-H2 (M3561, Dako)	Donkey anti-mouse Alexa Fluor™ 488 (A21202, Thermo Fisher)
Anti-synaptopodin antibody (65194, Progen)	Donkey anti-mouse Alexa Fluor™ 488 (A21202, Thermo Fisher)
Monoclonal Anti-PLA2R antibody (AMAB90772, Sigma Aldrich)	Donkey anti-mouse Alexa Fluor™ 488 (A21202, Thermo Fisher)
Human CD31/PECAM-1 Antibody (BBA7, R&D Systems)	Donkey anti-mouse Alexa Fluor™ 488 (A21202, Thermo Fisher)
PV-1 (PLVAP) antibody (HPA002279, Sigma-Aldrich)	Donkey anti-rabbit Alexa Fluor™ 568 (A10042, Thermo Fisher)
VE-cadherin (F-8) AC (sc-9989 AC, Santa Cruz Biotechnology)	Donkey anti-mouse Alexa Fluor™ 647 (A31571, Thermo Fisher)
Lotus Tetragonolobus Lectin (LTL) biotinylated (B-1325, Vector Laboratories)	Streptavidin, Alexa Fluor™ 405 conjugate (S32351, Thermo Fisher)
Anti-E-Cadherin clone 36 (610181, BD Biosciences)	Donkey anti-mouse Alexa Fluor™ 488 (A21202, Thermo Fisher)
GATA-3 (D13C9) monoclonal Antibody (5852, Cell Signaling)	Donkey anti-rabbit Alexa Fluor™ 647 (A31573, Thermo Fisher)
Flash Phalloidin™ Green 488 (424201, Biolegend)	n.a.
Anti-EHD3 antibody (HPA049890, Sigma-Aldrich)	Donkey anti-rabbit Alexa Fluor™ 568 (A10042, Thermo Fisher)

Table S3. Antibodies used for western blotting.

Primary antibody	Secondary antibody
Anti-Nephtrin (phosphor Y1176 + Y1193) Antibody (ab80299, Abcam)	Goat anti-rabbit, Alexa Fluor™ 680 (A-21076, Thermo Fisher)
Anti-Nephtrin antibody (sc-19000, Santa Cruz)	Donkey anti-goat, Alexa Fluor™ 680 (A-21084, Thermo Fisher)
Anti-γ-Tubulin antibody (T6557, Sigma)	Donkey anti-mouse, DyLight 800 (SA5-10172, Thermo Fisher)

Table S4. iPS-NPHS2 mutant CRISPR sequencing data**Sequencing data before CRISPR repair (NPHS2 exon 3):**

CAAGAGTATGAAAGAGTAATTATATTTCC**A**ACTGGGACATCTGCTTCCTGGAAGAGCCAAAGGCCCTGGT
 AAAAAAACACTCTTTTTTTTCTAAACACCTCTCTCCTGACTTGCCAATTTCTTCAACCCATGCAGATTT
 GTAATATGGACCTCAGATTAATGAAGTAACCTTGATTTCATGATATCTGAATTTTCCAATCTGTTACTTAT
 AGGTTATTCAAATATTCTTCAGAGACTATTACTACTAGGTCATAGGTAGCCAAGAGAGAGAATTGGTA
 CAGAGAGCCCATGATGCCAGGGCAAGGCTTGCTGGAATAGCAAGTTAGCTTAGGACCAATGGCTGGGG
 ACTGATTTGAGTACGATGTGTATATGGTCAGAGTCTGTGAATTTCTCAGAGAAAAGCTGAGCTAGTTT
 CCACTCCAGGTGCTCAGATCCAATCATGAGAAACAGGCAAAGTTCAGCATTCAAATAACAAGTTGC
 TCTTCAGTTATAGGGATTTAAAAAACATAGATTAAGCATTTCTTGTGGCTCA

Sequencing data after CRISPR repair (NPHS2 exon 3):

CAAGAGTATGAAAGGGTTATTATATTTCC**G**ACTGGGACATCTGCTTCCTGGAAGAGCCAAAGGCCCTG
 GTAAAAAACACTCTTTTTTTTCTAAACACCTCTCTCCTGACTTGCCAATTTCTTCAACCCATGCAGA
 TTTGTAATATGGACCTCAGATTAATGAAGTAACCTTGATTTCATGATATCTGAATTTTCCAATCTGTTAC
 TTATAGGTTATTCAAATATTCTTCAGAGACTATTACTACTAGGTCATAGGTAGCCAAGAGAGAGAATT
 GGTACAGAGAGCCCATGATGCCAGGGCAAGGCTTGCTGGAATAGCAAGTTAGCTTAGGACCAATGGC
 TGGGGACTGATTTGAGTACGATGTGTATATGGTCAGAGTCTGTGAATTTCTCAGAGAAAAGCTGAGCT
 AGTTCACCTCCAGGTGCTCAGATCCAATCATGAGAAACAGGCAAAGTTCAGCATTCAAATAACAA
 GTTGCTCTTCAGTTATAGGGATTTAAAAAACATAGATTAAGCATTTCTTGTGGCTCA

RED = sgRNA, *Cursive* = Homology Directed Repair (HDR) template, **BOLD** = mutation (edit)

The HDR Template contains silent mutations in the protospacer adjacent motif (PAM) region in order to prevent re-cutting after successful editing.

Original sequence:

CAAGAGTATGAAAGAGTAATTATATTTCCAACTGGGACATCTGCTTCCTGGAAGAGCCAAAGGCCCTG
 GTAAAAAACACTCTTTTTTTTCTAAACAC

Sequence after successful editing and incorporation of silent mutations:

CAAGAGTATGAAAGGGTTATTATATTTCCGACTGGGACATCTGCTTCCTGGAAGAGCCAAAGGCCCT-
 GTAAAAAACACTCTTTTTTTTCTAAACAC

Table S5. Reagent and resource table

Reagent or Resource	Source	Identifier
Antibodies		
anti-human nephrin, polyclonal sheep IgG	R&D Systems	Cat#AF4269
anti-human nephrin, polyclonal guinea pig	Progen	Cat#GP-N2
anti-human podocin, polyclonal rabbit IgG	Sigma Aldrich	Cat#P0372
anti-human WT1, monoclonal mouse IgG1	Dako	Cat#M3561
anti-human synaptopodin, monoclonal mouse IgG1	Progen Biotechnik	Cat#65194
anti-human PLA2R, monoclonal mouse IgG1	Sigma Aldrich	Cat#AMAB90772
anti-human CD31/PECAM-1, monoclonal mouse IgG1	R&D Systems	Cat#BBA7

Table S5. Continued

Reagent or Resource	Source	Identifier
anti-human PLVAP, polyclonal rabbit IgG	Sigma Aldrich	Cat#HPA002279
anti-human VE-cadherin, monoclonal mouse IgG1	Santa Cruz Biotechnology	Cat#sc-9989
biotinylated lotus tetragonolobus lectin (LTL)	Vector Laboratories	Cat#B1325
anti-human E-cadherin, monoclonal mouse IgG2a	BD Biosciences	Cat#610181
anti-human GATA-3, monoclonal rabbit IgG	Cell Signaling	Cat#5852
Flash Phalloidin™ Green 488	Biolegend	Cat#424201
anti-human EHD3, polyclonal rabbit IgG	Sigma Aldrich	Cat#HPA049890
anti-human nephrin (phospho Y1176/Y1193), monoclonal rabbit IgG	Abcam	Cat#ab80299
anti-human nephrin, polyclonal goat IgG	Santa Cruz Biotechnology	Cat#sc-1900
anti-human γ -tubulin, monoclonal mouse IgG	Sigma Aldrich	Cat#T6557
Alexa Fluor donkey anti-sheep IgG 647 (H+L)	Thermo Fisher	Cat#A21448
AffiniPure anti-guinea pig IgG Cy3 (H+L)	Jackson ImmunoResearch Laboratories Inc.	Cat#706-165-148
Alexa Fluor donkey anti-rabbit IgG 647 (H+L)	Thermo Fisher	Cat#A31573
Alexa Fluor donkey anti-rabbit IgG 568 (H+L)	Thermo Fisher	Cat#A10042
Alexa Fluor donkey anti-mouse IgG 647 (H+L)	Thermo Fisher	Cat#A31571
Alexa Fluor donkey anti-mouse IgG 488 (H+L)	Thermo Fisher	Cat#A21202
Streptavidin Alexa Fluor 405 conjugate	Thermo Fisher	Cat#S32351
Alexa Fluor goat anti-rabbit IgG 680	Thermo Fisher	Cat#A-21076
Alexa Fluor donkey anti-goat IgG 680	Thermo Fisher	Cat#A-21084
Donkey anti-mouse IgG DyLight 800 (H+L)	Thermo Fisher	Cat#SA5-10172
Chemicals, Peptides, and Recombinant Proteins		
Essential 8 FLEX Medium	Thermo Fisher	Cat#A2858501
Essential 6 Medium	Thermo Fisher	Cat#A1516401

Table S5. Continued

Reagent or Resource	Source	Identifier
UltraPure EDTA (0.5M) pH 8.0	Thermo Fisher	Cat#15575020
Geltrex LDEV-free, hESC-Qualified, reduced growth factor basement membrane matrix	Thermo Fisher	Cat#A1413302
Antibiotic-Antimycotic	Thermo Fisher	Cat#15240062
CHIR 99021	R&D Systems	Cat#4423/10
Recombinant Human FGF-9 Protein	R&D Systems	Cat#273-F9-025
Heparin sodium salt from porcine intestinal mucosa	Sigma Aldrich	Cat#H4784
Recombinant Human BMP-7 Protein	R&D Systems	Cat#354-BP-010
Human Activin A	Milteyni Biotec	Cat#130-115-010
Retinoic acid	Sigma Aldrich	Cat#2625-100MG
Human VEGF ₁₆₅	R&D Systems	Cat#293-VE-010/CF
MEM Non-Essential Amino Acids Solution	Thermo Fisher	Cat#11140050
TrypLE Select Enzyme, no phenol red	Thermo Fisher	Cat#12563-029
RevitaCell Supplement	Thermo Fisher	Cat#A2644501
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher	Cat#25300054
Corning® Transwell® polyester membrane cell culture inserts, 24 mm Transwell with 0.4 µm pore polyester membrane insert, TC-treated, sterile	Sigma Aldrich	Cat#CLS3450
Corning® Falcon® 96-well, clear flat bottom, TC-treated, sterile imaging microplate	Sigma Aldrich	Cat#353219
DMEM/F-12	Thermo Fisher	Cat#11320074
Human EGF	Sigma Aldrich	Cat#E9644
Protamine sulfate	Sigma Aldrich	Cat#P3369
Insulin-transferrin-sodium selenite media supplement	Sigma Aldrich	Cat#I1884-1VL
Penicillin-Streptomycin (5000 U/mL)	Thermo Fisher	Cat#15070063
Fetal Bovine Serum, heat inactivated	Thermo Fisher	Cat#16140071
Accutase® solution	Sigma Aldrich	Cat#A6964
PPACK dihydrochloride	Santa Cruz Biotechnology	Cat#sc-201291

Table S5. Continued

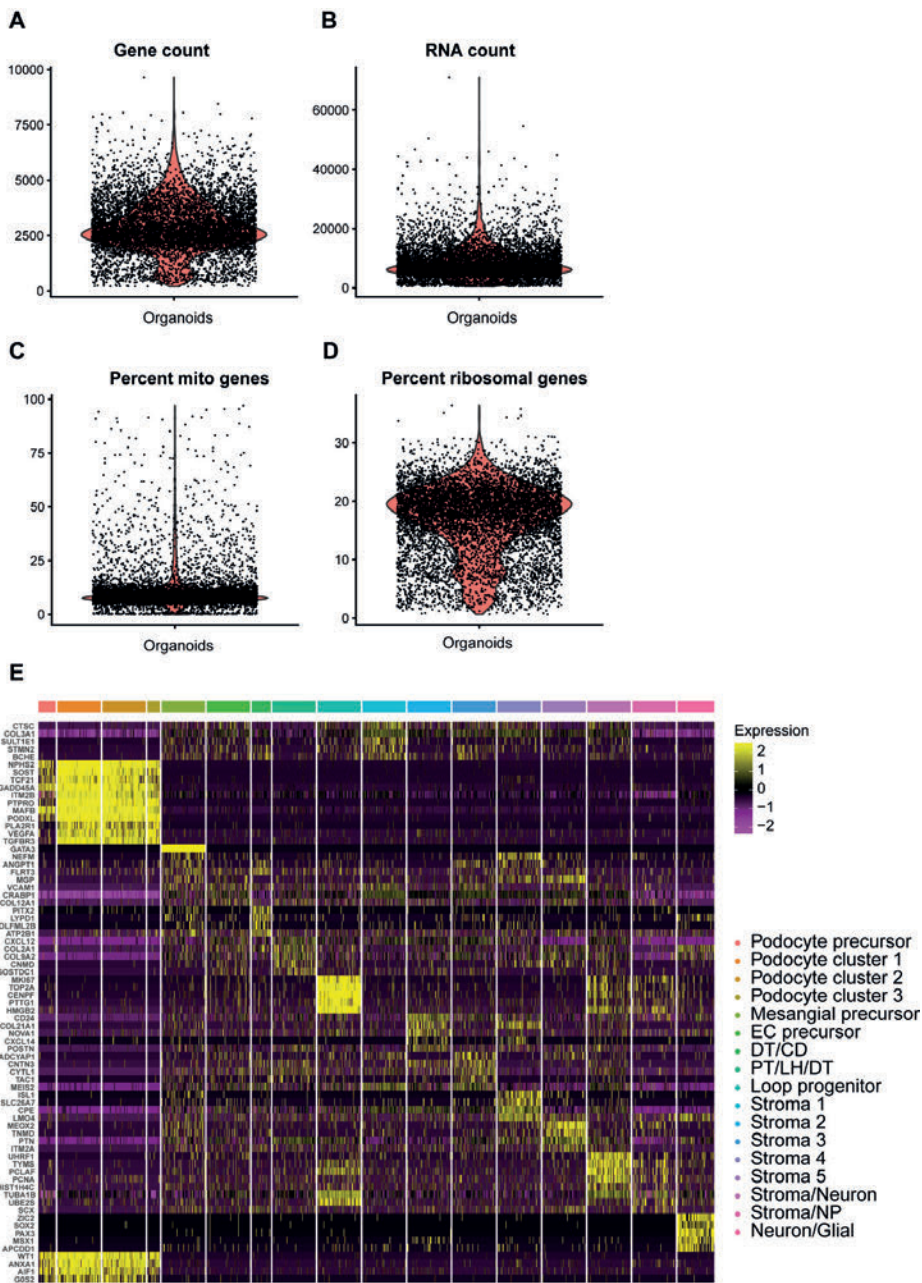
Reagent or Resource	Source	Identifier
Puromycin Aminonucleoside	MedChemExpress	Cat#HY-15695
Critical Commercial Assays		
PureLink RNA mini kit	Thermo Fisher	Cat#12183018A
PSC Cryopreservation Kit	Thermo Fisher	Cat#351-FS-050
RNAscope® Multiplex Fluorescent Reagent Kit v2 assay	Advanced Cell Diagnostics	Cat#323100
RNAscope® probe - Hs-COL4A3	Advanced Cell Diagnostics	Cat#4461861
RNAscope® probe - Hs- PECAM1-01-C2	Advanced Cell Diagnostics	Cat#487381-C2
RNAscope® probe - Hs-NPHS1-C3	Advanced Cell Diagnostics	Cat#416071-C3
RNAscope® 3-Plex negative control probe	Advanced Cell Diagnostics	Cat#320871
Chromium Next GEM Single Cell 3' Kit v3.1, 16 rxns	10x Genomics	Cat#1000268
Dual Index Kit TT Set A, 96 rxns	10x Genomics	Cat#1000215
Chromium Next GEM Chip G Single Cell Kit, 16 rxns	10x Genomics	Cat#1000127
Library Construction Kit 16 rxns	10x Genomics	Cat#1000190
NovaSeq 6000 S2 Reagent Kit v1.5 (100 cycles)	Illumina	Cat#20028316
Deposited Data		
scRNAseq data	This paper; deposited on Gene Expression Omnibus (GEO)	GSE181954: https:// www.ncbi.nlm.nih. gov/geo/query/acc. cgi?acc=GSE181954
Scripts and codes for data analysis	This paper; deposited on Zenodo	http://doi.org/10.5281/ zenodo.5156000
Experimental Models: Cell Lines		
Human induced pluripotent stem cell line iPS 15	SCTC Radboud UMC, The Netherlands	iPS 15
Human induced pluripotent stem cell line iPS 134	iPS core facility Erasmus MC, The Netherlands	iPS 134
Human induced pluripotent stem cell line iPS 19	SCTC Radboud UMC, The Netherlands	iPS19_91
Conditionally immortalized human podocyte cell line	Prof. Saleem, Bristol, UK	ciPOD

Table S5. Continued

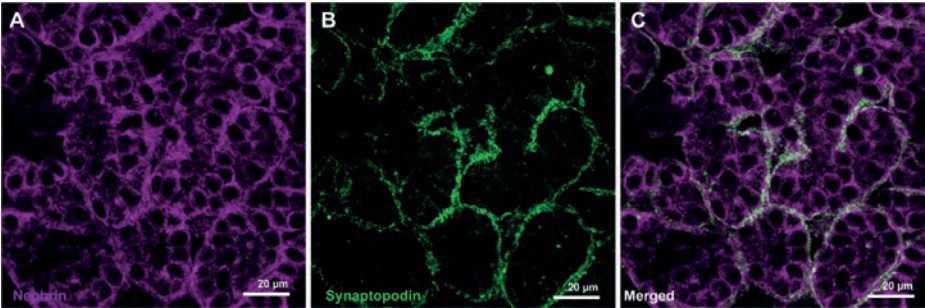
Reagent or Resource	Source	Identifier
Software and Algorithms		
ImageJ version Fiji 1.51n	National Institutes of Health, USA	https://imagej.nih.gov/ij/
ZEN 3.0 blue edition	Carl Zeiss	https://www.zeiss.de/mikroskopie/produkte/mikroskopsoftware/zen.html
Cell Ranger version 3.1.0	10x genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/release-notes/2-1
Seurat version 4.0.3	Hao and Hao et al. Integrated analysis of multimodal single-cell data. <i>Cell</i> (2021)	https://satijalab.org/seurat/
R 4.1.0	CRAN	https://www.R-project.org
Adobe InDesign CC2021	Adobe Systems Inc.	https://www.adobe.com/nl/products/indesign.html
Adobe Illustrator CC 2021	Adobe Systems Inc.	RRID:SCR_010279
Adobe Photoshop CC 2021	Adobe Systems Inc.	RRID:SCR_014199
GraphPad Prism version 9.0	GraphPad Software Inc.	RRID:SCR_002798
clusterProfiler 4.0.2	Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, Feng T, Zhou L, Tang W, Zhan L, Fu X, Liu S, Bo X, Yu G (2021). "clusterProfiler 4.0: A universal enrichment tool for interpreting omics data." <i>The Innovation</i> , 2(3), 100141. doi: 10.1016/j.xinn.2021.100141.	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html
Harmony 0.1.0	Korsunsky, I., Millard, N., Fan, J. et al. Fast, sensitive and accurate integration of single-cell data with Harmony. <i>Nat Methods</i> 16, 1289–1296 (2019). https://doi.org/10.1038/s41592-019-0619-0	https://portals.broadinstitute.org/harmony/articles/quickstart.html#session-info-1
PROGENy 1.14.0	Schubert, M., Klinger, B., Klünemann, M. et al. Perturbation-response genes reveal signalling footprints in cancer gene expression. <i>Nat Commun</i> 9, 20 (2018). https://doi.org/10.1038/s41467-017-02391-6	https://saezlab.github.io/progeny/

Table S5. Continued

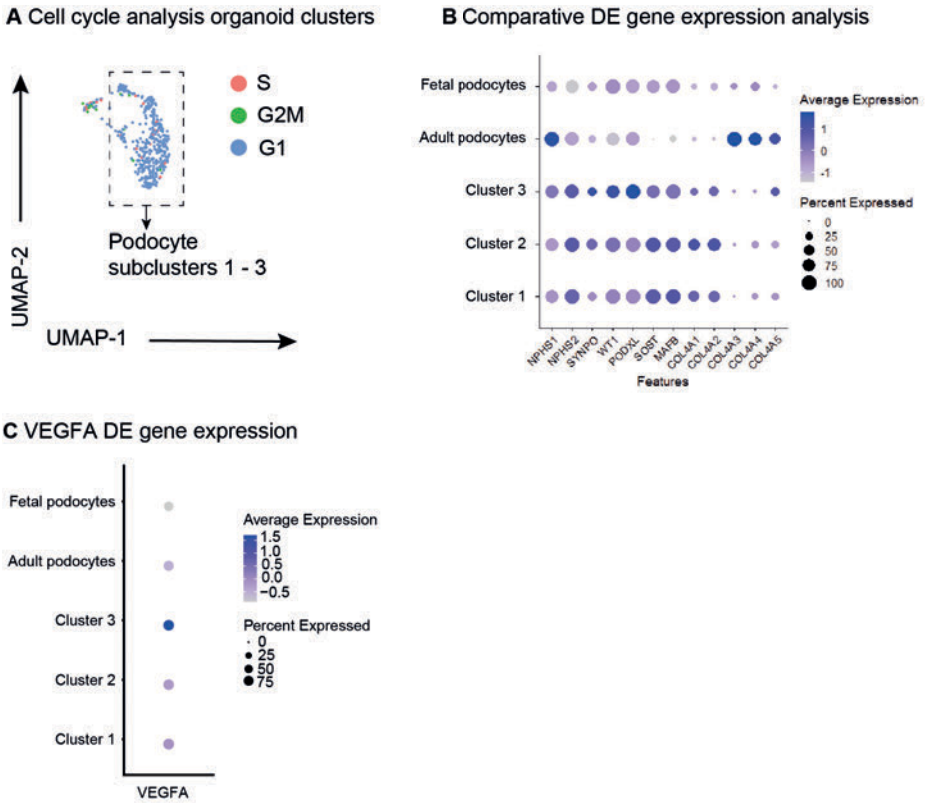
Reagent or Resource	Source	Identifier
DoRothEA 1.4.1	Garcia-Alonso L, Iorio F, Matchan A, et al. Transcription Factor Activities Enhance Markers of Drug Sensitivity in Cancer. <i>Cancer Research</i> . 2018 Feb;78(3):769-780. DOI: 10.1158/0008-5472.can-17-1679.	https://saezlab.github.io/dorothea/index.html
Pheatmap 1.0.12	Raivo Kolde (2019). Pheatmap: Pretty heatmaps.	https://CRAN.R-project.org/package=pheatmap
CrossTalker 1.0.2	CrossTalker: Analysis and Visualisation of Ligand Receptor Networks James S. Nagai, Nils B. Leimkühler, Rebekka K. Schneider, Ivan G. Costa bioRxiv 2021.01.20.427390; doi: https://doi.org/10.1101/2021.01.20.427390	https://github.com/CostaLab/CrossTalker
CellPhoneDB 2.0.5	Single-cell reconstruction of the early maternal-fetal interface in humans. Vento-Tormo R, Efremova M, et al., <i>Nature</i> . 2018 Nov;563(7731):347-353. doi: 10.1038/s41586-018-0698-6	https://www.cellphonedb.org/
msigdb 7.2.1	Arthur Liberzon, Aravind Subramanian, Reid Pinchback, Helga Thorvaldsdóttir, Pablo Tamayo, Jill P. Mesirov, Molecular signatures database (MSigDB) 3.0, <i>Bioinformatics</i> , Volume 27, Issue 12, 15 June 2011, Pages 1739–1740, https://doi.org/10.1093/bioinformatics/btr260	https://cran.r-project.org/web/packages/msigdb/index.html
DESeq2 1.32.0	Love, M.I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. <i>Genome Biol</i> 15, 550 (2014). https://doi.org/10.1186/s13059-014-0550-8	https://bioconductor.org/packages/release/bioc/html/DESeq2.html



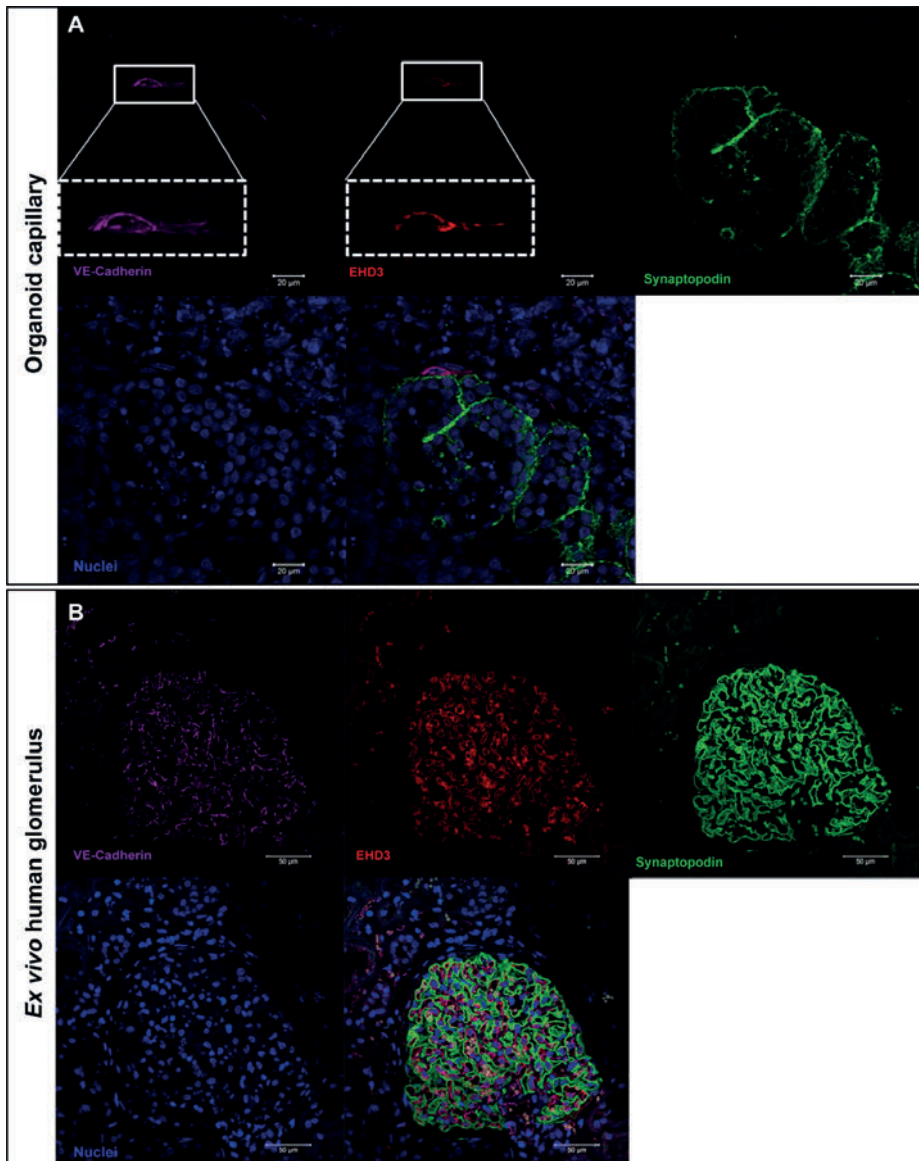
Supplemental figure 1, related to figure 1. Quality control data of scRNA seq data.(A) Number of genes, (B) total number of RNA molecules, percentage of (C) mitochondrial and (D) ribosomal genes in the organoids sample. (E) Heatmap showing a selection of 5 out of the top 20 differentially expressed (DE) genes per cluster. The full list of the top 20 DE genes per cluster is available in Table S1. CD, collecting duct; DT, distal tubule; EC, endothelial cells; LH, loop of Henle; PT, proximal tubule.



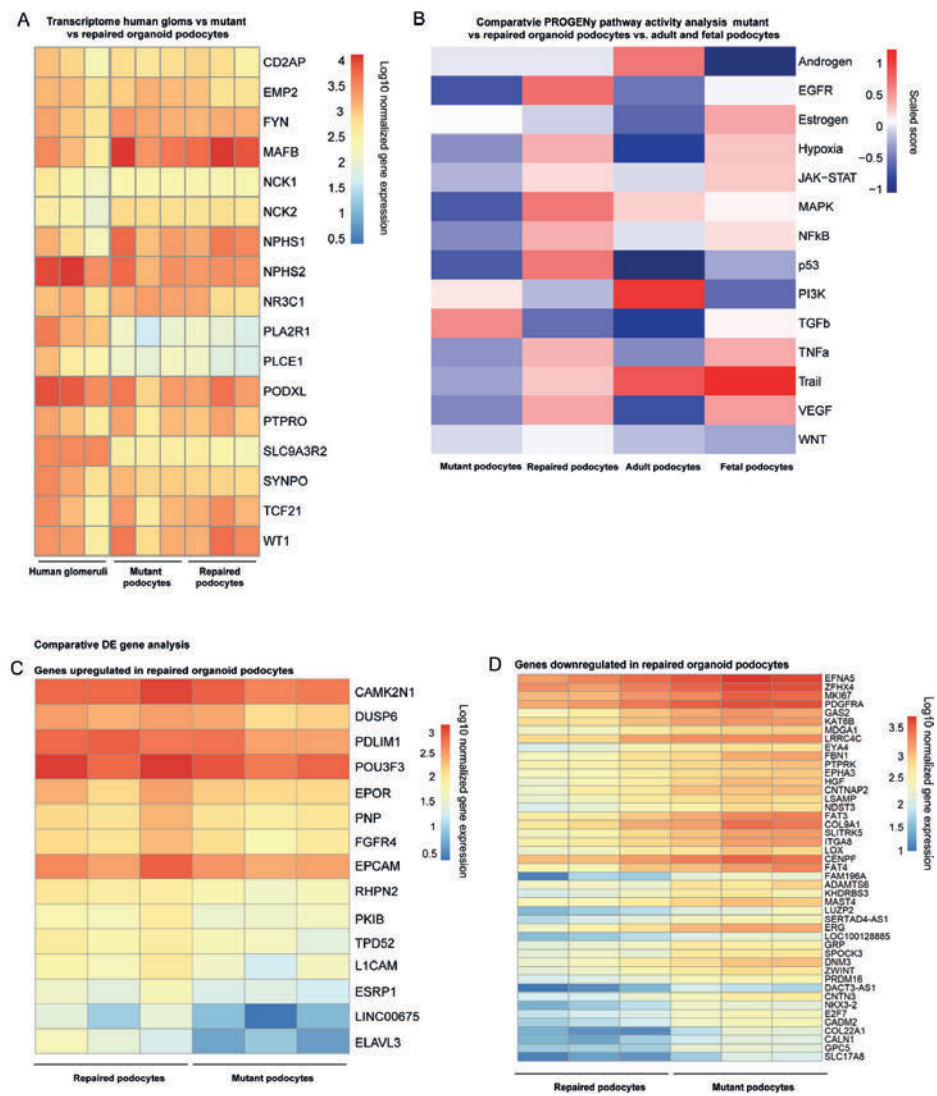
Supplemental figure 2, related to figure 1. Protein expression and localization of specific podocyte markers in kidney organoids. (A) Nephtrin (magenta) and (B) synaptopodin (green) expression in organoid podocytes (C, merged).



Supplemental figure 3, related to figure 2. Cell cycle analysis of the podocyte sub-clusters, comparative DE marker genes and comparative VEGFA expression of organoid podocyte sub-clusters, human fetal and adult podocytes. DE, differentially expressed.

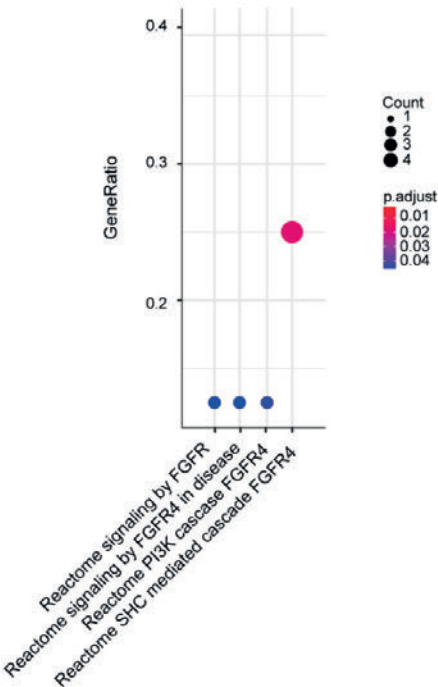


Supplemental figure 4, related to figure 2. Glomerular endothelial EHD3 expression in organoids and in human glomeruli. (A) VE-cadherin (magenta) and EHD3 (red) expression co-localized in an endothelial capillary in a kidney organoid, adjacent to synaptopodin expressing (green) podocytes. (B) VE-cadherin (magenta) and Eps15 Homology Domain-containing 3 (EHD3, red) expression co-localized in glomerular endothelium EHD3, in close proximity to podocytes as shown by synaptopodin expression.

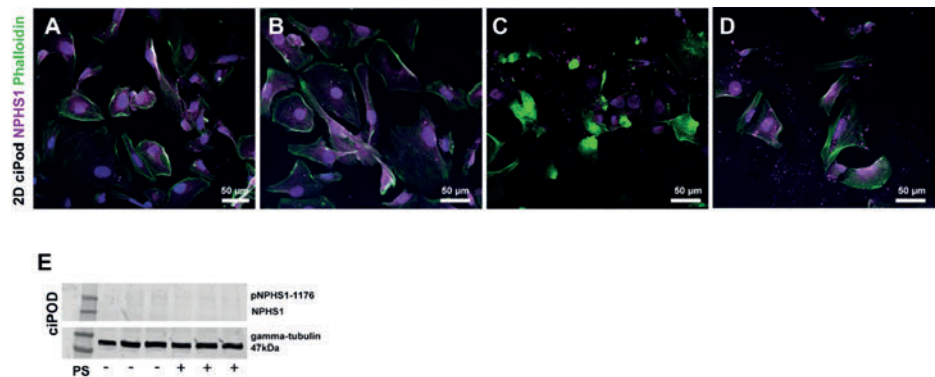


Supplemental figure 5, related to figure 4. Comparative RNA sequencing analysis of kidney organoid NPHS2 mutant podocytes, repaired organoid podocytes and normal human glomeruli-derived podocytes. (A) Comparative bulk transcriptomics of podocyte specific genes in mutant and repaired organoid podocytes versus isolated human glomeruli. (B) Comparative PROGENy pathway activity analysis mutant and repaired organoid podocytes versus adult and fetal podocytes. Heatmap showing the enriched up- (C) and downregulated (D) genes in NPHS2-repaired organoid-derived podocytes compared to mutant organoid-derived podocytes.

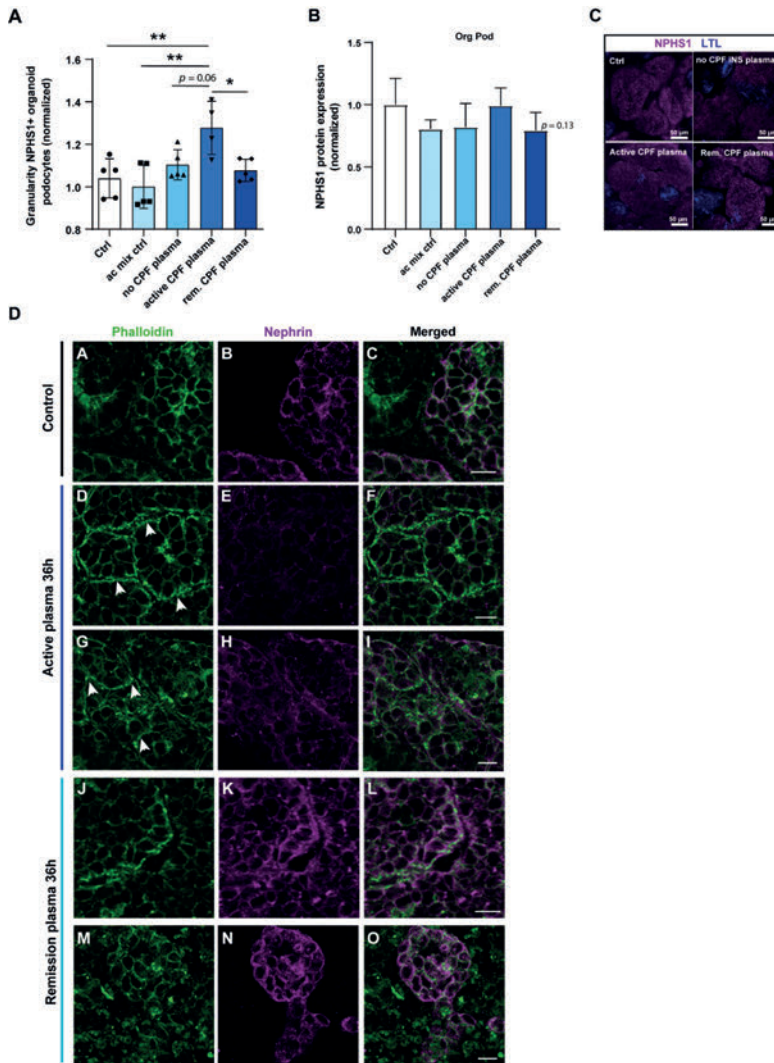
Comparative MSigDb C2 analysis - reactomes enriched in repaired organoid podocytes



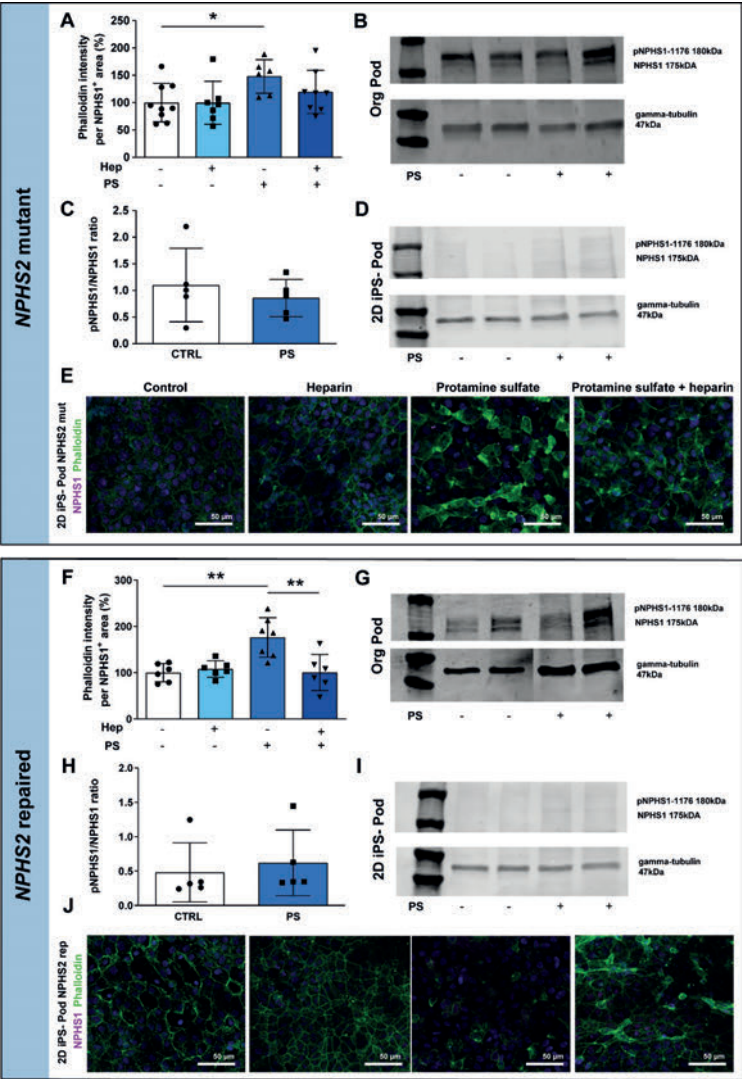
Supplemental figure 6, related to figure 4. Comparative Molecular Signature database Analysis C2 showing enhanced FGFR reactomes in repaired organoid podocytes.



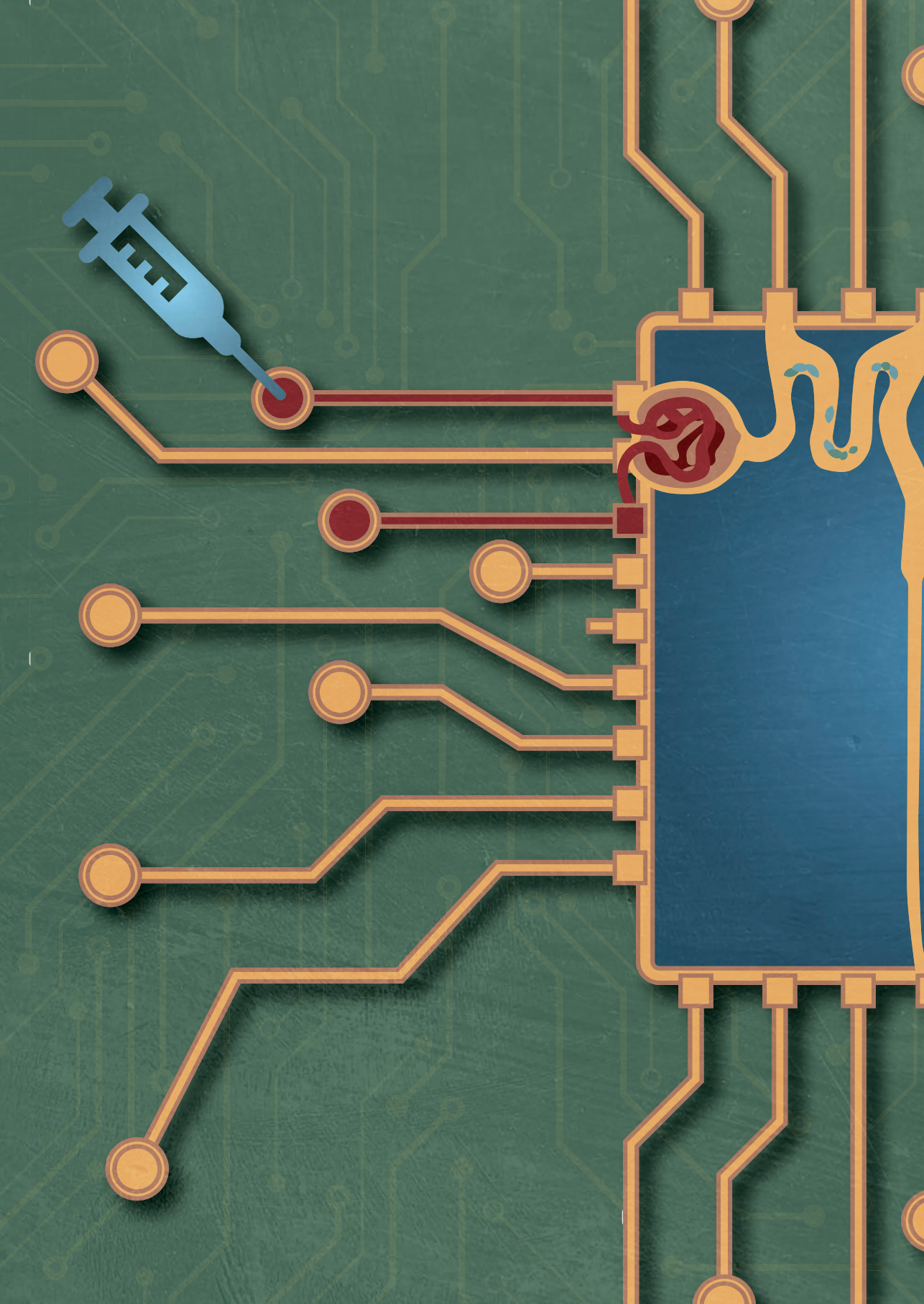
Supplemental figure 7, related to figure 5. Podocyte cytoskeleton analysis and slit diaphragm activation measurements in ciPODs following protamine sulfate treatment.(A-D) Phalloidin (green), NPHS1 (magenta)) of protamine sulfate-treated and heparin rescued ciPODs. (E) pNPHS1 and NPHS1 protein expression analysis of protamine sulfate-treated ciPODs. Gamma tubulin was used as a reference protein.



Supplemental figure 8, related to figure 5. Podocyte cytoskeleton analysis and slit diaphragm activation measurements in mutant and repaired kidney organoids. Quantification of phalloidin intensity in protamine sulfate-treated and heparin-rescued podocytes in (A) mutant and (F) repaired kidney organoids. $*=p<0.05$, $**=p<0.01$, using one-way ANOVA analysis followed by Tukey post-test. Data are presented as mean $\pm$ s.d. from three independent experiments using at least two biological replicates per conditions. pNPHS1 and NPHS1 protein expression analysis of protamine sulfate-treated (B) mutant and (G) repaired kidney organoids. Western blotting quantification pNPHS1/NPHS1 ratio of protamine sulfate-treated (C) mutant and (H) repaired kidney organoids. Data are presented as mean $\pm$ s.d. from three independent experiments using at least two biological replicates per conditions. pNPHS1 and NPHS1 protein expression analysis of protamine sulfate-treated 2D (D) mutant and (I) repaired iPSC-derived podocytes. Gamma tubulin was used as a reference protein. Podocyte cytoskeleton analysis (phalloidin (green)) in (E) mutant and (J) repaired 2D iPSC-derived podocytes (NPHS1 (magenta)) following protamine sulfate treatment and heparin rescue.



Supplemental figure 9. Podocyte injury analysis following CPF-containing and -free plasma after 4h and 36h. (A) Cytoplasmic granularity analysis in NPHS1-sorted organoid podocytes, isolated from kidney organoids treated with 10% (v/v) iNS plasmapheresis plasma (circulating permeability factor (CPF) free, active CPF and remission CPF plasma), control (E6 medium) and anti-coagulation mix (ac mix ctrl) for 4h (N=2 independent experiments, biological triplicates per condition). * $p < 0.05$, ** $p < 0.01$, using one-way ANOVA analysis followed by Tukey post-test. Data are presented as mean $\pm$ s.d. (B) Flow cytometry analysis of NPHS1 expression in organoid podocytes (Org Pod) treated with 10% (v/v) active and remission CPF plasma, CPF-free plasma, anticoagulation control (Ac mix ctrl) and E6 medium control (Control) for 4h. Data are presented as mean $\pm$ s.d. (C) NPHS1 (magenta) and LTL (blue) expression following 4h plasma treatments in kidney organoids. (D) Podocyte cytoskeleton analysis following CPF-containing (active) and -free (remission) plasma after 36h exposure. Arrowheads indicate actin condensation at the podocyte basement membrane. Scale bars 10 microns.



The podocyte as a direct target of glucocorticoids in nephrotic syndrome

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Abstract

Nephrotic syndrome (NS) is characterized by massive proteinuria; podocyte loss or altered function is a central event in its pathophysiology. Treatment with glucocorticoids is the mainstay of therapy. However, many patients experience one or multiple relapses and prolonged use may be associated with severe adverse effects. Recently, the beneficial effects of glucocorticoids have been attributed to a direct effect on podocytes in addition to the well-known immunosuppressive effects. The molecular effects of glucocorticoid action have been studied using animal and cell models of NS. This review provides a comprehensive overview of different molecular mediators regulated by glucocorticoids, including an overview of the model systems that were used to study them. Glucocorticoids are described to stimulate podocyte recovery by restoring pro-survival signalling of slit diaphragm related proteins and limiting inflammatory responses. Of special interest is the effect of glucocorticoids on stabilizing the cytoskeleton of podocytes, since these effects are also described for other therapeutic agents used in NS, such as cyclosporin. Current models provide much insight, but do not fully recapitulate the human condition since the pathophysiology underlying NS is poorly understood. New and promising models include the glomerulus-on-a-chip and kidney organoids, which have the potential to be further developed into functional NS models in the future.

Introduction

Nephrotic syndrome (NS) is characterized by massive proteinuria, hypoalbuminemia and edema¹. NS can be primary (idiopathic) or secondary to systemic diseases². Idiopathic NS is considered to be a primary podocytopathy, indicating that podocyte loss or altered function is a central event in the development of NS^{1,3}. The podocyte is a highly specialized cell type with little to no ability to regenerate. Together with a fenestrated endothelium and glomerular basement membrane, podocytes are part of the glomerular filtration barrier⁴. Podocytes have a large cell body with many long extensions that branch into so-called foot processes. Between interdigitating foot processes the slit diaphragm is formed, which is the final layer of the glomerular filtration barrier.

For over 60 years, treatment with glucocorticoids, mainly prednisolone, has been the mainstay of therapy for NS⁵. In children, NS is predominantly caused by minimal change NS (MCNS), and approximately 80-90% of patients achieve remission with glucocorticoid treatment^{1,5}. Adult NS is most frequently caused by membranous nephropathy (MN), followed by MCNS and focal segmental glomerulosclerosis (FSGS)². Remission with glucocorticoids is lower in adult patients with MCNS compared to children, with an adult remission rate of approximately 75%⁶. Not all patients with MN or FSGS are treated with glucocorticoids, and when they are used, the remission rate is lower than as seen in MCNS.

Although most patients initially respond to glucocorticoid treatment, a large number of patients will have one or multiple relapses and some may even develop resistance to glucocorticoid therapy^{1,5}. It is not yet possible to predict the response of an individual patient to glucocorticoid treatment, in part due to our limited knowledge on the mechanism of glucocorticoid action in NS. Hence, it is evident that treatment with glucocorticoids is unpredictable in NS. In addition, glucocorticoid use is associated with psychological changes and hypertension, whereas long-term use of glucocorticoids may be associated with obesity and osteoporosis¹. Therefore, more effective treatment options with less side effects are needed for the treatment of NS.

Since the immune system is regarded to be involved in the pathogenesis of NS, the beneficial effects of glucocorticoids have been attributed to their systemic anti-inflammatory and immunosuppressive actions. Both T and B cells have been implicated in the pathogenesis of NS and are likely affected by glucocorticoids to induce recovery. In addition to immune cells, glomerular

cells also express the glucocorticoid receptor and may be directly targeted by glucocorticoids⁷. In support of this hypothesis, podocyte specific knockout of the glucocorticoid receptor in model systems increases disease severity⁸. However, the exact mechanism of glucocorticoid-induced recovery and how this affects podocyte function remains poorly understood. Here, we will review the current knowledge of mechanisms of glucocorticoid action in podocytes. For an overview of glucocorticoid action on the immune system, which will not be addressed in the current review, we refer to an excellent recent review⁹. In addition, we will discuss the different *in vivo* and *in vitro* models that are used to study glucocorticoid action on podocytes and describe promising new models and techniques to further elucidate glucocorticoid action on podocytes.

Models used to study glucocorticoid effect on podocytes

To study the effects of glucocorticoids on podocytes in NS, *in vivo* and *in vitro* models are available, which are summarized in Table 1. It is well-known that *in vivo* models more closely mimic podocyte function and physiology compared to podocytes *in vitro*. Podocytes *in vivo* reside in a three-dimensional environment with a certain matrix stiffness and are in contact with other cell types via paracrine signalling, which are all essential parameters to maintain the filtration barrier. Furthermore, *in vivo* podocytes are subjected to fluid flow and an oxygen/nutrient gradient, conditions that are key for podocyte physiology but generally absent in *in vitro* models. Differences in the parameters mentioned above are responsible for aberrant characteristics of human or murine conditionally immortalized podocytes (ciPODs), which are widely used *in vitro* models^{16,17}. *In vitro* models are vital to study podocyte biology and learn more about specific cellular functions or signalling pathways in human disease. However, ciPOD behaviour and marker expression can differ between laboratories using similar ciPODs or depend on the ciPOD species, which hampers generalizability of such results¹⁸⁻²². Therefore, results from ciPOD studies should always be confirmed *in vivo* and/or using more accurate *in vitro* 3D glomerular models¹⁶. Finally, it is notable that dexamethasone is used in most ciPOD studies, which is known to have a stronger glucocorticoid effect than prednisolone that is generally used in clinics.

Table 1. Overview of model systems with advantages and limitations.

Model system	Advantages	Limitations	Ref
<i>In vivo</i> models, non-immune related podocyte damage			
Adriamycin rat or mouse model	Podocyte injury with foot process effacement and FSGS lesions. Podocyte injury and proteinuria decrease with glucocorticoid treatment.	Does not have albumin-selective proteinuria. Only partially steroid-sensitive. Only certain mice strains are susceptible to injury.	10,11
Protein overload rat model	Podocyte injury with foot process effacement. Podocyte foot process effacement is reversible.	Mechanism of podocyte injury only partially known.	10, 11
Puromycin amino-nucleoside rat model	Podocyte injury with foot process effacement. Podocyte injury and proteinuria decrease with glucocorticoid treatment.	Does not have albumin-selective proteinuria. Only partially steroid-sensitive.	10-12
Zebrafish	Short reproduction cycle for an <i>in vivo</i> system. Easy to perform genetic manipulation.	Podocyte restorative mechanisms present that are absent in humans. Not all genes in humans are also expressed in zebrafish.	13-15
<i>In vivo</i> models, immune related podocyte damage			
Heymann nephritis	Widely studied and therefore a variety of methods are available. Podocyte foot process effacement with GBM thickening.	Closely follows pathophysiology of MN, which is less sensitive to glucocorticoids.	11
LPS mouse model	Podocyte foot process effacement, which is reversible. Podocyte injury is induced by stimulation of TLR4.	Besides podocyte damage, mice experience sepsis and a high lethality is observed. Proteinuria is relatively low. LPS batches differ and each new batch requires pilot experiments to determine the optimal concentration.	10,11
Nephrotoxic serum model	Podocyte injury induced by injecting serum with anti-glomerulus antibodies. Crescentic glomerulonephritis is often induced.	Models crescentic glomerulonephritis which represents only a small population of NS patients with a specific phenotype.	7, 11
<i>In vitro</i>			
Human and murine ciPODs, general	Ability to standardize environment and analyse effects of specific environmental changes. Easy assessment of specific protein or gene function. Can be used in high-throughput assays.	Higher expression of cytoskeleton related proteins compared to podocytes <i>in vivo</i> . Lower expression of slit diaphragm related proteins and podocyte marker proteins compared to podocytes <i>in vivo</i> . Different morphology compared to podocytes <i>in vivo</i> .	16,22

Table 1. Continued

Model system	Advantages	Limitations	Ref
Murine ciPODs, Endlich lab	Stable nephrin expression and specific localization to focal adhesion sites.		59
Murine ciPODs, Mundel lab	Good overall podocyte marker expression on both protein and mRNA level.		60
Human ciPODs, Saleem lab	Human ciPODs, provide a more accurate phenotype in studying proteins involved in human disease than murine ciPODs.		61

Abbreviations: GBM, glomerular basement membrane; LPS, lipopolysaccharide; TLR4, toll-like receptor 4

Glucocorticoid action on podocytes

Glucocorticoids have been described to affect podocyte function by different mechanisms. In this review we have grouped these mechanisms into the effect on the podocyte slit diaphragm, the podocyte cytoskeleton and other mechanisms. An overview of all glucocorticoid regulated molecular changes that are discussed in the following sections, including their function under physiological conditions, is summarized in Table 2. Also, the effects of glucocorticoids on podocyte molecules is shown in Figure 1.

Glucocorticoid effects on the slit diaphragm

Nephrin is a key component of the slit diaphragm and consists of an intracellular and extracellular domain⁴. The extracellular domain of nephrin forms a zipper-like structure with nephrin molecules of neighbouring podocyte foot processes. Nephrin is anchored to the cell membrane by podocin and anchored to the cytoskeleton by CD2-associated protein (CD2AP). Besides its filtering function, the phosphorylation of nephrin has been shown to increase pro-survival signalling in the podocyte via CD2AP²³. Homozygous genetic mutations in nephrin lead to congenital nephrotic syndrome of the Finnish type, which illustrates the importance of nephrin for an intact filtration barrier. Correspondingly, nephrin expression is lost or decreased in some biopsy samples of patients with MCNS²⁴. Nephrin expression is also lost in PAN injured rats and is restored by glucocorticoid treatment²⁵. In the ciPOD model, the phosphorylation of nephrin was found to be decreased after injury and glucocorticoid treatment restored nephrin phosphorylation, as well as nephrin

expression²⁶⁻²⁸. Further studies revealed that glucocorticoids induce nephrin phosphorylation by activating serum and glucocorticoid-regulated kinase 1 (SGK1) and Src-family kinase^{28,29}.

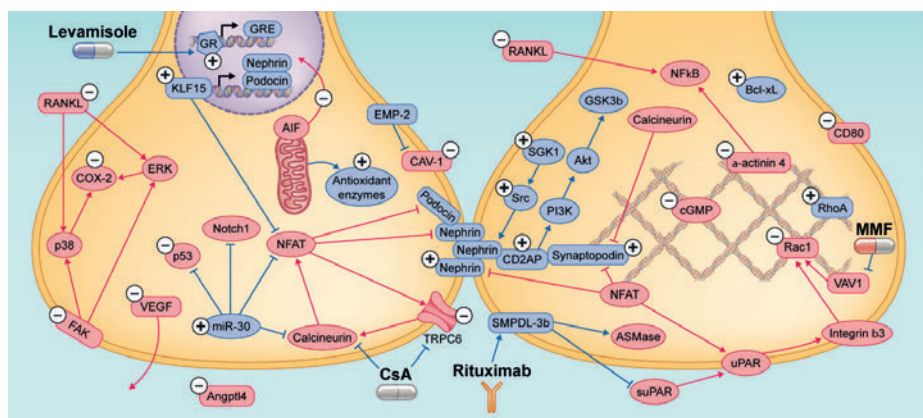


Figure 1. Schematic overview of proposed molecules affected by glucocorticoid treatment. Green arrows indicate a mechanism that favours podocyte survival, while red arrows indicate mechanisms that favour podocyte apoptosis. Molecules in green are beneficial for podocytes when expressed, whereas molecules in red are detrimental for podocyte health when highly expressed. An arrow headed line represents a stimulatory reaction, meaning that the interaction yields an increased expression or activity. A bar headed line indicates an inhibitory reaction, meaning that the interaction yields a decreased expression or activity. A circle with a minus means that this molecule is inhibited by glucocorticoids, whereas a circle with a plus means that this molecule is stimulated by glucocorticoids. The mechanisms of action of other NS therapeutic agents: rituximab, mycophenolate mofetil, cyclosporin A and levamisole are also indicated. Image created with Biorender. Abbreviations: AIF, apoptosis inducing factor; Akt, protein kinase B; Angptl4, angiopoietin-like 4; ASMase, acid sphingomyelinase; Bcl-xL, B-cell lymphoma-extra large; CAV-1, caveolin-1; CD2AP, CD2- associated protein; CD80, cluster of differentiation 80; COX-2, cyclooxygenase 2; cGMP, cyclic guanosine monophosphate; CsA, cyclosporine A; EMP-2, epithelial membrane protein 2; ERK, extra- cellular regulated kinase; FAK, focal adhesion kinase; GR, glucocorticoid receptor; GRE, glucocorticoid responsive elements; GSK3 β , glycogen synthase kinase 3 β ; KLF15, krüppel-like factor 15; MMF, mycophenolate mofetil; miR30, microRNA family member 30; NFAT, nuclear factor of activated T- cell; NFkB, nuclear factor kB; p38, p38 mitogen-activated kinase; p53, cellular tumour antigen p53; PI3K, phosphoinositol-3 kinase; Rac1, ras-related C3 botulinum toxin substrate 1; RANKL, receptor activator of nuclear factor kappa B ligand; RhoA, ras homology family member A; SGK1, serum and glucocorticoid-regulated kinase 1; SMPDL-3b, sphingomyelin phosphodiesterase acid-like 3b receptor; suPAR, soluble urokinase-type plasminogen activator receptor; TRPC6, transient receptor potential channel 6; uPAR, urokinase-type plasminogen activator receptor; VAV1, vav guanine nucleotide exchange factor 1; VEGF, vascular endothelial growth factor.

Gene expression of both nephrin and podocin is positively regulated by the transcription factor kruppel-like factor 15 (KLF15)³⁰. Less KLF15 expression was observed in biopsies of patients with FSGS and MCNS compared to control. Also, KLF15 and nephrin expression was increased in steroid-sensitive NS compared to steroid-resistant NS^{30,31}. In knockout mice and ciPODs, KLF15 was shown to be vital for podocyte recovery upon glucocorticoid treatment³⁰. Mechanistically, KLF15 decreases nuclear factor of activated T-cell (NFAT) signalling, which was previously shown to induce glomerulosclerosis and proteinuria by decreasing podocin, nephrin and synaptopodin expression^{31,32}.

Other molecules that are associated with the slit diaphragm are caveolin-1 (CAV-1) and transient receptor potential channel 6 (TRPC6)^{14,33}. TRPC6, together with podocin, is a mechanical stress sensor and enables podocytes to adjust to mechanical pressure changes⁴. A TRPC6 gain-of-function mutation is a monogenic cause of steroid-resistant NS. Similarly, mutations in TRPC6 are found in FSGS and TRPC6 expression was elevated in injured ciPODs, which was restored by glucocorticoid treatment³³. CAV-1 interacts with nephrin and CD2AP and is upregulated upon PAN administration in ciPODs or zebrafish and downregulated after glucocorticoid treatment¹⁴. CAV-1 upregulation after injury was only observed in epithelial membrane protein 2 (EMP-2) knockouts, by a yet unknown mechanism. Interestingly, EMP-2 mutations are also associated with human SRNS.

Glucocorticoid effects on cytoskeleton-associated proteins

The podocyte foot processes are structurally shaped by an extensive actin cytoskeleton²¹. Disruption of the cytoskeleton is known to cause podocyte foot process effacement and slit diaphragm dysfunction, followed by proteinuria. Synaptopodin, ras homology family member A (RhoA), and Ras-related C3 botulinum toxin substrate 1 (Rac1) are important regulators of actin cytoskeleton stability^{20,21,25}. Synaptopodin positively regulates RhoA activity, which is important for the stabilization of the cytoskeleton by promoting the production of actin stress fibres. In a small cohort of FSGS patients, low synaptopodin expression in kidney biopsies was associated with a poor response to glucocorticoid treatment³⁴. Similarly, PAN injury in rats decreases synaptopodin expression²⁵. Glucocorticoids increase synaptopodin expression in PAN injured rats and RhoA activity in ciPODs^{21,25}. In contrast, Rac1 activity and high cyclic guanosine monophosphate (cGMP) levels stimulate migration of the podocyte and actin cytoskeleton destabilization. In line with this, glucocorticoids decrease the activity of Rac1 and cGMP concentrations in a

ciPOD model^{20,35}. All of the glucocorticoid mediated changes mentioned above corresponded with an increased stability of the actin cytoskeleton^{20,21,25,35}.

Focal adhesion kinase (FAK) binds to actin and $\beta 1$ integrin and is able to deregulate cytoskeleton organization¹⁹. This regulation is mediated by receptor activator of nuclear factor kappa B ligand (RANKL) and activates downstream mediators that lead to podocyte apoptosis^{19,36}. The FAK/RANKL pathway increases cyclooxygenase 2 (COX-2) expression, which is considered a key regulator of kidney inflammation and hemodynamics³⁷. Glucocorticoid treatment decreases several molecules in the FAK/RANKL pathway in rat and ciPOD models, which are elevated when injury is induced^{19,25,36,37}.

Another protein that aids in stabilizing the cytoskeleton is α -actinin 4 (ACTN4)³⁸. However, overexpression of ACTN4 activates pro-apoptotic signalling pathways and ACTN4 has also been found to stimulate Angptl4 expression, which will be discussed later. Elevated ACTN4 levels are observed in human biopsy samples of patients with MN, specifically concentrated around subepithelial immune deposits³⁹. Correspondingly, glucocorticoids were found to lower the expression of ACTN4 in ciPODs injured with Adriamycin³⁸. In contrast, ACTN4 levels were not altered in MCNS biopsy samples. This illustrates that findings in a model system are not always representative for the entire disease spectrum.

Other molecules implicated in podocyte glucocorticoid response

MicroRNAs are a group of small non-coding RNAs that regulate post-transcriptional gene expression⁴⁰. Members of the miR-30 family of microRNAs (miR-30s) may increase podocyte survival by reducing apoptotic factors, including cellular tumour antigen p53 (p53). In accordance, expression of miR-30s was decreased in FSGS biopsies, PAN treated rats and ciPODs, when compared to controls⁴⁰. Glucocorticoid treatment restored miR-30a expression in rats and ciPODs. Similarly, in a study by Wada et al., glucocorticoids decreased the expression of p53 and several other pro-apoptotic factors⁴¹. Although Wada et al. did not examine the expression of miR-30s, it can be hypothesized that their observation was caused by glucocorticoid-dependent miR-30 upregulation. Contrary to the results described above, a study in murine ciPODs showed that the inhibition of miR-30a, a member of the miR-30 family, decreased podocyte apoptosis⁴². These results were found in murine ciPODs, whereas the previously mentioned study examined miR-30a expression in FSGS biopsies, PAN injured rats and human ciPODs. Therefore,

the function of miR-30a may be different in mice compared to its function in humans or rats. Another option is that this effect of miR-30a is an artefact of the murine ciPOD model systems. In addition to reducing apoptotic factors, miR-30 expression was also shown to benefit podocyte recovery by inhibiting NFATC signalling, leading to an increased cytoskeleton stability⁴³.

Angiopoietin-like 4 (Angptl4) expression was found to be elevated in a small number of human MCNS biopsy samples, as well as in PAN treated rats¹². Angptl4 has previously not been linked to proteinuria and is highly expressed in liver and adipose tissue upon fasting¹². Still, in PAN treated rats glucocorticoids decreased the elevated Angptl4 levels. Both, vascular endothelial growth factor (VEGF) and cluster of differentiation 80 (CD80) expression, are found to be increased in patients with MCNS^{27,44}. VEGF is exclusively expressed in podocytes in the glomerulus and is necessary to maintain glomerular filtration barrier function, whereas CD80 is a co-stimulatory molecule and is only expressed in podocytes that have been stimulated via toll-like receptor. In human ciPODs, both molecules were found to be downregulated by dexamethasone. Lastly, reactive oxygen species (ROS) have been shown to result in injury in the PAN rat model⁴⁵. Glucocorticoid treatment upregulated anti-oxidant enzymes that counteract ROS induced damage, resulting in a protective effect on podocytes.

Table 2. Molecules altered by glucocorticoid treatment.

Molecule	Model used	Regulation by glucocorticoids	Normal function	Ref
Slit diaphragm-related molecules				
CAV-1	Zebrafish and human ciPODs	↑ Expression	Component of caveolae, lipid rafts on the membrane where signalling molecules are present. Interacts with nephrin and CD2AP	14
CD2AP	Murine ciPODs	↑ Expression ↑ Akt and GSK3b activity	Anchors nephrin to the cytoskeleton and is able to induce pro-survival signalling via PI3K/Akt/GSK3β	23
KLF15	Human and murine ciPODs and transgenic mouse model	↑ Expression ↓ NFAT1c expression	Regulates transcription of podocyte-specific genes (such as nephrin/podocin). Decreases NFAT signalling by repressing NFAT1c	30, 31

Table 2. Continued

Molecule	Model used	Regulation by glucocorticoids	Normal function	Ref
Nephrin	PAN rat model and human ciPODs	↑ Expression and/or phosphorylation	Major component of the slit diaphragm	25-27
SGK1	Human ciPODs	↑ Activity	Positively regulates Src family kinase activity	28, 29
Src family kinase	Human ciPODs	↑ Activity	Phosphorylates nephrin when activated	28
TRPC6	Murine ciPODs	↓ Expression	Mediate Ca ²⁺ influx in foot processes, acts as mechanical stress sensor	33
Cytoskeleton-related molecules				
ACTN4	Murine ciPODs	↓ Expression	Binds and stabilizes actin cytoskeleton	38
	Human ciPODs	Co-factor for glucocorticoid receptor		62
cGMP	Murine ciPODs	↓ cGMP synthesis	High cGMP levels stimulate the disassembly of actin stress fibres and encourage migration	35
COX-2	PAN rat model, protein overload rat model and murine ciPODs	↓ COX-2 expression	Key regulator of kidney inflammation and haemodynamics	25, 37
FAK/ RANKL	Adriamycin rat model	↓ Activity of FAK, ERK, p38	Binds the cytoplasmic tail of β1 integrins and actin. Able to induce apoptotic signalling via FAK/RANKL/ERK/p38 or FAK/RANKL/NF-κB	19
Rac1	Human ciPODs	↓ Activity	Active Rac1 stimulates the motility of podocytes	20
RhoA	Murine ciPODs	↑ Activity	Active RhoA helps in stabilizing actin stress fibres	21
SYNPO	PAN rat model	↑ Expression	Actin-associated protein, linking CD2AP to actin and activating RhoA	25
Others				
Angptl4	PAN rat model	↓ Expression	Highly expressed in the liver, upon lipid metabolism	12
Apoptotic factors	Murine ciPODs	↓ p53 and AIF translocation ↓ Bcl-xL	P53 and AIF translocation induce apoptosis. Bcl-xL expression counteracts apoptosis	41

Table 2. Continued

Molecule	Model used	Regulation by glucocorticoids	Normal function	Ref
CD80	Human ciPODs	↓ Expression	CD80 functions as a co-stimulatory molecule on antigen presenting cells and is expressed by podocytes upon stimulation of toll-like receptor 3 and 4	44
miR-30	Human ciPODs and PAN rat model	↑ Expression	Important for regulating podocyte physiology, can inhibit p53 and Notch1 signalling. Additionally, decreases calcineurin/ NFAT signalling, leading to decreased uPAR/integrin β signalling. This pathway normally stimulates Rac1 and CDC42 activity	40
	Murine ciPODs	Inhibition prevents injury by PAN		42
VEGF		↓ Expression	Exclusively expressed by the podocyte within the glomerulus and needed for normal functioning	27

Abbreviations: ACTN4, α -actinin 4; AIF, apoptosis-inducing factor; Akt, protein kinase B; Angptl4, angiopoietin-like 4; AOE, anti-oxidant enzymes; Bcl-xL, B- cell lymphoma-extra large; CAV-1, caveolin-1; CD2AP, CD2-associated protein; CD80, cluster of differentiation 80; CDC42, cell division control protein 42 homolog; COX-2, cyclooxygenase 2; cGMP, cyclic guanosine monophosphate; ciPODs, conditionally immortalized podocytes; ERK, extra-cellular regulated kinase; GSK3 β , glycogen synthase kinase 3 β ; FAK, focal adhesion kinase; KLF15, krüppel-like factor 15; miR30, microRNA family member 30; NFAT, nuclear factor of activated T-cell; NF κ B, nuclear factor κ B; p38, p38 mitogen-activated kinase; p53, cellular tumour antigen p53; PAN, puromycin aminonucleoside; PI3K, phosphoinositol-3 kinase; Rac1, ras-related C3 botulinum toxin substrate 1; RhoA, ras homology family member A; ROS, reactive oxygen species; SGK1, serum and glucocorticoid-regulated kinase 1; SYNPO, synaptopodin; TRPC6, transient receptor potential channel 6; uPAR, urokinase-type plasminogen activator receptor; VEGF, vascular endothelial growth factor.

Common pathways found in the mechanism of action of other NS therapeutics

Patients with NS resistant to glucocorticoid treatment or in need of steroid-sparing treatment are treated with other immunosuppressive agents, including cyclophosphamide, levamisole, calcineurin inhibitors, mycophenolate mofetil (MMF), and rituximab⁶. In order to highlight important pathways for podocyte recovery, we reviewed the mechanism of action of these other agents. For instance, levamisole was found to increase glucocorticoid receptor activity and

is therefore hypothesized to act on similar pathways as glucocorticoids or to enhance glucocorticoid effects⁴⁶. Cyclosporine A (CsA), MMF and Rituximab were found to act on podocytes by stabilizing the podocyte actin cytoskeleton⁴⁷⁻⁵⁰. Similar to glucocorticoids, CsA treatment increases cytoskeleton stability by decreasing TRPC6 expression and NFAT signaling^{48,49}. In addition, CsA decreases calcineurin levels, which stimulate synaptopodin degradation when increased. MMF decreases the expression of vav guanine nucleotide exchange factor 1 (VAV1), which subsequently reduces Rac1 activity⁵⁰. Rituximab also counteracts podocyte cytoskeleton remodelling by binding and activating the sphingomyelin phosphodiesterase acid-like 3b receptor (SMPDL-3b)^{47,51}. This receptor increases acid sphingomyelinase (ASMase) activity, which stabilizes the actin cytoskeleton of podocytes. Furthermore, SMPDL-3b is considered to increase cytoskeleton stability by binding soluble urokinase-type plasminogen activator receptor (suPAR), which results in inhibition of Rac1 stimulation⁵². Altogether, since CsA, MMF and rituximab were found to increase podocyte cytoskeleton stability, SMPDL-3b is a target of special interest for podocyte recovery.

Functional glucocorticoid studies in podocytes: where do we go from here?

Several molecules that are involved in the slit diaphragm signalling, cytoskeleton organization or inflammatory reaction have been found to be regulated by glucocorticoids in podocytes. However, how these molecules affect podocyte function remains largely unknown. Unravelling the mechanisms of podocyte recovery after glucocorticoid treatment would allow the development of more specific treatment options for patients with NS. Although glucocorticoids seem to benefit not only podocytes, but also other renal cell types and the immune system, their beneficial effects come at a price. Namely, glucocorticoids are not specific enough leading to many severe side effects and they are still not always effective. With the introduction of cutting-edge techniques and models, such as single-cell sequencing and 3D kidney organoid cultures, key mechanisms and their effects on podocyte function may be unravelled in the near future.

The function of a molecule can be dependent on the injury model used. As an example, loss of Rac1 has been shown to either decrease or increase proteinuria in two different *in vivo* injury models⁵³. Similarly, ACTN4 was found to be upregulated in biopsies of patients with MN and in Adriamycin treated ciPODs, but not in biopsies of patients with MCNS³⁸. Therefore, confirmation or

validation studies in humans should be standard of practice. Validation studies may use for instance human biopsies, which was seen in several studies that were discussed in this review, or *ex vivo* isolated podocytes. In order to find more effective treatment option with less side effects, it is important that an *in vivo* model mimics steroid-sensitive NS as closely as possible. However, our current knowledge of NS pathophysiology is limited, which hampers the development of more accurate *in vivo* models. A recent study describes the workflow for single-cell sequencing of human biopsies, which may increase our understanding of MCNS pathophysiology⁵⁴. Even more, the combination of single-cell RNA and Assay for Transposase-Accessible Chromatin (ATAC) sequencing may present a more sensitive method to notice cellular fate changes that are not noticed at the level of transcription alone⁵⁵. ATAC sequencing is a novel technique to map the regions of DNA that are accessible for transcription and is therefore able to detect epigenetic changes, which are not detected on a transcriptional level. Therefore, the integration of new single-cell sequencing techniques has the potential to increase our understanding of NS pathophysiology. This will allow us to develop more physiologically relevant model systems, which ultimately lead to more effective treatment options with less severe side effects.

Recently established kidney organoid cultures and the glomerulus-on-a-chip aim to tackle limitations of ciPODs, while keeping the advantages of *in vitro* models, such as the ability to standardize the environment. A recent study describes the use of human podocytes in combination with human glomerular endothelial cells in a glomerulus-on-a-chip system⁵⁶. In their system, podocytes form a slit diaphragm, are subjected to fluid flow, have a three-dimensional conformation and interact with the endothelial cells to form a basement membrane like structure. The authors show that their system can be used to model diseases by using podocytes derived from patients with Alport syndrome or by administration of PAN or MN serum.

Another improved *in vitro* culturing system is the generation of kidney organoids from induced pluripotent stem cells. In kidney organoids, multiple cell types form a three-dimensional structure and interact with each other. The gene expression profile from organoid-derived podocytes has a better resemblance to the *in vivo* gene expression profile than that of ciPODs⁵⁷. Kidney organoids have the potential to be the future model system of choice to study diseases, although there are still hurdles to overcome. Challenges include the

immaturity of kidney organoid cell types, high batch-to-batch variation and the presence of off-target cells, such as neuronal or muscle-like cells⁵⁸.

Conclusion

Glucocorticoids may stimulate podocyte recovery by restoring slit diaphragm function, decreasing inflammatory responses and especially by stabilizing the actin cytoskeleton. However, the exact and key molecular mechanisms have not been elucidated yet. Findings in ciPODs or animal model systems need to be validated in human podocytes, by using kidney biopsies or isolated primary podocytes. New technology, such as single-cell sequencing, will aid in our understanding of disease pathology and thus the development of more relevant *in vivo* and *in vitro* models. *In vitro*, the kidney organoids and glomerulus-on-a-chip may present a more realistic model to mechanistically study targets observed in animal models or human biopsies. Combined, the improvement in disease modelling may lead to the identification of key molecules involved in podocyte recovery and thereby to the development of more effective therapies with less side effects.

Authors contribution

MB analysed and interpreted the data and drafted the manuscript. BS, JJ, MFS provided critical content for the design of the manuscript and revised the manuscript. All authors provided intellectual content of critical importance for the manuscript and approved the final version before submission.

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Declaration of interest

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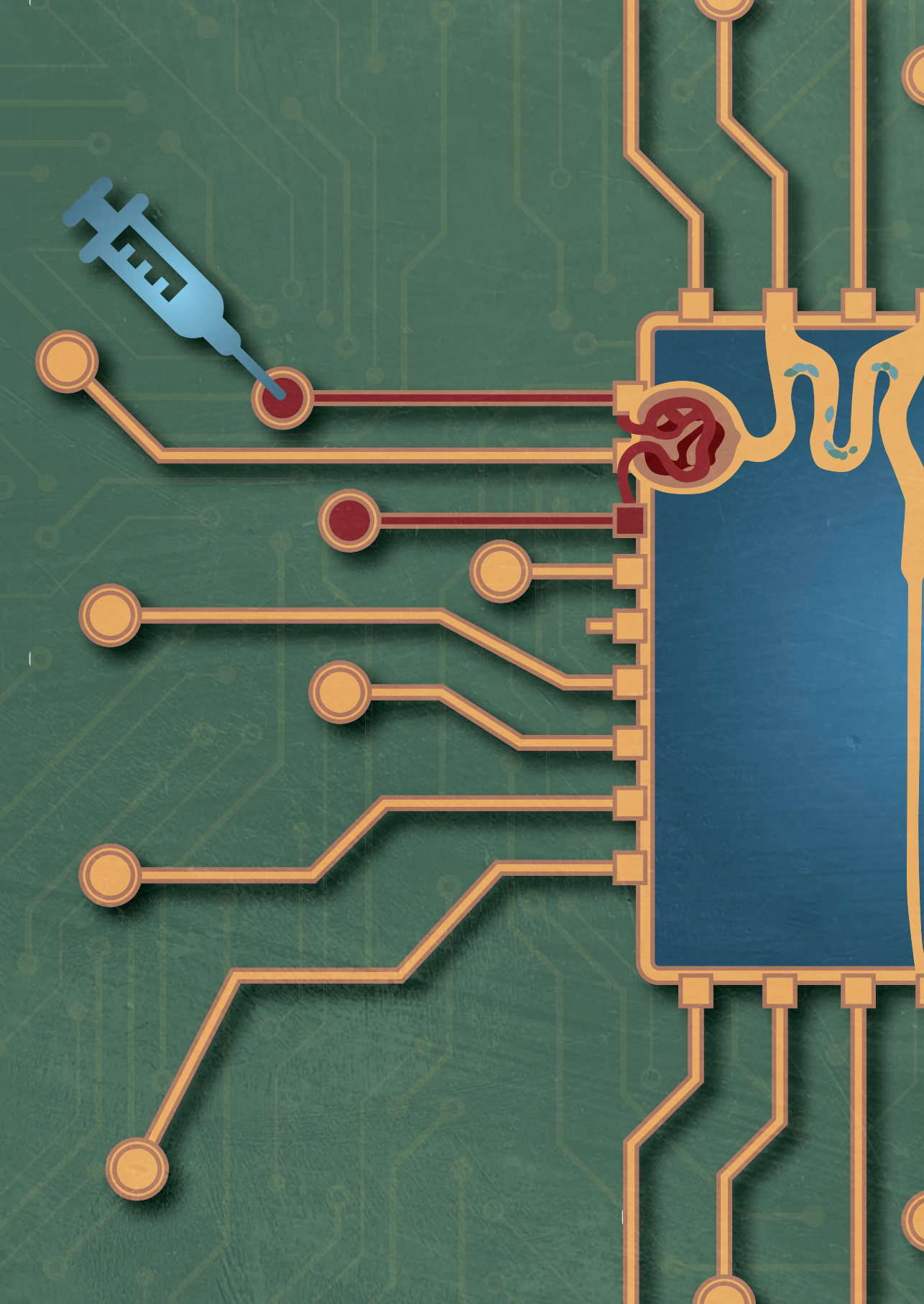
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Multimodal analysis of prednisolone effects on podocytes

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In preparation

Abstract

Idiopathic nephrotic syndrome (iNS) is the most common cause of glomerular disorders in children and leads to defective glomerular filtration and proteinuria. Glucocorticoids have been the mainstay of treatment for over 60 years, but glucocorticoids are not always effective and are associated with severe side effects. Although the mechanism of glucocorticoid-induced recovery is not fully understood, beneficial effects have been shown in podocytes, which are the main affected cell type in iNS. Identifying these direct effects of glucocorticoids on podocytes may lead to the development of more effective treatment options with less side effects for patients with iNS. In this study, the partially glucocorticoid-responsive puromycin aminonucleoside (PAN) rat model was used for single-nuclei RNA (snRNA) and assay for transposase accessible chromatin (snATAC) sequencing. Using integrated analysis of snRNA and snATAC, an increase in fatty acid metabolism was found in injured podocytes, observed by upregulation of carnitine palmitoyltransferase 1B (*Cpt1b*), pyruvate dehydrogenase kinase 4 (*Pdk4*) and PPARG coactivator 1 α (*Ppargc1a*). Prednisolone treatment increased expression of circadian rhythm related transcription factors and reduced fatty acid metabolism, by downregulation of *Cpt1b*, *Pdk4*, and *Ppargc1a*. Although circadian rhythm related transcription factors were strongly upregulated by prednisolone, they were not as strongly expressed in healthy podocytes, suggesting a role in recovery, but with a narrow therapeutic window. Inhibition of CPT1 using etomoxir in conditionally immortalized podocytes (ciPODs) reduced cell loss because of PAN injury, and showed a trend towards increased podocyte marker gene expression in podocytes from human induced pluripotent stem cell-derived kidney organoids. These pre-liminary *in vitro* studies demonstrated the potential of blocking fatty acid metabolism as a therapeutic target in iNS. In conclusion, this study indicates that fatty acid metabolism is increased in podocytes during iNS and lowering fatty acid metabolism may be further explored as potential treatment for iNS.

Keywords: glucocorticoids, nephrotic syndrome, podocytes, multi-omics, snRNA and snATAC

Introduction

Idiopathic nephrotic syndrome (iNS) is primarily caused by podocyte injury, leading to a defective glomerular filtration barrier and proteinuria¹. iNS is the most common cause of glomerular disorders in children and, for over 60 years, glucocorticoids have been the mainstay of treatment for iNS²⁻⁴. Even after decades of use, the exact mechanism of action for glucocorticoids in iNS is still elusive. Although a large proportion of patients with iNS respond to treatment, relapses are common, and some patients even become dependent on or resistant to glucocorticoid treatment⁵. Also, prolonged treatment with prednisolone is associated with several, sometimes severe, side effects. Evidently, patients with iNS need better options to treat relapses and thereby prevent side effects.

Glucocorticoids, of which prednisolone is used in childhood iNS, bind to the glucocorticoid receptor to transcribe or repress target genes⁶. Glucocorticoids are used in iNS for their anti-inflammatory effects, since a dysregulated immune system is suggested as a pathophysiological mechanism in iNS⁷⁻⁹. However, the glucocorticoid receptor is not only expressed in immune cells but also in many kidney cell types, including the podocyte, the parietal epithelial cell (PEC) and the proximal tubule epithelial cell (PTEC)^{10,11}. Many studies have shown that prednisolone has direct effects on the podocytes, and injured podocytes *in vitro* recover after glucocorticoid treatment in the absence of an immune system¹²⁻¹⁴. Unravelling these direct effects on the podocyte might enable the identification of new therapeutic targets that are more effective and specific, ultimately leading to novel drugs with fewer side effects.

Recently, the development of single-cell omics techniques has enabled dissecting transcriptomic, epigenetic, proteomic and metabolomic profiles of single cells in health and disease¹⁵⁻²⁰. In several kidney diseases, the combination of single-nucleus RNA (snRNA) and assay for transposase-accessible chromatin (snATAC) sequencing has led to the identification of cellular heterogeneity and key molecular changes during disease^{16,21,22}. However, datasets that focus on the single-cell landscape in iNS with high numbers of good quality podocytes have been lacking thus far²³⁻²⁵.

In this study, we used the puromycin aminonucleoside (PAN) rat model, which is partially responsive to glucocorticoids, to study iNS *in vivo* and to create snRNA and snATAC datasets²⁶. Integrative analysis of these datasets

revealed increased fatty acid metabolism in injured podocytes, with increased expression of carnitine palmitoyltransferase 1B (*Cpt1b*), which was reversed by prednisolone treatment. Injury in conditionally immortalized podocytes (ciPOD) was partially restored by inhibition of CPT1 using Etomoxir, demonstrating the potential of targeting fatty acid metabolism in iNS.

Results

Podocyte injury is partially restored by prednisolone *in vivo*

In order to establish an *in vivo* model of iNS that responds to prednisolone treatment, several concentrations of intravenously injected PAN (5, 10, or 15mg / 100g body weight, abbreviated as 5mg, 10mg, or 15mg) that have been used before to model iNS were tested (Figure 1A)²⁶⁻²⁹. A dose-dependent increase in proteinuria after intravenous PAN injection was observed on day 7, with significantly increased proteinuria levels when using 10 and 15mg PAN (Figure 1B). Using electron microscopy, a comparable dose-dependent increase in podocyte injury was observed (Figure 1C). Only small areas of podocyte foot processes effacement were observed when rats were treated with 5mg PAN, whereas larger areas of effacement were seen in rats treated with 10 or 15mg PAN (Figure 1C, red arrows). Administration of 15mg PAN resulted in pronounced podocyte injury, evidenced by the formation of podocyte pseudocysts (Figure 1C, red asterisk), a phenomenon previously reported by others and typically observed preceding podocyte detachment and loss^{30,31}. Since desmin is absent in healthy podocytes and expressed *de novo* in injured podocytes in rodent models, the amount of desmin protein was quantified using a semi-automated approach as detailed in the methods (Figure 1D, Supplemental figure 1)³²⁻³⁵. In accordance with proteinuria and electron microscopy, a dose-dependent increased desmin expression was observed in the podocytes following 10 and 15mg PAN administration (Figure 1D).

To induce remission in the injury model, rats receiving 5, 10, or 15mg of PAN were concurrently administered prednisolone (1.5mg / 100g body weight, abbreviated to 1.5mg prednisolone) via daily intraperitoneal injection, starting at the time of PAN administration (Figure 1A). Proteinuria only decreased in rats treated with 10mg PAN and prednisolone, but not in rats treated with either 5 or 15mg PAN and prednisolone (Figure 1E, Supplemental figure 2A-B). Based on these findings, rats treated with 10mg PAN were selected for further optimization of prednisolone therapy. Notably, increasing the prednisolone dose (to either 3mg

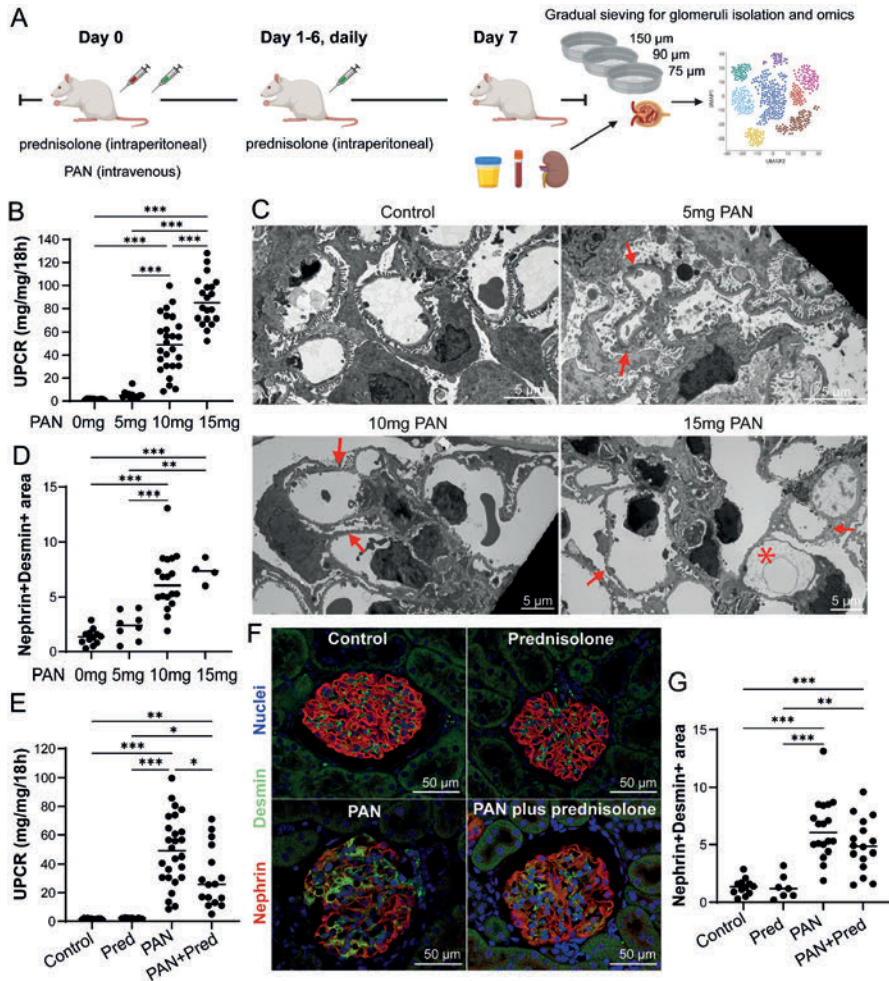


Figure 1. A glucocorticoid-sensitive nephrotic syndrome model is established using PAN and prednisolone in rats. **A**) Schematic overview of the animal study, including time points of injections and collection of materials for analysis. **B**) Urinary protein:creatinine ratio (UPCR) was used as a clinical hallmark of nephrotic syndrome. **C**) Electron microscopy images of glomeruli from rats in the different conditions. Arrows indicated foot process effacement and an asterisk is used to highlight pseudocyst formation. **D**) Quantification of the nephrin and desmin positive area of immunofluorescence stainings using a semi-automated method. An example image of a staining is provided in Supplementary figure 1. **E**) UPCR analysis and **F**) immunofluorescence images after prednisolone treatment in PAN injured rats. Podocytes (nephrin, red), injured podocytes (desmin, green), nuclei (blue). **G**) Quantification of the nephrin and desmin positive area of immunofluorescence stainings using a semi-automated method. An example image of a staining is provided in Supplementary figure 1. For statistics, ordinary one-way ANOVA was used with Tukey correction for multiple comparisons, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Abbreviations: PAN, puromycin aminonucleoside; Pred, prednisolone; UPCR, urinary protein:creatinine ratio.

or 5mg / 100g bodyweight) did not yield improved outcomes, as measured by reductions in proteinuria, compared to the 1.5mg dose (Supplemental figure 2C). Therefore, studies were conducted using 10mg PAN rats treated with 1.5mg prednisolone. Desmin levels in kidney sections seemed reduced in rats treated with prednisolone, consistent with the observed decrease in proteinuria (Figure 1F). However, desmin expression was heterogeneous and varied considerably per glomerulus, and therefore desmin quantification did not show a significant reduction (Figure 1G, Supplemental figure 2D). Finally, glomeruli were isolated to enrich for glomerular cells. Isolated glomeruli from the best responders, identified by the most pronounced proteinuria response to injury and treatment, were processed for snRNA and snATAC sequencing (Supplemental figure 2E).

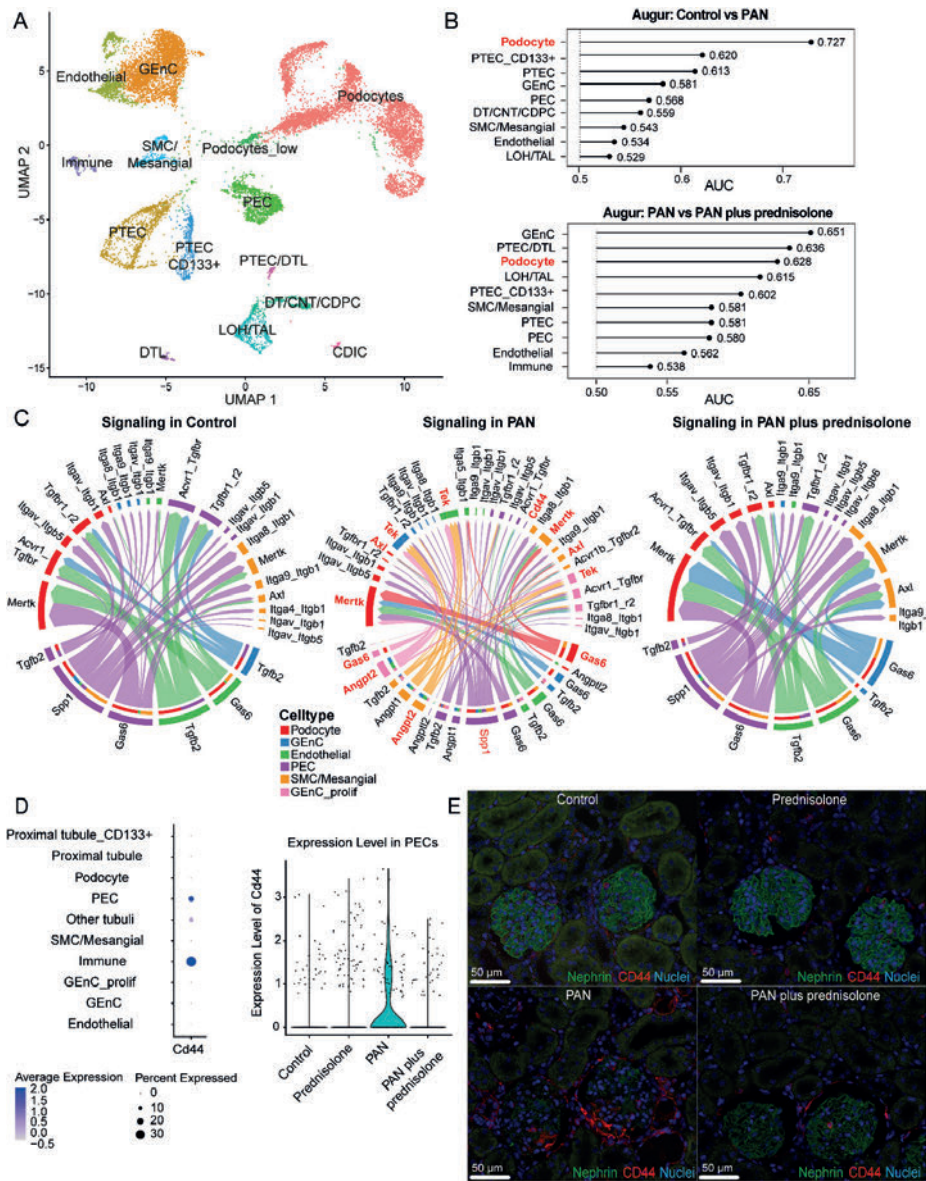
Prednisolone affects not only podocytes but also reduces CD44 expression in PECs.

Both the snRNA and snATAC sequencing datasets were prepared for analysis using the standard workflows provided by Seurat and Signac packages and annotated using previously described cell type markers (Supplemental figures 3, 4A-B)^{15,18,36,37}. Consistent with the presence of proteinuria, the PAN dataset showed a reduced proportion of healthy podocytes (46% versus 12%) and an increased proportion of injured podocytes (3% versus 6%) compared with the control condition (Supplemental figure 4C-D). Similarly, in the PAN plus prednisolone condition, injured podocytes were still present, but the proportion of healthy podocytes increased compared to the PAN dataset (27% versus 12%). As a final step in pre-processing the datasets for analysis, the snRNA and snATAC datasets were integrated using Seurat (Supplemental figure 5)^{36,37}. To enable the use of tools, such as Cellchat and Augur, which detect differences only within similarly annotated clusters, we merged podocytes, glomerular endothelial cells (GEnC) and PTEC clusters prior to analysis (Figure 2A).

Augur analysis was used to identify the most perturbed cells between the conditions (Figure 2B)³⁸. The podocyte was the most affected cell type between the control and PAN conditions. However, some other cell types were affected, like PTECs, GEnCs, PECs and mesangial cells. These changes could be caused by PAN directly, or could be secondary to podocyte injury. Interestingly, the podocyte was not the most affected cell type when comparing the PAN and PAN plus prednisolone conditions. This suggests that prednisolone may exert significant effects on other cell types besides podocytes as well, or that effects in podocytes have a substantial impact on surrounding cells. To investigate

cellular crosstalk, Cellchat was used to perform ligand-receptor interaction analysis and identify upregulated signalling pathways in the PAN condition (Supplemental figure 6). Upregulated ligands/receptors such as angiopoietin 2 (*Angpt2*)/TEK receptor tyrosine kinase (*Tek*), secreted phosphoprotein 1 (*Spp1*)/*Cd44* and growth arrest specific 6 (*Gas6*)/AXL receptor tyrosine kinase (*Axl*) identified in the PAN condition have been described in various glomerular diseases (Figure 2C, in red)³⁹⁻⁴³.

Interestingly, increased expression of *Cd44* was seen in PECs in the PAN condition (Figure 2D), which has been described in activated PECs during focal segmental glomerulosclerosis (FSGS)^{41,42}. In the PAN condition, PECs showed hypertrophy, and an increased expression of CD44 (Figure 2E). PECs also showed a change in localization, with more presence on the glomerular tuft rather than only at Bowman's capsule⁴⁴. The CD44 expression was decreased and similar to the control condition in the PAN plus prednisolone condition. Both the GEnCs and PECs showed injury-associated cell signalling in the PAN condition that was restored by prednisolone. In order to estimate which cell types were affected by prednisolone, the expression of several previously mentioned prednisolone-responsive genes was compared between different cell types (Supplemental figure 7)^{45,46}. The podocytes seemed to have higher RNA expression of some of the markers in the PAN plus prednisolone and prednisolone condition (glucocorticoid-induced leucine zipper (*Tsc22d3*), FKBP Prolyl Isomerase 5 (*Fkbp5*), Kruppel-like factor 15 (*Klf15*)), although the gene accessibility did not change that much (Supplemental figure 7). On RNA expression level, both GEnCs and PECs showed a much weaker response to *Tsc22d3*, *Fkbp5* and *Klf15*, however, on gene accessibility in the ATAC dataset, both cell types showed a slight increase in the prednisolone conditions (*Tsc22d3* for both and *Klf15* for PECs only). Therefore, it is still questionable whether the changes in GEnCs and PECs are primary or also induced secondary to changes in podocytes by prednisolone. Altogether, multiple cell types were affected by the injury induced by PAN and showed a response to prednisolone, with the podocyte as the only cell type that showed glucocorticoid-induced gene expression.

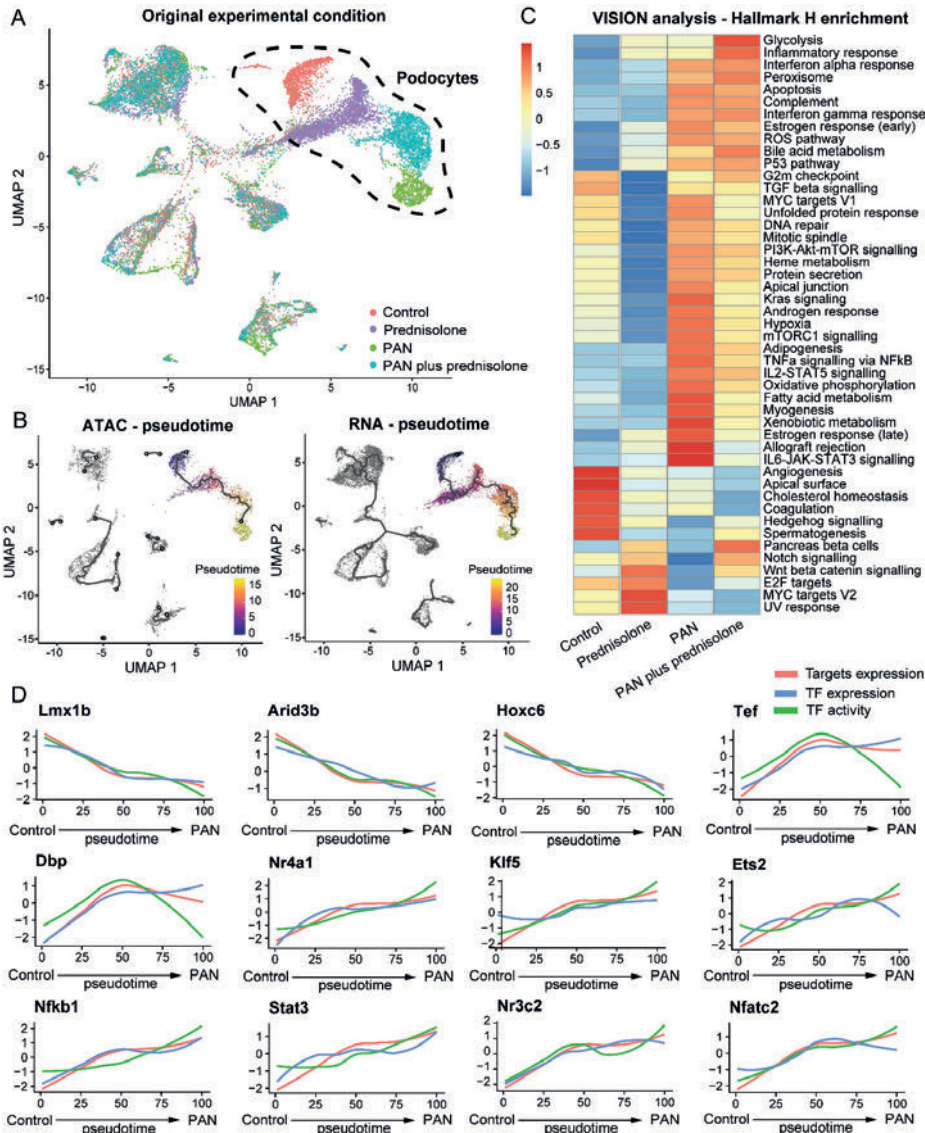


< Figure 2. PAN stimulates injury signalling in multiple glomerular cell types, including CD44 expression in PECs, which is downregulated by prednisolone. A) UMAP of cell clusters obtained by integration of snRNA and snATAC datasets. B) Augur analysis results indicating which cell types are most affected between two conditions for control versus PAN and PAN versus PAN plus prednisolone. C) Visualization of ligand-receptor interactions in control, PAN and PAN plus prednisolone for ligand-receptor interactions that are upregulated in the PAN condition. Some of the signalling ligands/receptors are previously described to be involved in various kidney diseases and highlighted in bold. D) Dotplot showing Cd44 expression in immune cells and PECs. Violin plot showing that mainly PECs from the PAN condition express Cd44, with very low expression in PECs from control, prednisolone or PAN plus prednisolone condition. E) Representative images CD44 immunofluorescent staining in rat kidneys. Podocytes (nephrin, green), activated PECs (CD44, red), nuclei (blue). Each image shows a representative view that was found in the conditions in three independent rat kidney slides. Abbreviations: Acvr1, Activin A receptor type 1; Angpt1, angiotensinogen 1; Angpt2, angiotensinogen 2; Angptl2, angiotensinogen-like 2; Axl, AXL receptor tyrosine kinase; Cd44, CD44 molecule; CDIC, collecting duct intercalated cell; CDPC, collecting duct principal cell; CNT, connecting tubule; DT, distal tubule; DTL, descending thin limb; Gas6, growth arrest specific 6; GEnC, glomerular endothelial cell; Itga4, integrin subunit α 4; Itgav, integrin subunit α 5; Itga8, integrin subunit α 8; Itga9, integrin subunit α 9; Itgb1, integrin subunit β 1; Itgb5, integrin subunit β 5; LOH, loop of Henle; Mertk, MER proto-oncogene tyrosine kinase; PAN, puromycin aminonucleoside; PEC, parietal epithelial cell; PTEC, proximal tubule epithelial cell; snATAC, single-nucleus assay for transposase-accessible chromatin; snRNA, single-nucleus RNA; SMC, smooth muscle cell; Spp1, secreted phosphoprotein 1; TAL, thick ascending limb; Tek, TEK receptor tyrosine kinase; Tgfb2, transforming growth factor β 2; Tgfr, transforming growth factor β receptor; Tgfr1, transforming growth factor β receptor 1; Tgfr2, transforming growth factor β receptor 2; UMAP, uniform manifold approximation and projection.

Prednisolone decreases podocyte injury signalling pathways and strongly activates circadian rhythm associated genes.

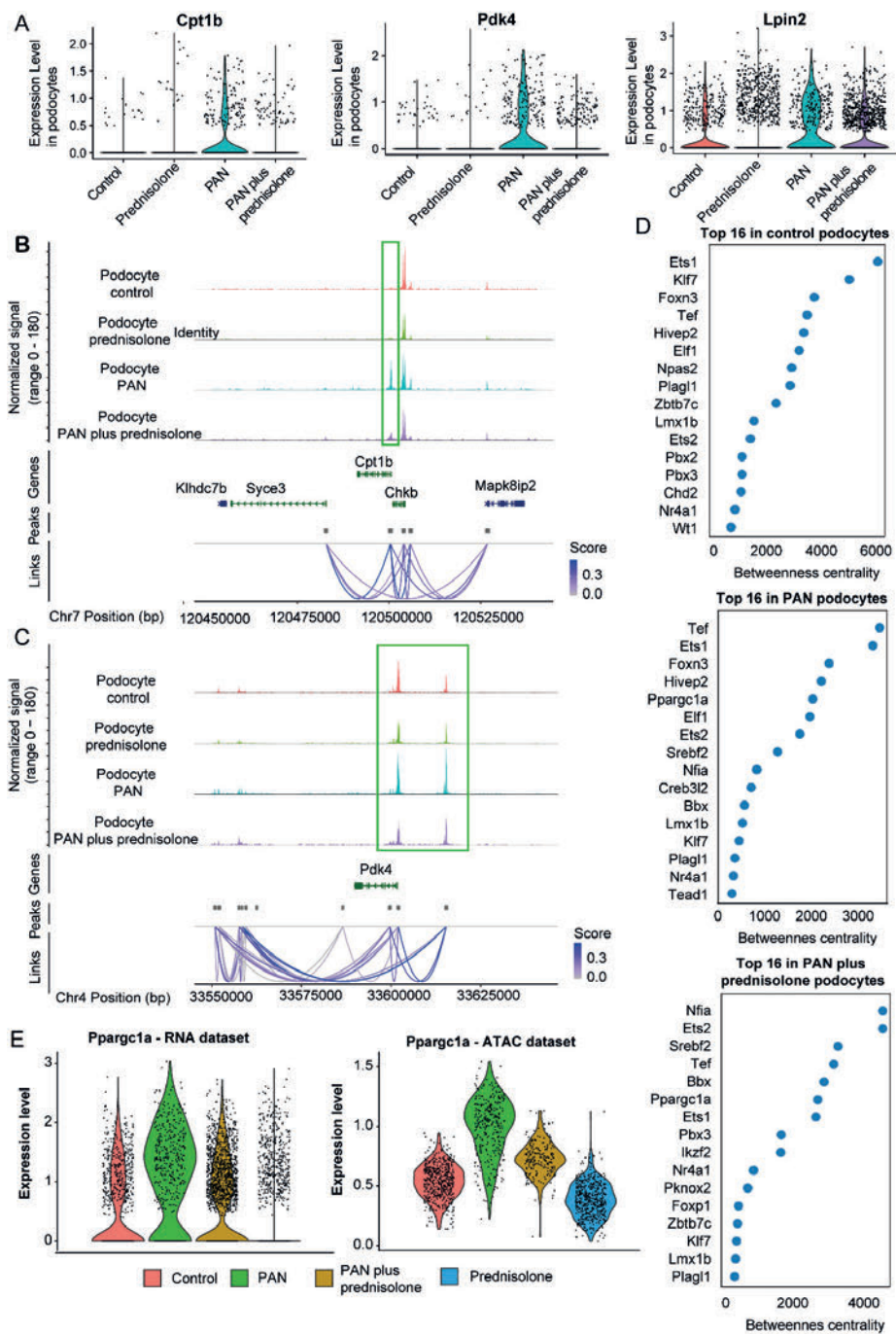
Following integration of the snRNA and snATAC sequencing datasets, we divided the podocyte cluster into four distinct but closely related subclusters, each defined by the experimental condition (Figure 3A). Using monocle3, pseudotime was identified in the integrated dataset, starting in the control podocytes and ending in the PAN podocytes (Figure 3B). To investigate the mechanisms regulating podocyte transition from an injured to a healthy phenotype, overrepresentation analysis was performed using cluster markers identified in the snRNA and snATAC datasets, as well as genes associated with pseudotime (Supplemental tables 1-4). Previously described markers of podocyte injury like: MAPK activated protein kinase 2 (*Mapkapk2*), insulin like growth factor binding protein 7 (*Igfbp7*) and interleukin-6 janus kinase-signal transducer and activator of transcription 3 (IL6 JAK-STAT3) signalling were upregulated in PAN-injured podocytes (Figure 3C, Supplemental tables 1-2)⁴⁷⁻⁵⁰. *Mapkapk2* and p38 signalling have been linked to Rac family small GTPase 1 (Rac1) activation and in line with this mechanism, the pseudotime analysis revealed various genes that were associated with Ras homolog family (Rho) and Rac signalling

(Supplemental figure 8A, Supplemental tables 3-4). Rho and Rac signalling is associated with podocyte cytoskeletal changes, corresponding to increased expression of genes associated with regulation of the actin cytoskeleton (Supplemental figure 8B). Altogether, the identification of these genes and pathways confirms valid injury responses in the PAN-injured podocytes, which are restored by prednisolone treatment. However, Rac signalling has been shown to have divergent roles in podocyte maintenance and injury, and may therefore be less attractive as a therapeutic target⁵¹.



< Figure 3. *Prednisolone reverses the molecular injury phenotype in podocytes and induces upregulation of circadian rhythm related transcription factors. A) UMAP of the integrated RNA and ATAC dataset. Podocytes were subsetted per experimental condition, resulting in 4 subclusters B) Monocle3 pseudotime analysis was performed on the RNA and ATAC datasets. In both, podocytes followed a trajectory starting from control podocytes, passing through prednisolone and PAN plus prednisolone podocytes, and ending with PAN podocytes C) VISION analysis on the podocytes using the cells from the RNA dataset and Hallmark H gene sets. D) GRN analysis using scMEGA. Relative visualization of: transcription factor mRNA expression, transcription factor ATAC activity, transcription factor target genes mRNA expression over pseudotime. At pseudotime 0, healthy podocytes from the control condition are present, halfway PAN plus prednisolone podocytes are encountered and at the end of pseudotime the PAN podocytes are located. Abbreviations: Arid3b, AT-rich interaction domain 3B; Dbp, D-box binding PAR BZIP transcription factor; Ets2, ETS proto-oncogene 2; GRN, gene regulatory network; Hoxc6, homeobox C6; Klf5, krüppel like factor 5; Lmx1b, LIM homeobox transcription factor 1β; Nfatc2, nuclear factor of activated T cells 2; Nfkb1, nuclear factor κB subunit 1; Nr3c2, nuclear receptor 3 c2; Nr4a1, nuclear receptor subfamily 4 group A member 1; PAN, Puromycin aminonucleoside; ROS, reactive oxygen species; Stat3, signal transducer and activator of transcription 3; Tef, TEF transcription factor, PAR BZIP family member; TF, transcription factor; UMAP, uniform manifold approximation and projection.*

To further dissect gene regulation in the injured and treated podocytes, we used chromVAR to estimate transcription factor activity, and we inferred a gene regulatory network (GRN) using the tool scMEGA (Figure 3D, Supplemental table 5)⁵². To identify GRNs that were important for recovery of podocytes, podocyte pseudotime was used as previously identified (Figure 3B). Since we were interested in the changes from injured to recovering to healthy podocytes, the podocytes treated with prednisolone alone were not included in this analysis. GRNs controlled by transcription factors nuclear receptor subfamily 4 group a member 1 (*Nr4a1*), kruppel-like factor 5 (*Klf5*) and nuclear factor κB subunit 1 (*Nfkb1*) were active during injury (Figure 3D, Supplemental figure 9). Prednisolone reduced the activity of these transcription factors in podocytes and stimulated LIM homeobox transcription factor 1β (*Lmx1b*), AT-rich interaction domain 3B (*Arid3b*) and homeobox C6 (*Hoxc6*), which are active in healthy podocytes. Interestingly, GRN analysis identified some circadian rhythm related transcription factors, such as D-box binding PAR BZIP transcription factor (*Dbp*) and TEF transcription factor, PAR BZIP family member (*Tef*), which were upregulated by prednisolone but were not active in healthy or injured podocytes. These circadian transcription factors were also upregulated in the prednisolone condition and increased accessibility was seen surrounding the *Dbp*, HLF transcription factor, PAR BZIP family member (*Hlf*) and *Tef* motifs sequence in the podocytes from prednisolone and PAN plus prednisolone conditions, indicating increased transcription factor activity (Supplemental figure 10). Therefore, activation of circadian



< Figure 4. Fatty acid metabolism is upregulated in injured podocytes and downregulated by prednisolone to induce podocyte recovery. A) Violin plots showing the expression of *Cpt1b*, *Pdk4* and *Lpin2* in podocytes from control, prednisolone, PAN or PAN plus prednisolone condition. B-C) Visual representation of the genomic accessibility surrounding the (B) *Cpt1b* and (C) *Pdk4* genes. Differential accessible locations are highlighted with the green squares. Co-accessible links of DNA are added, calculated by Cicero. D) Centrality scores of transcription factors in the gene regulatory network from control and PAN podocytes. E) RNA and ATAC expression of *Ppargc1a* in the podocytes from the different experimental conditions. Abbreviations: *Bbx*, BBX high mobility group box domain containing; *Chd2*, chromodomain helicase DNA binding protein 2; *Chkb*, choline kinase β ; *Cpt1b*, carnitine palmitoyltransferase 1B; *Creb3l2*, cAMP responsive element binding protein 3 like 2; *Elf1*, E74 like ETS transcription factor 1; *Ets1*, ETS proto-oncogene 1; *Ets2*, ETS proto-oncogene 2; *Foxn3*, forkhead box N3; *Foxp1*, forkhead box P1; *Hivep2*, HIVEP zinc finger 2; *Ikzf2*, IKAROS family zinc finger 2; *Klf7*, kruppel-like factor 7; *Klhd7b*, kelch domain containing 7B; *Lmx1b*, LIM homeobox transcription factor 1B; *Mapk8ip2*, mitogen-activated protein kinase 8 interacting protein 2; *Nfia*, nuclear factor I A; *Npas2*, neuronal PAS domain protein 2; *Nr4a1*, nuclear receptor subfamily 4 group A member 1; *PAN*, puromycin aminonucleoside; *Pbx2*, PBX homeobox 2; *Pbx3*, PBX homeobox 3; *Pdk4*, pyruvate dehydrogenase kinase 4; *Pknox2*, PBX/knotted 1 homeobox 2; *Plagl1*, PLAG1 like zinc finger 1; *Ppargc1a*, PPARG coactivator 1 α ; *Srebf2*, sterol regulatory element binding transcription factor 2; *Syce3*, synaptonemal complex central element protein 3; *Tead1*, TEA domain transcription factor 1; *Tef*, TEF transcription factor, PAR BZIP family member; *Wt1*, Wilms tumour 1; *Zbtb7c*, zinc finger and BTB domain containing 7C.

rhythm transcription factors by prednisolone may help to induce podocyte recovery. However, activation of these transcription factors is not seen in healthy podocytes, indicating a narrow window of effect (i.e. activation in injured podocytes is beneficial, but stimulation in healthy podocytes may lead to adverse effects).

Altogether, we observed changes in Rho and Rac signalling in the PAN condition that have been previously described in podocyte injury and were reversed by prednisolone treatment. Prednisolone strongly induced circadian rhythm-related transcription factors, which may help to induce podocyte recovery.

Podocyte free fatty acid metabolism increases after injury and is restored by prednisolone

Recently, metabolism has acquired increased attention in glomerular diseases, and targeting metabolic dysregulation has been proposed as an attractive target⁵³. Increased expression of genes associated with fatty acid metabolism (pyruvate dehydrogenase kinase 4 (*Pdk4*), Lipin 2 (*Lpin2*), and *Cpt1b*) was observed in the injured podocytes, and was decreased by prednisolone treatment (Figure 4A, supplemental tables 1-2). The increased *Cpt1b* and *Pdk4* expression in injured podocytes is in line with a recent study by Bhayana et al.

and with publicly available minimal change disease (MCD)/FSGS patient RNA sequencing data^{29,54}. Both *Cpt1b* and *Pdk4* also had increased DNA accessibility at the promoter region in injured podocytes, which was less accessible in the PAN plus prednisolone condition (Figure 4B-C). CellOracle was also used to infer a GRN in the dataset, using the pre-built GRN constructed from a rat promoter database provided by CellOracle⁵⁵. Among others, an increased activity of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a*; PGC-1 α) transcription factor was observed in the PAN condition (Figure 4D). Interestingly, PGC-1 α has been demonstrated to stimulate *Cpt1b* and *Pdk4* expression⁵⁶. Although the PGC-1 α GRN was still found in the PAN plus prednisolone condition, an increase in RNA expression and gene accessibility for *Ppargc1a* was observed in the PAN condition, which decreased in the PAN plus prednisolone condition (Figure 4E, supplemental table 1). Overall, increased expression of *Ppargc1a*, *Pdk4* and *Cpt1b* was observed in injury and restored by prednisolone, which may lead to altered fatty acid metabolism.

CPT1 inhibition prevents podocyte injury in vitro

To validate the effects of fatty acid metabolism on podocyte injury and recovery, ciPODs were injured with PAN and treated with inhibitors for CPT1, PDK4 and PGC-1 α (Figure 5A-B, Supplemental figure 11). The ciPODs were largely lost after PAN injury for 40 hours, visualized by staining of the nucleus and the actin cytoskeleton, and counting the cells. Simultaneous treatment with PAN and the CPT1 inhibitor etomoxir resulted in reduced cell loss compared to PAN treatment alone. CPT1 inhibition significantly increased the number of podocytes surviving treatment with 25 μ g/mL PAN, and a similar effect was observed when using 50 μ g/mL PAN (Figure 5B). Even though PDK4 and PGC-1 α were found to be involved in podocyte injury in the snRNA and snATAC datasets, the inhibition of PDK4 or PGC-1 α did not result in the prevention of podocyte loss (Supplemental figure 11). Human kidney organoids were similarly exposed to 25 μ g/mL PAN and treated using the 50 μ M etomoxir and bulk RNA sequencing was performed on fluorescence activated cell sorting (FACS) podocytes from the organoids. Using DESeq2 analysis, we identified some known podocyte markers to be significantly downregulated in organoid podocytes that were treated with PAN, such as *MAGI2*, *DACH1* and *TGFBR3*⁵⁷⁻⁶⁰. However, no significant rescue of these markers was seen after PAN plus etomoxir treatment. Although no significant differences were observed, these podocyte markers did show slightly increased expression in the PAN plus CPT1 inhibitor condition (Figure 5C). The same trend was seen with other podocyte markers, such as *NPHS1*, *WT1* and organoid podocyte

markers PTPRO, SOST and CLIC5^{57,58}. Overall, the inhibition of CPT1 may present a more specific target for inducing podocyte recovery, but these proof-of-concept experiments should be validated in future studies.

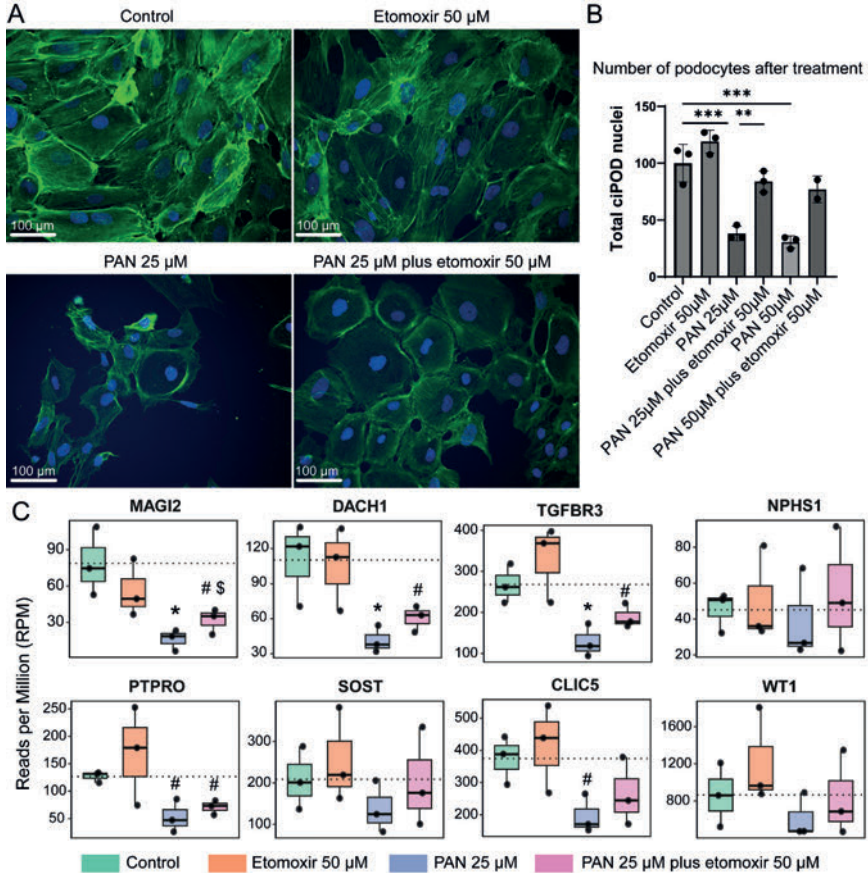


Figure 5. Inhibition of CPT1 with etomoxir protects podocytes in vitro from PAN injury. A) Immunofluorescent imaging of ciPODs at 10x magnification in control condition or treated with PAN, CPT1 inhibitor etomoxir or both. Nuclei (hoechst33342, blue) and actin cytoskeleton (phalloidin, green) staining is shown B) Quantification of cell numbers in three independent wells per condition, performed in three or two (PAN and etomoxir 50 µM) biological replicates. C) Reads per million (RPM) for several podocyte markers from literature in the organoid-podocyte samples from control, etomoxir, PAN and PAN plus etomoxir condition. A dotted line is drawn at the mean RPM value of the control samples. * indicates that gene expression is lower compared to control with a adjusted p-value < 0.05, # indicates that expression is lower compared to control with an adjusted p-value > 0.05 but raw p-value < 0.05 and \$ indicates that gene expression is higher compared to the PAN condition with adjusted p-value > 0.05 but p-value < 0.05. Statistics by ordinary one-way ANOVA with Tukey correction for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: ciPOD, conditionally immortalized podocyte; CPT1, carnitine palmitoyltransferase I; PAN, puromycin aminonucleoside; RPM, reads per million.

Discussion & Conclusion

In this study, snRNA and ATAC sequencing were utilized in an iNS model treated with prednisolone to elucidate the mechanisms underlying podocyte recovery facilitated by prednisolone. A model of iNS based on PAN exposure that was partially rescued by prednisolone was established and used for snRNA and snATAC analysis. Multiple cell types were affected by PAN and prednisolone, with for instance increased *Cd44* gene expression in PECs in injury, which was decreased by prednisolone. An increase in fatty acid metabolism genes was found in injured podocytes, which was restored by prednisolone treatment. Using GRN analysis, we identified activation of the transcription factor *Ppargc1a* in the PAN condition, which coincided with increased mRNA expression and chromatin accessibility of the *Cpt1b* and *Pdk4* genes. Inhibition of CPT1 but not PDK4 or PGC-1 α prevented podocyte loss *in vitro*. Altogether, this study presents the first snRNA and snATAC dataset of experimental iNS and its treatment with prednisolone and discovered an important role for fatty acid metabolism in podocyte injury and recovery following prednisolone treatment.

The snRNA and snATAC datasets are in line with results from previous studies describing prednisolone effects on podocytes, supporting the validity of the model used in this study. For example, changes in genes regulating the Rho/Rac pathway were shown between injury and recovery with prednisolone as previously described by McCaffrey et al.^{61,62}. Furthermore, prednisolone treatment downregulated pro-inflammatory tumour necrosis factor α (TNF α) and NF κ B pathways that have been extensively mentioned as downstream targets of prednisolone⁶³.

The aim of this study was to investigate the prednisolone-induced changes in the podocyte as a key cell type in iNS development and recovery^{5,12}. However, using Augur and ligand-receptor signalling analysis, various changes in other cell types during injury and recovery were found as well. For instance, increased expression of CD44 in PECs in the injury condition was observed, which decreased after prednisolone treatment, on both protein and transcriptomic level. CD44 expression in PECs of PAN rats has been previously reported, suggesting that the PAN rat model may be most reflective of early FSGS, since in patients, no CD44 staining is observed in MCD^{42,64}. An interesting question would be whether these changes are induced directly by prednisolone action on the other cell types or indirectly by changes in the podocyte. Mesangial cells have been shown to respond to glucocorticoids,

and glucocorticoid receptor knockouts in GEnCs and PECs specifically change the progression of kidney diseases^{11,65,66}. However, based on the unsupervised clustering of the snRNA and snATAC data, the podocytes cluster separately from each other, whereas the other cell types are not separated by the experimental condition. This indicates that experimental condition changes in podocytes are much more significant than in other cell types. Second, expression of the prednisolone-responsive gene *Tsc22d3* was only found in the podocytes after prednisolone treatment⁴⁵. Similarly, the circadian rhythm genes identified in this study are much more strongly expressed in the podocytes compared to the other cell types. Altogether, podocytes seem to be most affected by PAN and prednisolone directly, and effects seen in other cell types are more likely to be secondary to changes in the podocytes.

Previous studies have already shown stimulation of circadian rhythm transcription factors by the glucocorticoid dexamethasone in human podocytes⁶⁷. In other organs, a link has been described regarding tissue sensitivity to circulating natural glucocorticoids, and the sensitivity to glucocorticoids can change during the day^{68,69}. Interestingly, studies have shown that if metabolism is important to target, the treatment regimen can significantly change the effects of prednisolone, with sometimes even a complete loss of effect when the time of administration is changed^{70,71}. Considering these results, future studies should take the time of administration into account when investigating the effects of prednisolone on metabolism.

We observed an increase in *Pdk4*, *Cpt1b* and *Ppargc1a* in injured podocytes, indicative of increased fatty acid oxidation⁷². In the injured podocytes treated with prednisolone, we identified differentially upregulated genes associated with glycolysis. Glycolysis has been implicated to be important for the foot processes of podocytes, since mitochondria are mainly located in the cell body and key glycolytic enzymes are more abundant in the foot processes⁷³. Indeed, by knocking out several genes involved in oxidative phosphorylation in podocytes specifically, no phenotype was observed, indicating that podocytes are not reliant on oxidative phosphorylation⁷⁴. In contrast, the knockdown of *Pkm2*, a kinase important in glycolysis, led to more severe albuminuria in a diabetic nephropathy mouse model, and the activation of PKM2 could reverse podocyte injury⁷⁵. The authors also show that PKM2 activation establishes podocyte recovery at least in part by increasing PGC-1 α expression and mitochondrial biogenesis. Other studies have similarly shown the beneficial effects of PGC-1 α , and lower levels of PGC-1 α are associated with kidney disease^{76,77}. However, overexpression of

PGC-1 α induces collapsing glomerulopathy in a rodent model, and in cultured podocytes, it leads to increased *Pdk4* expression and fatty acid consumption, together with an injured phenotype⁷⁷. In summary, these results indicate a tightly regulated dual role for PGC-1 α . The upregulation of PGC-1 α could lead to podocyte injury, as seen in our podocyte model, whereas downregulation does not actively lead to podocyte injury, although it is associated with podocyte injury. However, the downregulation of PGC-1 α alone should not induce injury and could therefore be investigated as a target for treatment in podocyte injury accompanied by PGC-1 α overexpression.

The induced expression we observed of *Cpt1b* in the injured condition, with a downregulation by prednisolone has not been described before for podocytes. However, a recent article described the upregulation of *Cpt1a* as protective for high glucose induced podocyte injury, which mimics diabetic nephropathy⁷⁸. This may indicate that different CPT1 isoforms have diverging roles in the podocytes that can be both beneficial and detrimental. However, using the non-specific CPT1 inhibitor etomoxir, beneficial effects on podocytes during injury were found in this study, suggesting that the mechanism of injury may be more likely to cause this discrepancy.

A recent study showed that etomoxir is capable of binding many other proteins involved in fatty acid metabolism and transport in hepatocytes and is therefore not suitable as an inhibitor of mitochondrial fatty acid β -oxidation specifically⁷⁹. Still, our results in ciPODs with etomoxir provide a first proof-of-concept that fatty acid metabolism is deregulated in iNS and inhibition of metabolism might rescue podocytes. In the organoids, a less clear rescue was observed, which may be caused by heterogeneity in the cell population that was isolated by FACS or the concentration of etomoxir might be suboptimal. For future studies, other fatty acid metabolism inhibitors, glycolysis stimulators or a combination of them may be tested to find more effective metabolic treatment strategies. Also, future studies may focus on including metabolomics analysis to further dissect the mechanism of injury and recovery in iNS.

In conclusion, we present the first single-nuclei multi-omics atlas for iNS with prednisolone treatment as a valuable resource for the field. An upregulation of fatty acid metabolism was identified in injured podocytes, which was decreased by prednisolone. Prednisolone also strongly induced circadian rhythm-related transcription factor activity that might contribute to decreasing fatty acid metabolism in podocytes. Inhibition of fatty acid metabolism leads

to lower podocyte loss after PAN injury *in vitro*. Together, these results propose an important role for metabolism in podocyte injury and recovery and provide proof-of-concept that targeting metabolism in iNS might lead to new therapeutic strategies.

Methods

Animal study

All of the animal procedures described were in accordance with the guidelines of the Animal Ethics Board of the Radboud University Nijmegen. Six week old male Wistar rats were purchased from Charles-River and housed in the RadboudUMC central animal research facility according to local regulations. Rats were acclimatized for 1 week to their new housing before starting the experiment by intravenous injection of PAN (MedChemExpress, Solentuna, Sweden). Treatment with prednisolone was achieved by daily intraperitoneal injection (in the morning) with methylprednisolone sodium succinate: Solu-medrol (Pfizer, Capelle a/d IJssel, the Netherlands).

Urine collection and proteinuria measurement

For urine collection, rats were placed in a metabolic cage for 18 hours, after which urine was collected. The total amount of urinary protein and creatinine was determined by the RadboudUMC central laboratory for diagnostics. Urinary protein levels were determined using a turbidometric assay using both an Ethylenediaminetetraacetic acid (EDTA) containing alkaline solution and benzothionium chloride. Urinary creatinine levels were obtained using the enzymatic kinetic colorimetric assay (creatinine PAP). The urinary protein-to-creatinine ratio was determined as mg of protein per mg of creatinine.

Collection and processing of kidney tissue

Kidneys were collected by sedating the rats using inhalation of isoflurane. The left kidney was clamped and removed, keeping the renal artery clamped afterwards. Next, a heart puncture followed by blood collection was performed, after which whole body perfusion was done by inserting a blunt needle directly into the heart and clamping the needle. First, the circulation was flushed with 50 mL NaCl 0.9% and afterwards the organs were fixed using 50 mL of 3% paraformaldehyde (PFA). The right kidney was collected after perfusion and immediately processed for paraffin embedding. The left kidney that was removed, before whole body fixation, was immediately transferred to ice and a small piece

was used to snap freeze. Another small piece of the kidney was fixated with 2.5% (w/v) glutaraldehyde dissolved in 0.1M sodium cacodylate overnight at 4°C. The next day, the solution was replaced by 0.1M sodium cacodylate to store the tissue at 4°C until further processing for electron microscopy. The remaining kidney tissue was used for glomerulus isolation, using a sieving approach, as described before⁸⁰. Briefly, kidney tissue was minced and forced through a sieve with pores of 150 µm. Material was collected on a sieve with pores of 90 µm, forced through and collected on a sieve with pores of 75 µm. Isolated glomeruli were collected with Phosphate Buffered Saline (PBS, Gibco, Thermo Fisher, Ochten, the Netherlands) + 1% bovine serum albumin (BSA, Sigma-Aldrich, Zwijndrecht, the Netherlands) and collected in 15mL tubes. Glomeruli were left for 5 minutes on ice to allow them to settle to the bottom, after which the supernatant was removed as much as possible and the glomeruli were either resuspended in lysis buffer (Qiagen RNeasy kit, Qiagen, Venlo, the Netherlands) or used for snRNA and ATAC sequencing. For snRNA and ATAC sequencing, the glomeruli were washed once with PBS + 1% BSA and after removal of the majority of the liquid, snap frozen and stored at -80°C for later use.

Histological analysis of kidney tissue

Kidney tissue was either fixed with PFA and formalin for immunofluorescent or histological staining or with glutaraldehyde for transmission electron microscopy. For immunofluorescent staining, 2 µm thick sections were used and deparaffinated and rehydrated using xylene and ethanol. Next, antigen retrieval was performed using microwave boiling for 10 minutes in tris-EDTA buffer, pH 8 (VWR, Amsterdam, the Netherlands). After cooling down, slides were washed with PBS and incubated overnight at 4°C with primary antibody solution (table 1) in PBS + 1% BSA. The next day, slides were washed three times with PBS and incubated with secondary antibody (table 1), for 1 hour at room temperature. Afterwards, slides were washed again three times with PBS and mounted using Fluoromount-G with DAPI mounting medium (Southern Biotech, Birmingham, AL, USA) and visualized using a Leica DMI6000B high-content microscope or a Panoramic MIDI II digital scanner. For electron microscopy, tissue that was stored at 4°C was used and fixed for 1 h using 2% OsO₄ buffered in Palade's buffer [0.23 % (w/v) CH₃COONa, 0.58% (w/v) C₈H₁₁N₂NaO₃ dissolved in MilliQ (pH 7.3)]. Tissue was dehydrated and embedded in Epon 812 (Sigma-Aldrich). Ultra-thin sections (90 nm) were cut using a Leica Ultracut and mounted on copper grids and contrasted at room temperature with uranyl acetate and lead citrate. Sections were assessed using a Jeol JEM 1400 electron microscope at 60 kV. For image collection, a digital camera was used (Gatan).

Table 1. Antibodies used and their dilutions

Primary antibodies (1:100 diluted)	Secondary antibody (1:200 diluted)
Mouse anti-desmin, Immunologic, clone D33, ILM5116	Donkey anti-mouse IgG (H+L), Alexa Fluor 488, Thermo Fisher, A21202
Sheep anti-nephrin, R&D systems, AF4269	Donkey anti-sheep IgG (H+L), Alexa Fluor 647, Thermo Fisher, A21448
Mouse anti-CD44, clone 8E2, Cell Signalling, 5640	Donkey anti-rabbit IgG (H+L), Alexa Fluor 568, Thermo Fisher, A10042

Semi-automated quantification of desmin and nephrin staining

Slides were scanned using the Panoramic MIDI II scanner. In every section, 12 glomeruli were randomly selected to be analysed and the separate desmin and nephrin images were made at the same position and stored. To avoid the inclusion of extra-glomerular desmin, which is also present in arteries/veins in the kidney, a circle was drawn surrounding the glomerular tuft. In an automated way in Fiji, this selection was used to select the nephrin positive area, the desmin positive area, and calculate the overlapping areas, with both nephrin and desmin present (Supplemental figure 1).

Single-nuclei RNA and ATAC sequencing

Nuclei isolation was performed as described in the online manual from 10X Genomics: Nuclei Isolation from Complex Tissues for Single Cell Multiome ATAC + Gene Expression Sequencing (CG000375 • Rev C), with a few adaptations. After the addition of NP40 lysis buffer, samples were incubated on ice for 5 minutes and the protocol was followed as instructed. After FACS sorting, nuclei suspension were obtained with: 2400 nuclei/ μ L (control), 4110 nuclei/ μ L (prednisolone), 665 nuclei/ μ L (PAN), 2130 nuclei/ μ L (PAN plus prednisolone), which was enough to load approximately 16,000 nuclei per 10X Genomics chip and continue with the Next GEM Single Cell 3' v3.1 kit according to the instructions in manual: CG000204 Rev D. For ATAC sequencing, approximately 10,000 nuclei were loaded per chip and processed with the Next GEM Single Cell ATAC kit v2 according to the instructions in manual: CG000496 Rev B. snRNA sequencing samples were sequenced on an Novaseq6000 system (Illumina, San Diego, USA), using a SP Flowcell (Illumina) with run settings 50-8-16-50 cycles for snATAC sequencing libraries an S2 Flowcell (Illumina) was used with run settings 28-10-10-90 cycles.

***In vitro* experiments using human pluripotent induced stem cell-derived kidney organoids**

iPSCs were generated from human fibroblasts like previously described by Jansen et al.⁵⁸ Similarly, maintenance culture, passaging and seeding of the iPSC was performed like described by Jansen et al.⁵⁸ For 3D organoid differentiation, iPSCs were seed on Geltrex-coated cell culture plates (Gibco) in complete Essential 8 (E8) medium (Gibco) supplemented with E8 supplement and 0.5% (v/v) antibiotic-antimycotic and 1x RevitaCell at a density of 26,000 cells/cm². After 24 hours, the medium was replaced by E6 medium supplemented with 6 μ M CHIR 99021 (Tocris, Bio-Techne). Half of the wells were cultured in E6 medium supplemented with 200 ng/ml fibroblast growth factor 9 (FGF9, R&D Systems) and 1 μ g/ml heparin (Sigma-Aldrich) from day 3 until day 7 (AM). The other half of the wells was cultured with 6 μ M CHIR until day 5 and was then cultured with E6 plus FGF9 plus heparin from day 5 until day 7 (PM). At day 7, cells were harvested using trypsin-EDTA and the cell suspension was mixed to contain 1:2 (AM:PM) ratio. Cells aggregates, consisting of 300,000 cells, were transferred to Transwell plates (Corning) and incubated for at least 1 hour at 37°C, 5% (v/v) CO₂, with E6 medium supplemented with 5 μ M CHIR 99021. Five days later, E6 medium supplemented with epidermal growth factor (EGF, 10 ng/ml, R&D Systems) and bone morphogenic protein 7 (BMP7, 50 ng/ml, R&D Systems) and stromal derived factor 1 β (SDF1 β , 10ng/mL, R&D Systems) was used to induce maturation of the organoids. Organoids were used for experiments at day 7+18 by changing the medium to experimental medium. Experimental medium consisted of E6 medium supplemented with EGF, BMP7 and SDF1 β for the control plus addition of 50 μ M Etomoxir (HY-50202, MedChemExpress) and/or 25 μ g/mL PAN (HY-15695, MedChemExpress). After 24 hours organoids were harvested for podocyte isolation using FACS.

FACS isolation of organoid podocytes and preparation for bulk RNA sequencing

Organoids were disassociated with Accutase and prepared for FACS as described by Jansen et al.⁵⁸ Briefly, organoids were disassociated into single-cell suspension, strained and stained with primary antibody against nephrin (1:100, R&D Systems, AF4269) for 45 minutes at 4°C. After washing, incubation with secondary antibody was performed (1:200, donkey anti-sheep Alexa Fluor™ 647, Thermo Fisher, A-21448) for 30 minutes at 4°C. DAPI was added 10 minutes before sorting on the fluorescence cell sorter (BD Biosciences; FACSARIA™ II SORP 18-color). DAPI⁻/Nephrin⁺ cells were collected RLT buffer

(RNeasy, Qiagen) and RNA was isolated according to the manufacturer's instructions. Sequencing was performed at Single Cell Discoveries, a (single cell) sequencing provider located in The Netherlands, using an adapted version of the CEL-seq protocol and is described by Jansen et al.⁵⁸ In short, an adapted version of the scRNA-seq protocol of CEL-Seq was used for bulk mRNA sequencing^{81,82}. Briefly, samples were barcoded with CEL-seq primers during a reverse transcription and pooled after second strand synthesis. The resulting cDNA was amplified with an overnight *in vitro* transcription reaction. From this amplified RNA, sequencing libraries were prepared with Illumina Truseq small RNA primers. Paired-end sequencing was performed on the Illumina Nextseq500 platform.

Bioinformatic analysis

Bulk RNA sequencing of organoid podocytes

For bulk RNA sequencing, read 1 was used to identify the Illumina library index and CEL-Seq sample barcode. Read 2 was aligned to the GRCh38 human reference genome using STARsolo (v2.7.11b)⁸³. Reads that mapped equally well to multiple locations were discarded. Mapping and generation of count tables was carried out using the MapAndGo script1. Samples were next normalized to reads per million, dividing the gene count by the total library size and multiplied by a million to generate plots in Figure 5 using ggplot2 (v3.5.2)⁸⁴. The raw read counts table was used to perform DESeq2 differential gene expression analysis⁸⁵. Scripts are available under "Data and code availability".

snRNA sequencing of rat glomeruli

Briefly, raw sequencing reads were processed with CellRanger pipeline version cellranger-7.1.0, using the mRatBN7 reference transcriptome to produce count matrices in the h5-format. Seurat (v5.1.0) was used to filter for cells containing more than 200 features per cell, but not more than 4000 and containing less than 20% mitochondrial reads (Supplemental figure 3)³⁷. Doublets were removed using DoubletFinder (v2.0.6) and filtered datasets were saved and are also provided in the GEO submission (Supplemental figure 3)⁸⁶. Next, integration of the 4 datasets was done with harmony (v1.2.1), and manual annotation was performed based on cell type markers collected from literature as shown in Supplemental figure 4⁸⁷.

snATAC of rat glomeruli

Raw snATAC sequencing reads were processed with the CellRanger pipeline `cellranger-atac-2.1.0` using the `mRatBN7` reference genome. snATAC datasets were processed using the Signac package (v1.14.0) according to the online tutorials provided³⁶. Briefly, CellRanger output was processed as `ChromatinAssay` and then into a Seurat object. After initial quality filtering for transcription start site (TSS) enrichment, the fraction of the reads in peaks, the number of peaks and nucleosome signal, normalization was performed using term frequency – inverse document frequency (TF-IDF), and linear dimensionality reduction using singular value decomposition (SVD). Since the clusters obtained in ATAC data are more difficult to annotate, we performed label transfer from the RNA sequencing data to the ATAC data and integrated the RNA and ATAC dataset. After integration of the datasets for better annotation, the datasets were either used separately or combined to perform downstream analyses, since some downstream analyses are only capable of handling one modality. We identified one cluster that was difficult to annotate, with some low level expression of podocyte markers. On inspection, we noted a very low number of features in this cluster (annotated as `podocyte_low`) and we removed the cluster from the dataset to focus the analysis on high quality cells.

snRNA downstream processing

To identify which clusters change in the several conditions, Augur analysis (v1.0.3) was performed by subsetting cells per condition and making pairwise comparisons³⁸. For ligand-receptor interactions, either all cell types or only glomerular cell types from all the conditions were used as input and Cellchat (v2.1.2) was used to identify upregulated signalling pathways in the different conditions⁸⁸. Using VISION (v3.0.2), the mouse hallmark H terms were used to assess their activity in the different podocyte clusters⁸⁹. Using the ClusterProfiler (v4.12.6) and the ReactomePA (v1.48.0) packages, GO/KEGG/Reactome enrichment analysis was performed on differential genes identified by differential gene expression analysis between clusters, or genes identified by monocle3 (v1.3.7) that influence the differentiation of podocytes from the control conditions to the PAN condition⁹⁰⁻⁹².

snATAC downstream processing

The accessibility of interesting targets was visualized using the "CoveragePlot" function in Signac, after performing cicero (v1.3.9) co-accessibility analysis⁹³. For the identification of differentially accessible regions, the "FindMarkers" function from Seurat was used. Furthermore, monocle3 (v1.3.7) pseudotime

analysis was also performed in the ATAC datasets to identify changes in the epigenome. Transcription factor activity was determined using ChromVAR (v1.26.0) with the cisBP mouse transcription factor database, as provided by Kartha et al. and using the transcription factor activities, differential transcription factor activity was found using the "FindMarkers" function^{94,95}. For transcription factor footprint plots, the "Footprint" function from Signac was used.

Gene regulatory network analysis

In order to combine RNA and ATAC data and analyse differential GRNs in healthy, injured and treated conditions CellOracle (v0.20.0) and scMEGA (v1.1.0) were used, according to the instructions provided by the authors^{52,55}. For scMEGA, briefly, the cells originating from the RNA and ATAC datasets were paired and matched to obtain cells with RNA and ATAC data. Next, transcription factor activity and transcription factor expression were correlated, similar as gene expression and gene accessibility to keep only transcription factors and genes that are consequently expressed in both the RNA and ATAC data. Using these transcription factor, genes and accessibility a GRN is then constructed and correlated to the pseudotime/trajectory that has been established in the object. In order to use CellOracle, the datasets needed to be in an Anndata object instead of a Seurat object. Because the conversion of a Seurat object to Anndata did not work properly, the pre-processing steps of the dataset were repeated using scanpy to create a correctly functioning Anndata object with the snRNA sequencing dataset⁹⁶. The podocytes from the control, PAN and PAN plus prednisolone conditions were used for the GRN analysis. The pre-built GRN based on a rat promoter database was used according to the instructions from the makers of CellOracle.

In vitro experiments using conditionally immortalized podocytes

The ciPOD line used in this study was kindly provided by Prof. Saleem (Bristol, UK)⁹⁷. Maintenance culture was done in uncoated 75cm² culture flasks (Greiner, Alphen aan den Rijn, the Netherlands) at 33°C with 5% (v/v) CO₂ in Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12, Gibco) supplemented with 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium (Sigma-Aldrich), 10% heat inactivated fetal calf serum (FCS, Gibco) and 1% (v/v) penicillin/streptomycin (Gibco). Passaging and seeding of cells, maximally at passage 38, was achieved using Accutase (Sigma-Aldrich). ciPODs were seeded at 10,000/cm² in black flat-bottom 96-wells plates (Corning, Corning, NY, USA). After seeding, ciPODs were cultured at 37°C to

initiate differentiation. After 7 days of differentiation, ciPOD medium was replaced with medium containing: nothing as a control, CPT1 inhibitor etomoxir (HY-50202, MedChemExpress), PDK4 inhibitor PDK4-IN-1 (HY-135954A, MedChemExpress) or PGC-1 α inhibitor SR-18292 (HY-101491, MedChemExpress), PAN (HY-15695, MedChemExpress) and combinations of PAN with the inhibitors and cultured at 37°C. After 40 hours, cells were washed once with Hank's balanced salt solution (HBSS) and fixed using 2% PFA at 4°C. After 20m, PFA was removed, cells were washed with PBS and incubated for 2 hours at room temperature in blocking buffer: PBS containing 10% (v/v) donkey serum (GeneTex, Huissen, the Netherlands) and 0.6% (v/v) Triton X-100 (Sigma). Afterwards, ciPOD cytoskeleton was stained with 1:100 Flash Phalloidin™ Green 488 (424201, Biolegend, Amsterdam, the Netherlands) diluted in the same blocking buffer, including 1:1000 hoechst33342 (H3570, Thermo Fisher Scientific), and incubated overnight at 4°C. Phalloidin solution was removed, cells were washed three times with PBS and Fluoromount-G (Southern Biotech) was added before visualization on the Leica DMI6000B high-content microscope. All cell lines used were tested negative for contamination, including Mycoplasma.

Statistical analysis

Statistical analysis was performed using Graphpad prism using appropriate statistical tests. For both UPCR and immunofluorescent desmin quantification statistics, data followed a Gaussian distribution, and comparison between several conditions was performed using a one-way ANOVA with multiple testing and Tukey correction for multiple testing. Only for statistical testing between control, 5mg PAN and 5mg PAN plus prednisolone (supplemental figure 2A), data did not follow a Gaussian distribution and the Kruskal-Wallis test was used with Dunn's correction for multiple comparisons. *In vitro* experiments were performed three times with at least three replicates per experiment.

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Author contributions

M.v.d.B. collected experimental data, lead the analysis of the data, drafted the first version of the manuscript and took responsibility for the final draft of the manuscript.

B.T.v.d.B. assisted with data collection and provided feedback on the data analysis and the final manuscript.

G.L.V.d.G. assisted with data collection and provided feedback on the final manuscript.

F.M.M. contributed by helping with data collection and providing feedback on the final manuscript.

B.W. assisted with data collection and provided feedback on the final manuscript.

A.B. provided essential support in planning and performing the 10x Genomics experiments and gave feedback on the final manuscript.

R.S.K. assisted with sequencing the libraries and provided feedback on the final manuscript.

R.K. provided logistical support for the 10x Genomics experiments and feedback on the final manuscript.

M.F.S. contributed to project conceptualization, assisted with data collection, provided feedback on data analysis, and reviewed all manuscript drafts.

B.S. contributed to project conceptualization, supported data collection, provided feedback on data analysis, and reviewed all manuscript drafts.

J.J. conceptualized the project, secured funding, assisted with data collection, provided feedback on data analysis, supplied infrastructure for the experiments and computational work, and reviewed every manuscript draft.

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Data and code availability

The code used to analyse the RNA and ATAC datasets is deposited on Zenodo under: <https://doi.org/10.5281/zenodo.15715854>. The raw and processed sequencing data are deposited online in GEO under accession numbers: GSE304586 & GSE304592.

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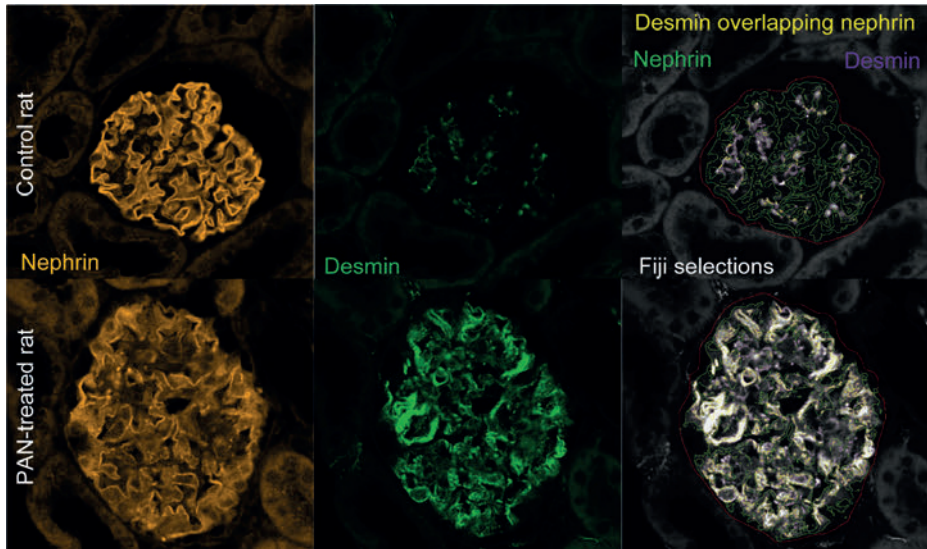
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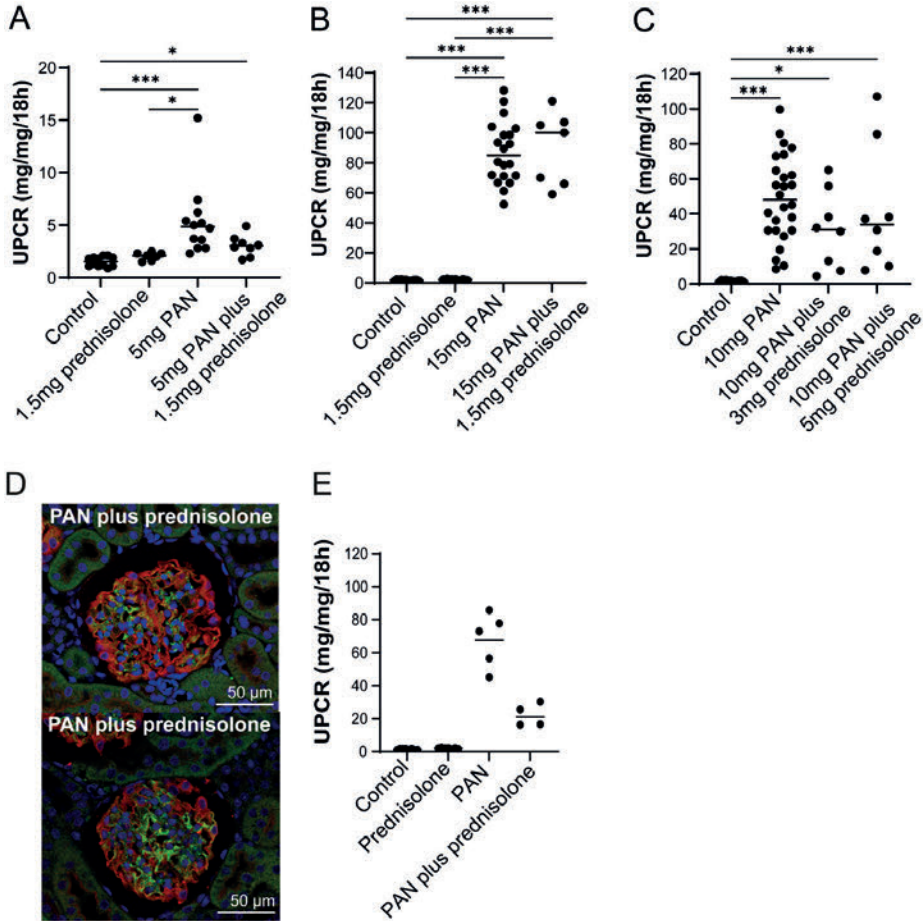
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Supplementary Files and Data

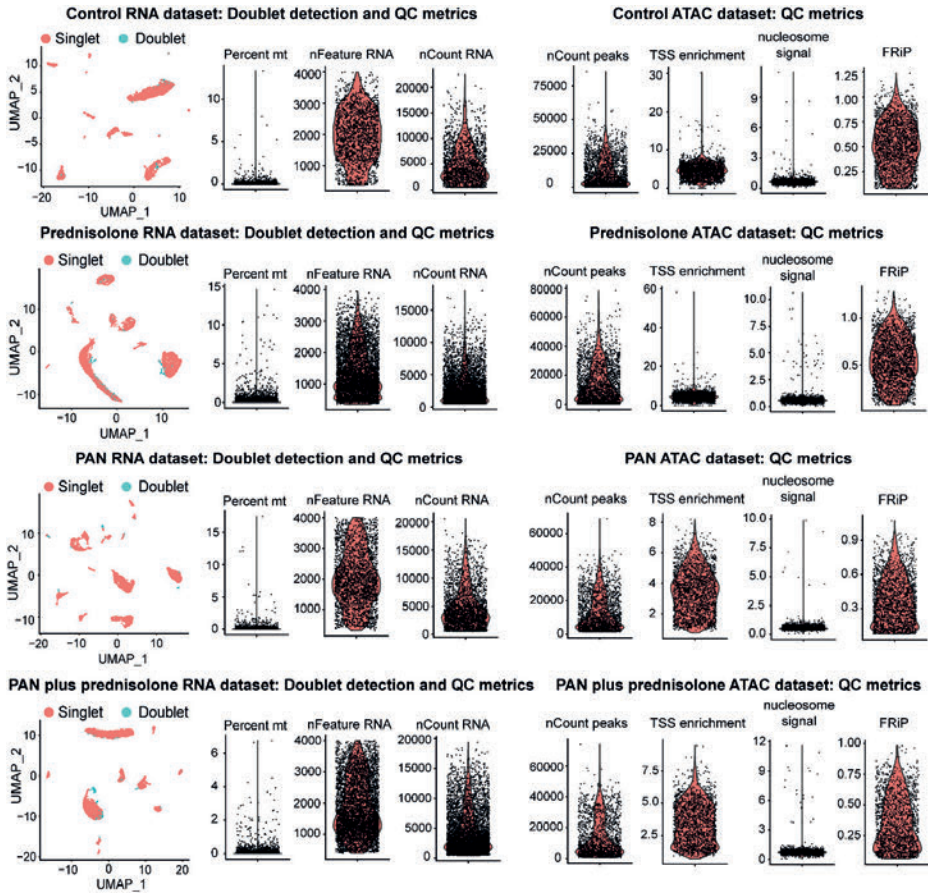
The full supplementary information (including supplementary tables 1-6) will be accessible online after publishing on bioRxiv.



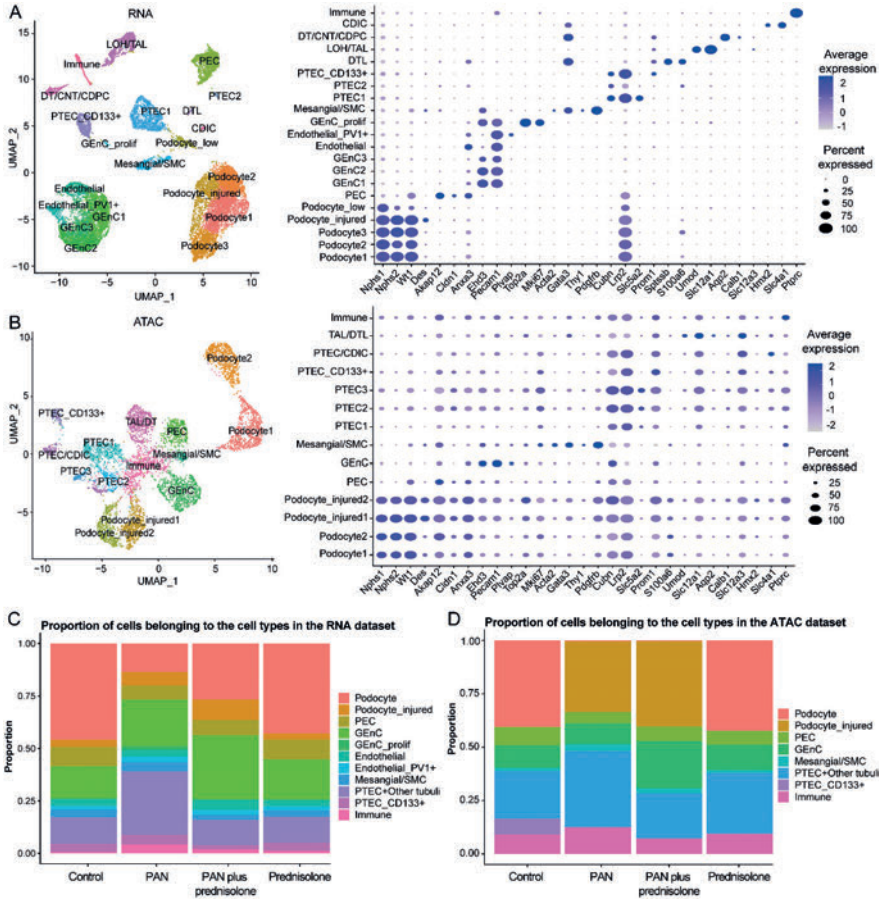
Supplemental figure 1. Quantification of nephrin and desmin immunofluorescent signal using a semi-automated approach in Fiji. Single-color images are depicted for nephrin and desmin with on the right the semi-automated selection of the immunofluorescence signal surface. Based on these selections, overlapping nephrin with desmin surface was determined within the pre-determined glomerular tuft surface, indicated by the red line. Abbreviations: PAN, puromycin aminonucleoside.



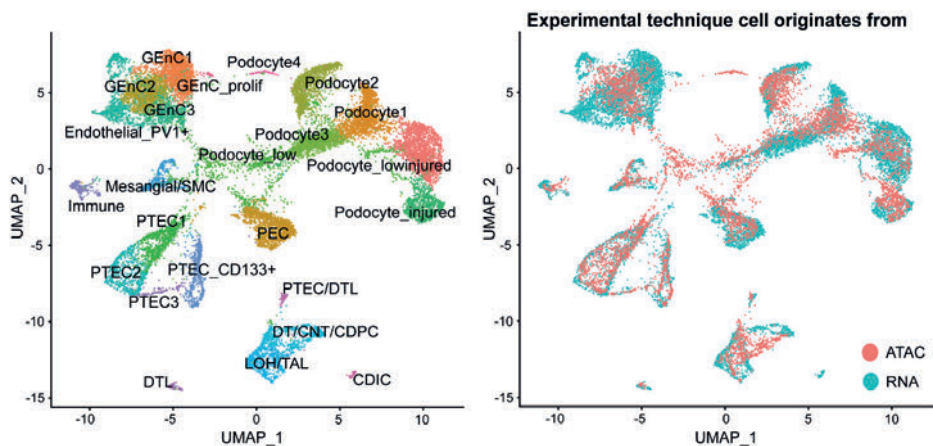
Supplemental figure 2. Quantification of disease severity using proteinuria and desmin quantification. Urinary protein:creatinine ratio (UPCR) as measure of proteinuria for rats treated with A) 5mg/100g body weight PAN and 1.5mg/100g body weight prednisolone, B) 15mg/100g body weight PAN and 1.5mg/100g body weight prednisolone or C) 10mg/100g body weight PAN and either 3 or 5 mg/100g body weight prednisolone. D) Immunofluorescent staining of two glomeruli in the same kidney of a rat treated with PAN and prednisolone. Podocytes (nephtrin, red), injured podocytes (desmin, green), nuclei (blue). E) UPCR values for the rats that were selected to be used for snRNA and snATAC sequencing. 6 rats were selected for control and prednisolone, 5 for PAN and 4 for PAN plus prednisolone. Data displayed in A did not follow a normal distribution and therefore, a Kruskal-Wallis test was used with Dunn's correction for multiple comparison. For the data presented in B, C, D and E, an ordinary one-way ANOVA was used with Tukey test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: UPCR, urinary protein:creatinine ratio; PAN, puromycin aminonucleoside; snRNA, single-nuclei RNA; snATAC, single-nuclei assay for transposase accessible chromatin.



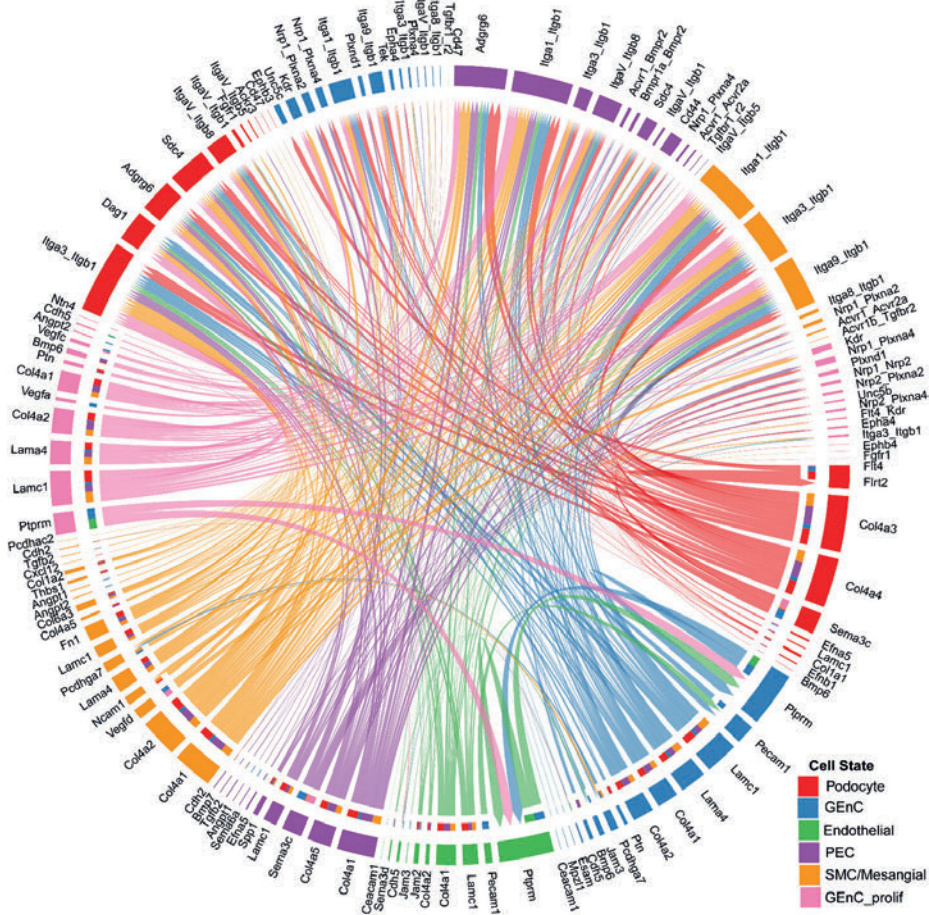
Supplemental figure 3. Preprocessing results for the snRNA and snATAC datasets. Overview of quality controls metrics for the snRNA and snATAC datasets. For RNA, doublet detection is provided, with identified doublets in blue and singlets in red. Furthermore, violin plots with the percent of mitochondrial reads (percent mt), the number of features detected per cell (nFeature RNA) and the total UMIs detected per cell (nCount RNA) are shown. For the snATAC dataset violin plots with the number of peaks detected per cell (nCount peaks), enrichment of peaks around TSS (TSS enrichment), nucleosome signal and fraction of reads in peaks (FRiP) are provided. Abbreviations: FRiP, fraction of reads in peak; PAN, puromycin aminonucleoside; QC, quality control; snRNA, single-nuclei RNA; snATAC, single-nuclei assay for transposase accessible chromatin; TSS, transcription start site; UMAP, uniform manifold approximation and projection; UMI, unique molecular identifier.



Supplemental figure 4. Clustering and markers used for annotations of the snRNA and snATAC datasets. UMAP projection of the A) snRNA dataset and the B) snATAC datasets, annotated based on the selected marker expression on the right. Also visualization of the proportion of the cell types present in the C) snRNA dataset and the D) snATAC dataset. Abbreviations: Acta2, actin- α 2; Akap12, A-kinase anchoring protein 12; Anxa3, annexin A3; Aqp2, aquaporin 2; Calb1, calbindin 1; CDIC, collecting duct intercalated cell; CDPC, collecting duct principal cell; Cldn1, claudin 1; CNT, connecting tubule; Cubn, cubilin; Des, desmin; DT, distal tubule; DTL, descending thin limb; Ehd3, EH domain containing 3; Gata3, GATA binding protein 3; GEnC, glomerular endothelial cell; Hmx2, H6 family homeobox 2; LOH, loop of henle; Lrp2, LDL receptor related protein 2; Mki67, marker of proliferation Ki-67; Nphs1, nephrin; Nphs2, podocin; PAN, puromycin aminonucleoside; Pdgfrb, platelet derived growth factor receptor β ; PEC, parietal epithelial cell; Pecam1, platelet and endothelial cell adhesion molecule 1; Plvap, plasmalemma vesicle associated protein 1; Prom1, prominin 1; PTEC, proximal tubule epithelial cells; Ptprc, protein tyrosine phosphatase receptor type C; PV1, plasmalemma vesicle associated protein 1; S100a6, S100 calcium binding protein A6; Slc4a1, solute carrier family 4 member 1; Slc5a2, solute carrier family 5 member 2; Slc12a1, solute carrier family 12 member 1; Slc12a3, solute carrier family 12 member 3; SMC, smooth muscle cell; snRNA, single-nucleus RNA; snATAC, single-nucleus assay for transposase accessible chromatin; Sptssb, serine palmitoyltransferase small subunit B; TAL, thick ascending limb; Thy1, Thy-1 cell surface antigen; Top2a, DNA topoisomerase II α ; UMAP, uniform manifold approximation and projection; Umod, uromodulin; Wt1, Wilms tumour 1.

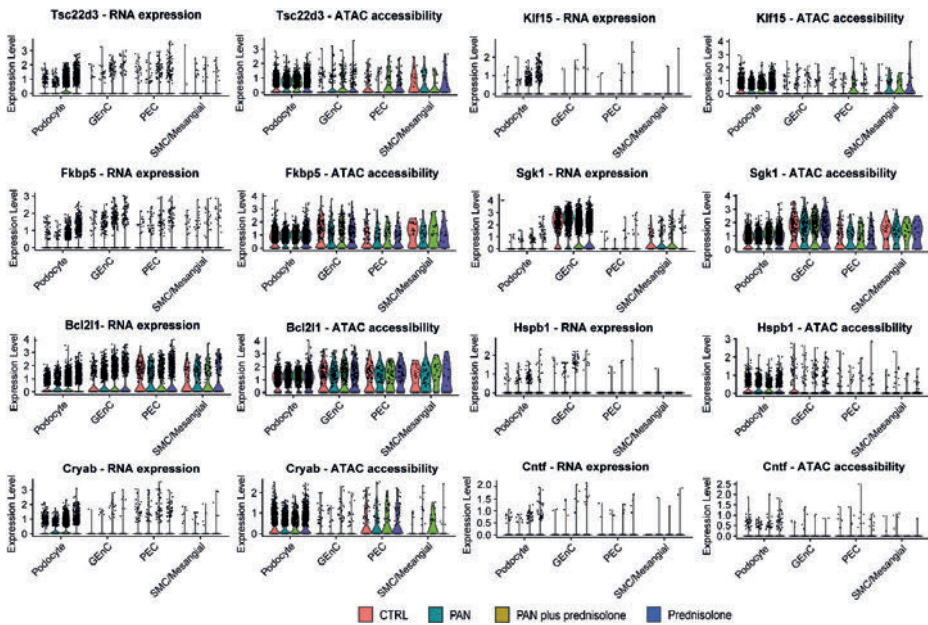


Supplemental figure 5. UMAP of the integrated snRNA and snATAC datasets object including the indication of their origin. Abbreviations: CDIC, collecting duct intercalated cell; CDPC, collecting duct principal cell; CNT, connecting tubule; DT, distal tubule; DTL, descending thin limb; GEnC, glomerular endothelial cell; LOH, loop of Henle; PEC, parietal epithelial cell; PTEC, proximal tubule epithelial cell; PV1, plasmalemma vesicle protein 1; snATAC, single-nucleus assay for transposase-accessible chromatin; snRNA, single-nucleus RNA; SMC, smooth muscle cell; TAL, thick ascending limb; UMAP, uniform manifold approximation and projection.

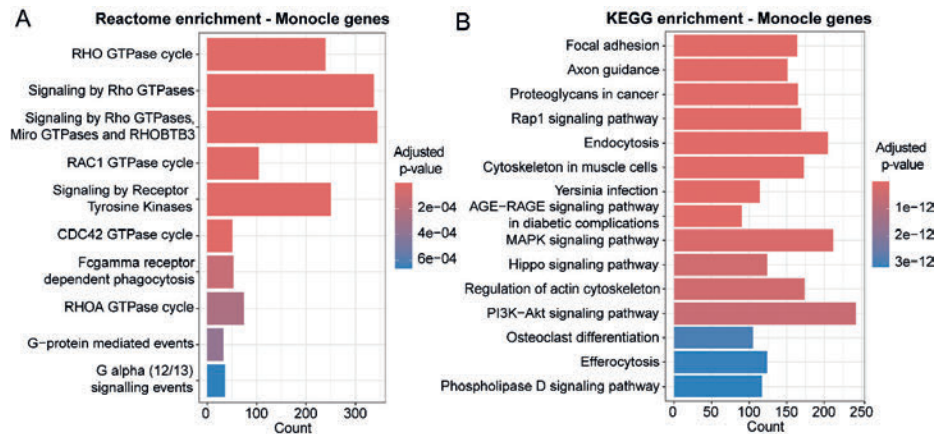


Supplemental figure 6. Cellchat ligand-receptor interactions identified to be upregulated in the PAN condition in glomerular cell types. Abbreviations: Akr3, atypical chemokine receptor 3; Acvr1, activin A receptor type 1; Acvr1b, activin A receptor type 1B; Acvr2a, activin A receptor type 2A; Adgrg6, adhesion G protein-coupled receptor G6 ; Angpt1, angiopoietin 1; Angpt2, angiopoietin 2; Bmp6, bone morphogenetic protein 6; Bmp7, bone morphogenetic protein 7; Bmpr1a, bone morphogenetic protein receptor type 1A; Bmpr2, bone morphogenetic protein receptor type 2; Ceacam1, CEA cell adhesion molecule 1; Chd2, chromodomain helicase DNA binding protein 2; Chd5, chromodomain helicase DNA binding protein 5; Col1a1, collagen type 1 α chain 1; Col1a2, collagen type 1 α chain 2; Col4a1, collagen type 4 α chain 1; Col4a2, collagen type 4 α chain 2; Col4a3, collagen type 4 α chain 3; Col4a4, collagen type 4 α chain 4; Col4a5, collagen type 4 α chain 5; Col6a3, collagen type 6 α chain 3; Cxcl12, C-X-C motif chemokine ligand 12; Dag1, dystroglycan 1; Efna5, ephrin A5; Efnb1, ephrin B1; Epha4, EPH receptor A4; Ephb3, EPH receptor B3; Ephb4, EPH receptor B4; Esam, endothelial cell adhesion molecule; Fgfr1, fibroblast growth factor receptor 1; Flrt2, fibronectin leucine rich transmembrane protein 2; Flt4, FMS related receptor tyrosine kinase 4; Fn1, fibronectin 1; GENc, glomerular endothelial cell; GENc_prolif, proliferating glomerular endothelial cell; Itga1, integrin subunit α 1; Itga3, integrin subunit α 3; Itgav, integrin subunit α 5; Itga8, integrin subunit α 8; Itga9, integrin subunit α 9; Itgb1,

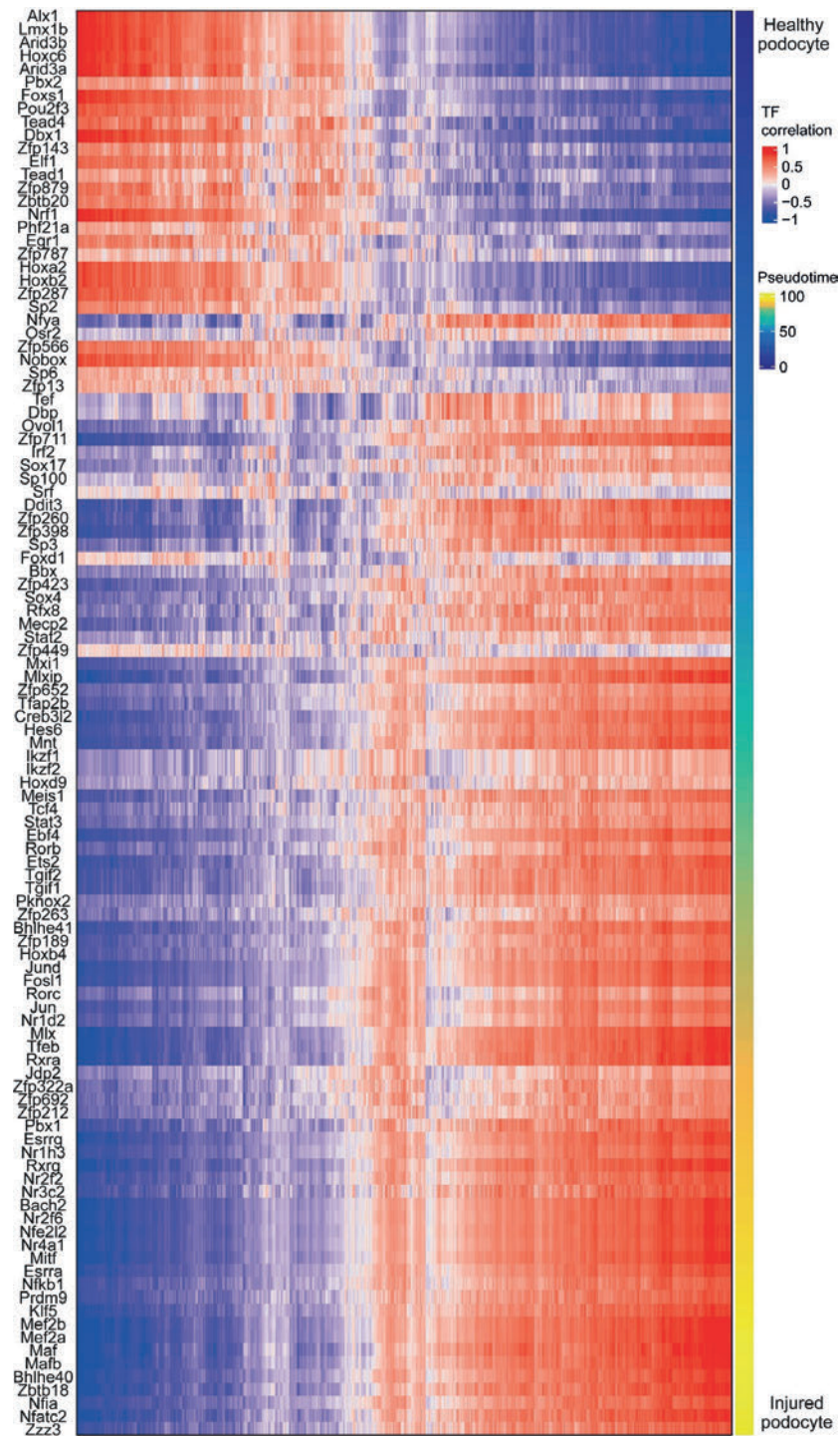
integrin subunit β 1; Itgb5, integrin subunit β 5; Itgb8, integrin subunit β 8; Jam1, F11 receptor; Jam2, junctional adhesion molecule 2; Jam3, junctional adhesion molecule 3; Kdr, kinase insert domain receptor; Lama4, laminin subunit α 4; Lamc1, laminin subunit γ 1; Mpzl1, myelin protein zero like 1; Ncam1, neural cell adhesion molecule 1; Nrp1, neuropilin 1; Nrp2, neuropilin 2; Ntn4, netrin 4; PAN, puromycin aminonucleoside; Pcdhac2, protocadherin α subfamily C2; Pcdhga7, protocadherin γ subfamily A7; PEC, parietal epithelial cell; Pecam1, platelet and endothelial cell adhesion molecule 1; Plxna2, plexin A2; Plxna4, plexin A4; Plxnd1, plexin D1; Ptn, pleiotrophin; Ptpm, protein tyrosine phosphatase receptor type M; PV1, plasmalemma vesicle protein 1; Sdc4, syndecan 4; Sema3c, semaphorin 3C; Sema3d, semaphorin 3D; Sema6a, semaphorin 6A; SMC, smooth muscle cell; Spp1, secreted phosphoprotein 1; Tek, TEK receptor tyrosine kinase; Tgfb2, transforming growth factor β 2; Tgfb1, transforming growth factor β receptor 1; Tgfb2, transforming growth factor β receptor 2; Thbs1, thrombospondin 1; Unc5b, Unc-5 netrin receptor B; Unc5c, Unc-5 netrin receptor B; Vegfa, vascular endothelial growth factor A; Vegfc, vascular endothelial growth factor C; Vegfd, vascular endothelial growth factor D.



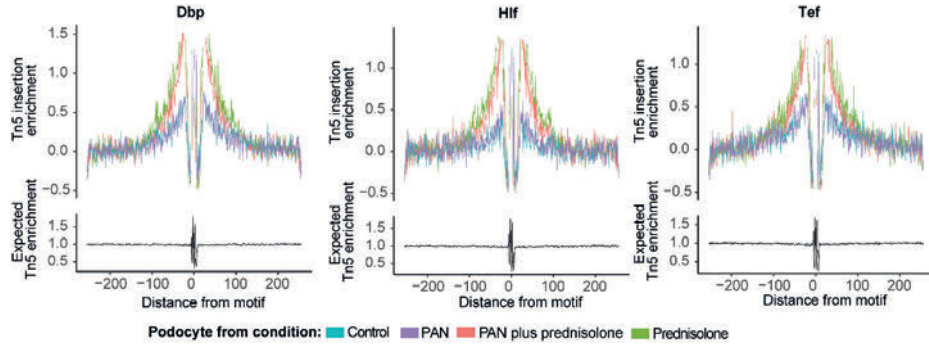
Supplemental figure 7. Expression of the glucocorticoid-induced leucine zipper *Tsc22d3* gene in different cell types. Abbreviations: ATAC, assay for transposase accessible chromatin; GENc, glomerular endothelial cell; PEC, parietal epithelial cell; PV1, plasmalemma vesicle associated protein 1; SMC, smooth muscle cell; *Tsc22d3*, TSC22 domain family member 3.



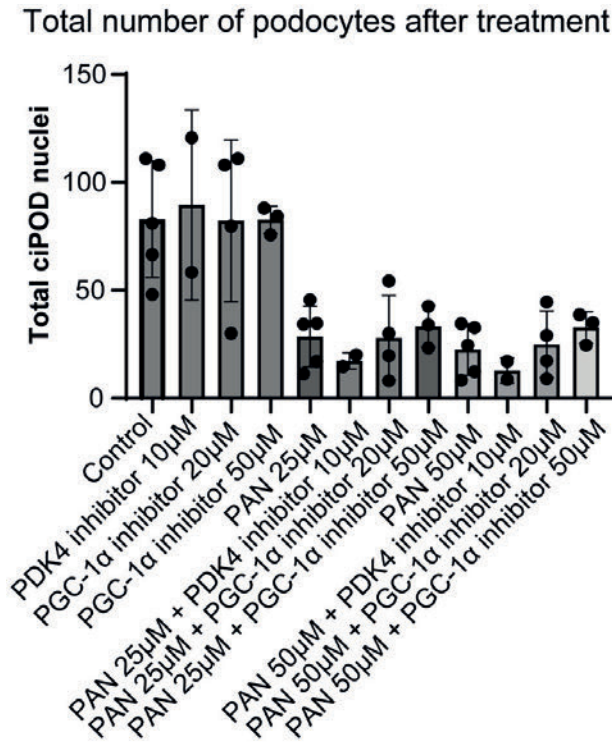
Supplemental figure 8. Enrichment analysis for pseudotime associated genes using Reactome, KEGG and Hallmark H gene sets. Abbreviations: AGE-RAGE, advanced glycation end products – receptor for advanced glycation end products; CDC42, cell division cycle 42; GTPase, guanosine triphosphatase; KEGG, Kyoto encyclopedia of genes and genomes; MAPK, mitogen-activated protein kinase; PI3K-Akt, phosphoinositide-3 kinase – AKT serine/threonine kinase; RAC1, Rac family small GTPase 1; Rap1, Ras-associated protein 1; RHO, Ras homolog; RHOTB3, Rho related BTB domain containing 3.



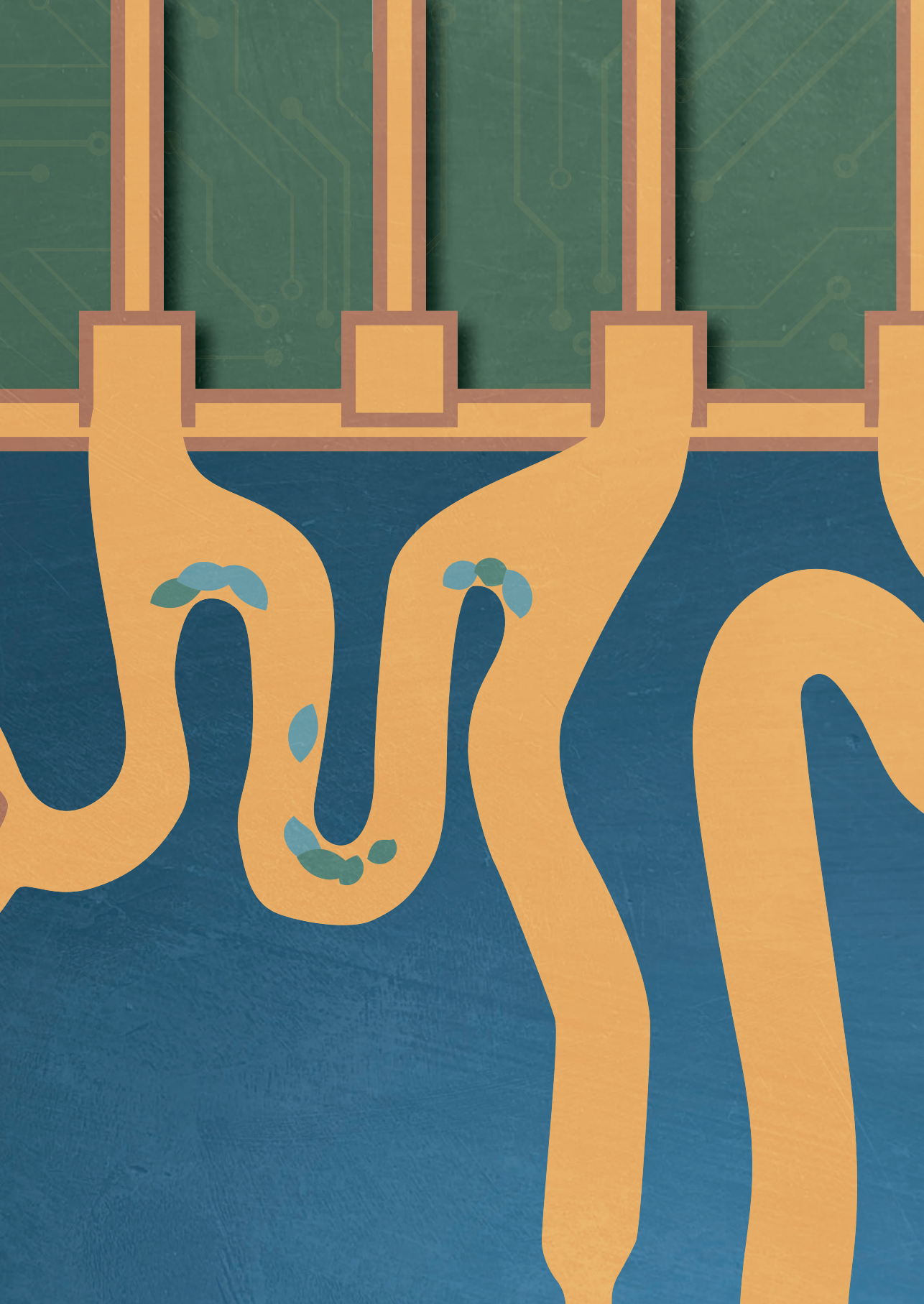
< Supplemental figure 9. Heatmap showing the correlation of the transcription factor activity with pseudotime. Abbreviations: *Alx1*, ALX homeobox 1; *Arid3a*, AT-rich interaction domain 3A; *Arid3b*, AT-rich interaction domain 3B; *Bach2*, BTB domain and CNC homolog 2; *Bbx*, BBX high mobility group box domain containing; *Bhlhe40*, basic helix-loop-helix family member E40; *Bhlhe41*, basic helix-loop-helix family member E41; *Creb3l2*, CAMP responsive element binding protein 3 like 2; *Dbp*, D-box binding PAR bZIP transcription factor; *Dbx1*, developing brain homeobox 1; *Ddit3*, DNA damage inducible transcript 3; *Ebf4*, EBF family member 4; *Egr1*, early growth response 1; *Elf1*, E74 like ETS transcription factor 1; *Esrra*, estrogen related receptor α ; *Esrrg*, estrogen related receptor γ ; *Ets2*, ETS proto-oncogene 2; *Fosl1*, FOS like 1 AP-1 transcription factor subunit; *Foxd1*, forkhead box D1; *Foxs1*, forkhead box S1; *Hes6*, hes family BHLH transcription factor 6; *Hoxa2*, homeobox A2; *Hoxb2*, homeobox B2; *Hoxb4*, homeobox B4; *Hoxc6*, homeobox C6; *Hoxd9*, homeobox D9; *Ikzf1*, IKAROS family zinc finger 1; *Ikzf2*, IKAROS family zinc finger 2; *Irf2*, interferon regulatory factor 2; *Jdp2*, jun dimerization protein 2; *Jun*, Jun proto-oncogene, AP-1 transcription factor subunit; *Jund*, JunD proto-oncogene, AP-1 transcription factor subunit; *Klf5*, krüppel-like factor 5; *Lmx1b*, LIM homeobox transcription factor 1 β ; *Maf*, MAF bZIP transcription factor; *Mafb*, MAF bZIP transcription factor B; *Mecp2*, methyl CpG binding protein 2; *Mef2a*, myocyte enhancer factor 2A; *Mef2b*, myocyte enhancer factor 2B; *Meis1*, meis homeobox 1; *Mitf*, melanocyte inducing transcription factor; *Mlx*, MAX dimerization protein MLX; *Mlxip*, MLX interacting protein; *Mnt*, MAX network transcriptional repressor; *Mxi1*, MAX interactor 1, dimerization protein; *Nfatc2*, nuclear factor of activated T cells 2; *Nfe2l2*, NFE 2 like bZIP transcription factor 2; *Nfia*, nuclear factor I A; *Nfkb1*, nuclear factor κ B 1; *Nfya*, nuclear transcription factor Y subunit α ; *Nobox*, NOBOX oogenesis homeobox; *Nr1d2*, nuclear receptor subfamily 1 group D member 2; *Nr1h3*, nuclear receptor subfamily 1 group H member 3; *Nr2f2*, nuclear receptor subfamily 2 group F member 2; *Nr2f6*, nuclear receptor subfamily 2 group F member 6; *Nr3c2*, nuclear receptor subfamily 3 group C member 2; *Nr4a1*, nuclear receptor subfamily 4 group A member 1; *Nrf1*, nuclear respiratory factor 1; *Osr2*, Odd-skipped related transcription factor 2; *Ovol1*, Ovo like transcriptional repressor 1; *Pbx1*, PBX homeobox 1; *Pbx2*, PBX homeobox 2; *Phf21a*, PHD finger protein 21A; *Pknox2*, PBX/knotted 1 homeobox 2; *Pou2f3*, POU class 2 homeobox 3; *Prdm9*, PR/SET domain 9; *Rfx8*, regulatory factor X8; *Rorb*, RAR related orphan receptor B; *Rorc*, RAR related orphan receptor C; *Rxra*, retinoid X receptor α ; *Rxrg*, retinoid X receptor γ ; *Sox4*, SRY-box transcription factor 4; *Sox17*, SRY-box transcription factor 17; *Sp2/Sp3/Sp6*, Sp2/Sp3/Sp6 transcription factor; *Sp100*, SP100 nuclear antigen; *Srf*, serum responsive factor; *Stat2*, signal transducer and activator of transcription 2; *Stat3*, signal transducer and activator of transcription 3; *Tcf4*, transcription factor 4; *Tead1*, TEA domain transcription factor 1; *Tead4*, TEA domain transcription factor 4; *Tef*, TEF transcription factor, PAR bZIP family member; *TF*, transcription factor; *Tfap2b*, transcription factor AP-2 β ; *Tfeb*, transcription factor EB; *Tgif1*, TGFB induced factor homeobox 1; *Tgif2*, TGFB induced factor homeobox 2; *Zbtb18*, zinc finger and BTB domain containing 18; *Zbtb20*, zinc finger and BTB domain containing 18; *Zfp*, zinc finger protein; *Zzz3*, zinc finger ZZ-type containing 3.



Supplemental figure 10. Transcription factor footprints of circadian rhythm related transcription factors are higher in prednisolone treated podocytes. Abbreviations: Dbp, D-box binding PAR bZIP transcription factor; Hlf, HLF transcription factor, PAR bZIP family member; PAN, puromycin aminonucleoside; Tef, TEF transcription factor, PAR bZIP family member.



Supplemental figure 11. Exposure of ciPODs to PAN and inhibitors for PDK4 and PGC-1 α . Quantification of cell numbers in three independent wells per condition, performed in two-five biological replicates. Example of phalloidin and nuclei stained ciPODs used for quantifications are provided in Figure 5. Abbreviations: ciPOD, conditionally immortalized podocyte; PAN, puromycin aminonucleoside; PDK4, pyruvate dehydrogenase kinase 4; PGC-1 α , PPARG coactivator 1 α .



Human scattered tubular cells represent a heterogeneous population of glycolytic dedifferentiated proximal tubule cells

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Abstract

Scattered tubular cells (STCs) are a phenotypically distinct cell population in the proximal tubule that increase in number after acute kidney injury. We aimed to characterize the human STC population. Three-dimensional human tissue analysis revealed that STCs are preferentially located within inner bends of the tubule and are barely present in young kidney tissue (<2 years), and their number increases with age. Increased STC numbers were associated with acute tubular injury (kidney injury molecule 1) and interstitial fibrosis (alpha smooth muscle actin). Isolated CD13+CD24-CD133- proximal tubule epithelial cells (PTECs) and CD13+CD24+ and CD13+CD133+ STCs were analysed using RNA sequencing. Transcriptome analysis revealed an upregulation of nuclear factor kappa-B, tumour necrosis factor alpha and inflammatory pathways in STCs, whereas metabolism, especially the tricarboxylic acid cycle and oxidative phosphorylation, was downregulated, without showing signs of cellular senescence. Using immunostaining and a publicly available single-cell sequencing database of human kidneys, we demonstrate that STCs represent a heterogeneous population in a transient state. In conclusion, STCs are dedifferentiated PTECs showing a metabolic shift towards glycolysis, which could facilitate cellular survival after kidney injury.

Keywords: Scattered tubular cells, proximal tubule, kidney regeneration, acute kidney injury, (mal-) adaptive repair

Introduction

Acute kidney injury (AKI) is a common clinical problem, estimated to occur in 1 in 5 adults and 1 in 3 children that are hospitalized with acute illness, and is associated with high morbidity and mortality rates^{1,2}. AKI can be defined as an abrupt decrease in glomerular filtration, in most cases caused by acute ischemic and/or toxic insults with the proximal tubule as the main site of injury, characterized by a loss of tubular cell function. Previously, it was shown that the proximal tubule has the capacity to regenerate and regain its function, depending on the degree of injury³. Despite the regenerative capacity of the tubules, kidney function does not always return to baseline level after repair, which can be indicative of progression to chronic kidney disease (CKD).

Scattered tubular cells (STCs) have a scattered distribution throughout the proximal tubule of normal human kidneys, show a less-differentiated phenotype and may play a role in (mal-) adaptive repair processes⁴⁻⁸. Previously, we and others have shown that STCs express the progenitor markers CD133 and CD24, have a higher proliferation index, become more numerous after AKI, and are more resistant to hypoxic injury^{4,6,7,9-11}. Lineage tracing experiments using mouse models of AKI indicated that STCs are not a fixed progenitor cell population but a transient phenotype that can arise from any proximal tubule epithelial cell (PTEC) after injury^{5,12}. However, debate is ongoing about whether STCs are progenitor/stem cells or if these cells are dedifferentiated PTECs^{4-7,13,14}.

Next to tubular regeneration, STCs have also been linked to processes promoting failed repair. Earlier studies using mouse models showed that a subpopulation of injured dedifferentiated PTECs become arrested at the G2/M cell cycle phase and adopt a senescence-associated secretory phenotype that may drive inflammation and fibrosis in the kidney. Single-cell transcriptome analysis of PTEC subpopulations at different time points during ischemia-reperfusion injury (IRI) revealed a 'failed-repair' population¹⁵.

The majority of studies contributing to our knowledge of STCs were performed using animal models. However, the mouse findings may not be directly translatable to humans because STCs are only detected during injury and are absent under healthy conditions in mice. Also, the cell phenotype can be associated with a certain disease stage since disease processes start at a single time point, whereas in human nephrectomies the still unknown trigger

may be chronic and cells may therefore be in different stages of injury and repair. Therefore, this study aimed to characterize the human STC population to gain a better understanding of their origin, location, heterogeneity, and function using immunohistochemical, immunofluorescence, and transcriptome analysis of human samples.

Results

Scattered tubular cells are mainly present in groups at inner bends of the proximal tubule

The term scattered tubular cell refers to the observed distribution of the cells scattered within the human kidney cortex. We questioned whether the STCs were distributed randomly or if there was a structural location (niche) harbouring STCs, similar to permanent stem or progenitor cell niches described in other organs¹⁶. We performed 3D tissue analysis, which enabled accurate analysis of STC tissue distribution, and we determined that STCs were located at the inner bends of the proximal tubule and located in groups consisting of two or more (up to 12) STCs (Figure 1A-C; Supplementary video 1). Although some STCs seemed to be in a straight part of the proximal tubule in single sections (Figure 1A), 3D reconstruction of aligned serial sections often revealed a bend in a higher or lower z-level in the tissue (Supplementary video 1). These results demonstrated that the location of STCs was not random; there was a preferred location within the sharp inner bends of the tubule.

Additionally, we identified phosphofructokinase-platelet (PFKP) as a novel STC marker, confirmed by co-expression with vimentin (Figure 1D). STCs stained for PFKP or vimentin were observed in aquaporin-1 (AQP1), glucose transporter 2 (GLUT2) and megalin (LRP2) expressing proximal tubule segments (Supplemental figure 1, A-C*). GLUT2 and LRP2 are expressed in the proximal tubule segments S1 and S2^{17,18}, whereas AQP1 is highly expressed in the proximal straight tubules and shows a less abundant expression in the convoluted part of the proximal tubule¹⁹. These data indicate the presence of STCs in all segments of the proximal tubule.

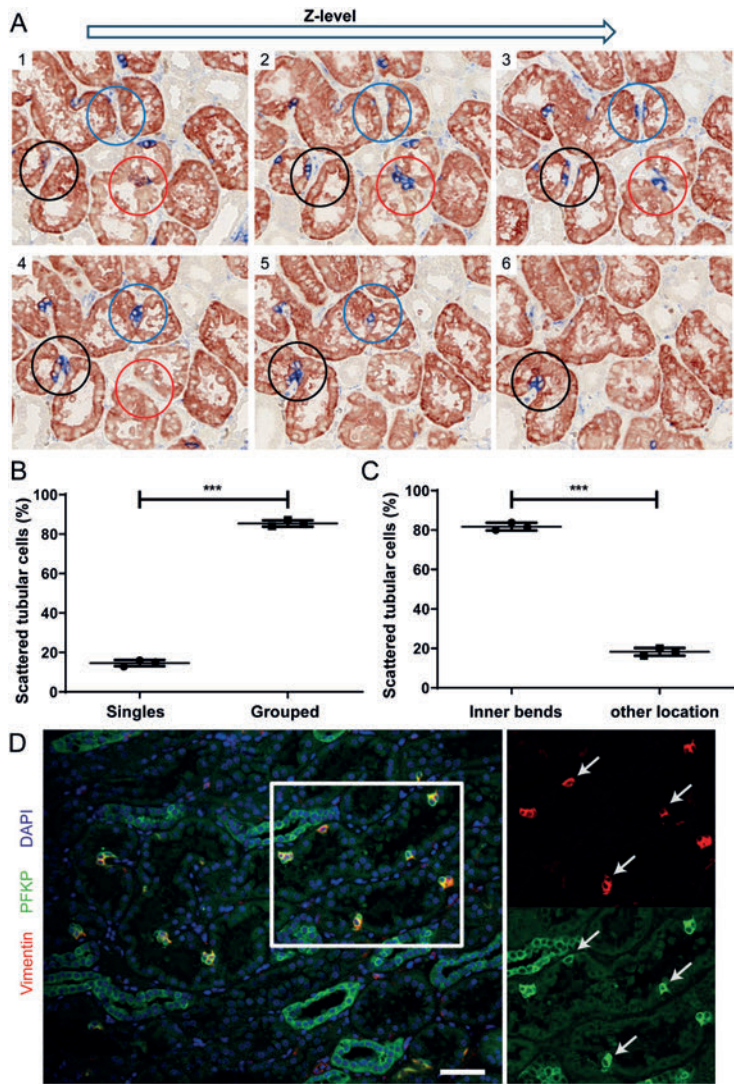


Figure 1. Scattered tubular cells are mainly present in groups located at the inner bends of the proximal tubule. (A) Immunohistochemistry staining for AQP-1 (red) as PTEC marker and vimentin (blue) as STC marker. Microscopic images 1-6 show staining of serial slides of 2 μ m from high z-plane (image 1) to low z-plane (image 6). The blue, black and red circles show areas in which STCs can be detected in groups located at inner bends of the proximal tubule. Manual scoring of the STCs was performed in 3 different normal human tissues for 10 randomly chosen cortex areas of 20 z-planes of serial slides per kidney. (B) The percentage STCs present as single cells or in groups of more than two cells in each normal human kidney. (C) The percentage of STCs at the inner bends of the proximal tubule and located elsewhere inside the proximal tubule in each normal human kidney. (D) Immunofluorescence staining of normal human kidney for vimentin (red) and PFKP (green). STCs show co-expression of both markers (white arrows). Scale bar: 50 μ m. *** = $p < 0.0001$, unpaired two-tailed t-test. AQP-1, aquaporin-1; PTEC, proximal tubule epithelial cell; STC, scattered tubular cell; PFKP, phosphofructokinase-platelet.

Scattered tubular cell number increases with age and kidney injury

In our 3D analysis, STCs seemed to be a structural part of the inner bends of the proximal tubule, suggesting these bends might be a niche harbouring STCs. We hypothesized that if STCs were dedifferentiated PTECs, they would not be present in young kidney tissue and increase with age, whereas a pre-existing progenitor cell population would be present and might decrease with age. Analysis revealed that the number of STCs (PFKP⁺, AQP1⁺) was negligible in children of age 0-2 years, whereas the number of STCs increases with age, arguing in favour of a dedifferentiated PTEC population rather than a pre-existing progenitor cell population ($R^2 = 0.3526$, Figure 2A-B). For all age groups, samples showing alpha-smooth muscle actin (α -SMA) staining of more than 5% of the tissue surface area were excluded for the age group clustering because they could be classified as pathologically suspicious, showing marked interstitial fibrosis (Supplemental figure 2A). STC numbers did not differ between male and female, but did show a difference between age groups (child-young adults, adults, middle-aged, and elderly) (Supplemental figure 2B-C).

To study the relationship between the number of STCs and kidney injury, we analysed the amount of fibrosis (α -SMA⁺) present in normal human kidney tissue and the presence of acute tubular injury by the presence of kidney injury molecule 1 (KIM-1). An increased number of STCs (PFKP⁺) was found in tissues with increased fibrosis ($R^2 = 0.3893$) or KIM-1 presence (Figure 2C-D, Supplemental figure 2D-F). In summary, these results suggest a relation between the number of STCs and interstitial fibrosis as well as tubular injury and indicate that the presence of STCs is related to kidney injury.

Scattered tubular cells show no marker expression associated with cellular senescence

To test whether STCs express markers associated with cell senescence, immunostaining of three different normal human kidney tissues and three sections per tissue was performed. Lamin B1 (LMNB1) is located around the nucleus and degraded in senescent cells. In annexin A2 (ANXA2)-positive STCs, normal LMNB1 surrounding the nucleus can be found (Figure 2E). Tumour suppressors p53 and p16, both known to be upregulated in senescent cells, were not detected in STCs (Figure 2, F-G), suggesting that STCs are not senescent cells.

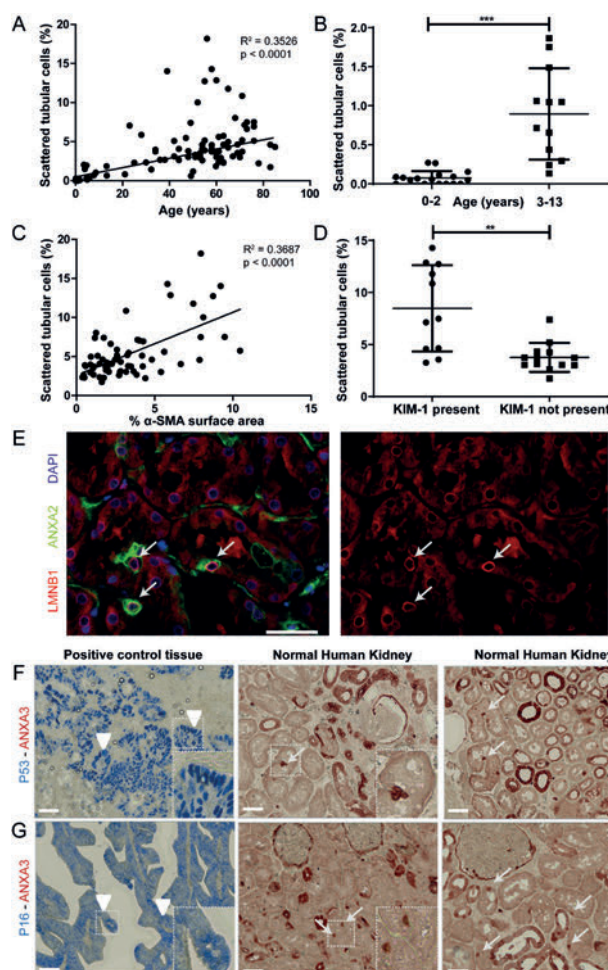


Figure 2. Scattered tubular cell number increases in renal aging and injury but cannot be associated with cellular senescence. (A) Regression analysis of STC number versus age in normal human kidney tissue from tumour nephrectomies using the unaffected pole of 37 patients (48-85 years), 29 renal transplant biopsies from donor kidneys prior to transplantation (age 23-76) and 41 kidney tissues obtained from autopsy (age 0-61 years). (B) STC numbers in children aged 0-13. Children of age 0-2 years show mean $0.076 \pm \text{s.d. } 0.09$ STCs ($N=17$), ages 3-13 years show mean $0.90 \pm \text{s.d. } 0.59$, ($N=12$). (C) Regression analysis of STC number and α -SMA expression surface area in normal human kidneys from tumour nephrectomy. (D) The relationship between KIM-1 and number of STCs in normal human kidney tissue derived from tumour nephrectomy. (E) Representative immunofluorescence LMNB1 (red) and STC marker ANXA2 (green). A normal to high level of LMNB1 around the nucleus (blue, DAPI staining) is found in STCs (arrow). (F) Immunohistochemistry of p53 (blue) and ANXA3 (red). Arrowheads show p53 in positive control tissue, arrows indicate STCs. (G) p16 immunohistochemistry (blue) and ANXA3 (red). Arrowheads indicate positive control tissue and arrows show STCs in normal human kidneys. Scale bar: F-G) $50 \mu\text{m}$. ** = $p < 0.001$, *** = $p < 0.0001$. STC, scattered tubular cell; α -SMA, alpha-smooth muscle actin; KIM-1, kidney injury molecule-1; LMNB1, lamin B1; ANXA2, annexin A2; ANXA3, annexin A3.

Comparative transcriptome profiling of sorted PTEC and STC

To compare the transcriptomic profile of the STC (CD13⁺CD24⁺ and CD13⁺CD133⁺) population to the PTEC (CD13⁺CD24⁻CD133⁻) population, we performed bulk RNA sequencing of the sorted populations (Supplemental figure 3A-D). Exploratory data analysis showed that PTECs and STCs from patients 6 and 7 deviated from the other patients and therefore these patients were not used for downstream analyses (Supplemental figure 3E,F). Next, differential gene expression (DGE) analysis was performed and confirmed the successful isolation of PTECs and STCs (Figure 3A). In addition, genes that were significantly upregulated in the STCs compared to PTECs were also expressed at the protein level in human STCs, such as ANXA3 and A-kinase anchoring protein 12 (AKAP12) (Supplemental figure 4A,B). Overrepresented terms in PTECs using Hallmark H gene sets are in line with normal PTEC functioning, for instance, PTECs have active mTORC1 signalling, reactive oxygen species pathway, and xenobiotic, heme, bile acid, and fatty acid metabolism (Figure 3B)²⁰⁻²³.

The majority of over-represented terms and pathway activities in the STC population are related to inflammatory and fibrosis-associated pathways, such as tumour necrosis factor alpha (TNF α) and nuclear factor kappa B (NF κ B) signalling, interferon-alpha (IFN- α) and interferon-gamma (IFN- γ) (Figure 3B-C). Of note, STC showed pathways associated with repair are active, such as epidermal growth factor receptor (EGFR) and Wnt signaling^{24,25}.

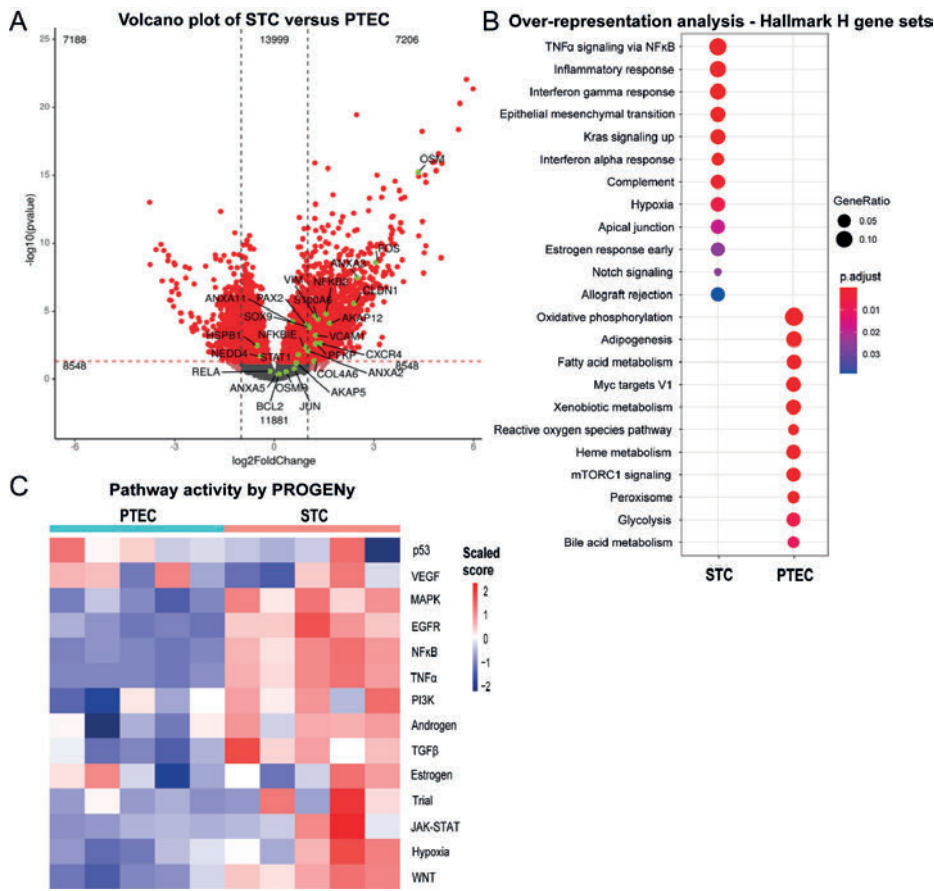


Figure 3. Transcriptome and pathway analysis shows significant differences between STCs and proximal tubular cells. (A) Volcano plot of PTEC versus STC transcriptomes; y-axis indicates statistical significance ($-\log_{10}(p\text{-value})$) and magnitude of change on the x-axis ($\log_2\text{FoldChange}$). Statistically significant different genes are highlighted in red. Known STC markers (based on protein expression⁴) are highlighted in green. (B) Over-representation analysis using the Hallmark H gene sets for the significantly upregulated genes in the PTEC and STC population. (C) Pathway activity by PROGENy in the PTEC and STC population for the 14 included pathways. PTEC, proximal tubular epithelial cell; STC, scattered tubular cell.

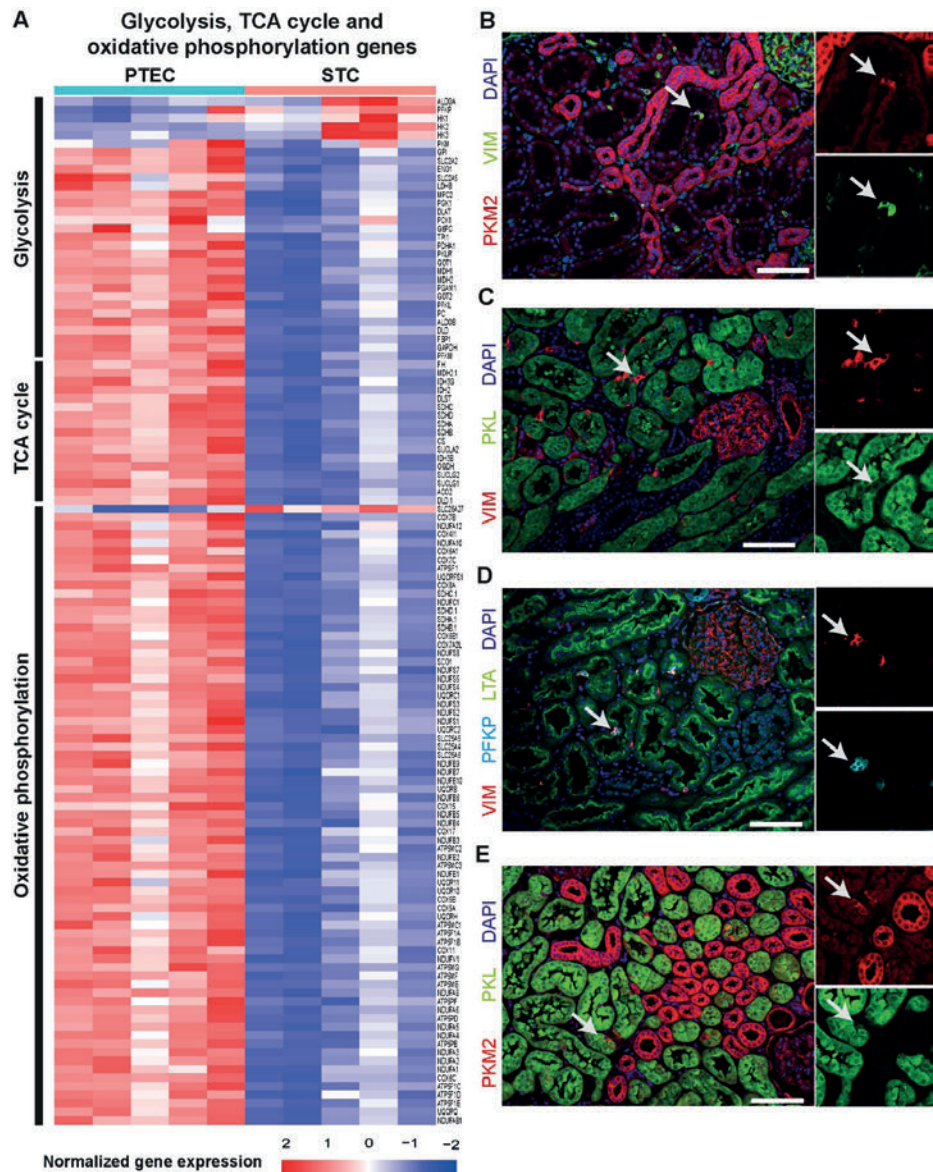


Figure 4. Downregulation of mitochondrial respiration in STCs. (A) Heatmap of genes involved in glycolysis, electron transport chain and TCA cycle (Krebs or citric acid cycle). Heatmaps show the row-wise z-score per sample per gene for PTECs versus STCs. (B) Immunofluorescence for PKM2 (red) and vimentin (green). (C) Immunofluorescence of vimentin (red) and PKL (green). Arrow indicate STCs. (D) Representative image of immunofluorescence for PFKFB (cyan) and vimentin (red) in STCs (arrows) located inside the proximal tubule (LTA, green). (E) Double staining for PKM2 (red) and PKL (green). Arrow indicates STCs. Scale bars: B-E) 100 μ m. TCA, tricarboxylic acid; PTEC, proximal tubular epithelial cell; STC, scattered tubular cell; PKM2, pyruvate kinase M2; PKL, pyruvate kinase L; LTA, Lotus tetragonolobus lectin.

Scattered tubular cells show a metabolic switch towards glycolysis

To further dissect the metabolic changes in STCs, we visualized the normalized gene expression of the oxidative phosphorylation, tricarboxylic acid (TCA) cycle and glycolysis pathways. The majority of genes involved in these pathways are downregulated in STCs (Figure 4A). As previously described, electron microscopic images showed a high density of mitochondria in PTECs, whereas STCs showed nearly no mitochondria inside the cytoplasm (Supplemental figure 5A,B)⁴, which is in accordance with the downregulation of genes involved in TCA cycle and oxidative phosphorylation. Although several genes of glycolysis are downregulated in STCs, genes of key regulators of glycolysis, such as hexokinase and phosphofructokinase (PFK) are upregulated (Figure 4A, Table S3). Furthermore, we observed a switch in isoform expression of PFK and pyruvate kinase (PK). The PTECs of normal healthy kidneys showed exclusive expression of PK-Liver(L), whereas STCs (vimentin+) expressed PK-Muscle 2(M2) and PFK-Platelet(P) (Figure 4D-G)²⁶. RNA sequencing data showed downregulation of *PFKL* and *PKLR* and an upregulation of *PFKP* in STCs (Tables S3 and S4). Furthermore, two lactate transporters encoded by *SLC16A3* (monocarboxylate transporter 4) and *SLC16A7* (monocarboxylate transporter 2) were also significantly upregulated in STCs compared to PTECs (Table S3)²⁷. Altogether, these results suggest a glycolytic switch in STCs compared to PTECs, although future research should focus on functional studies to confirm this glycolytic switch.

Scattered tubular cells show a heterogeneous phenotype

Heterogeneous expression of several STC markers was regularly observed in neighbouring cells (Figure 5A-C). As such, STCs solely expressed ankyrin repeat domain 2 (ANKRD2), only vimentin, or both (Figure 5A). To resolve the heterogeneous profile and driving factors of STC subtypes, we used the whole human kidney single-cell RNA-seq dataset from Muto et al.²⁸ and identified 4 clusters of STC (Figure 5D). We evaluated the markers ANKRD2, vimentin, c-JUN, fibroblast growth factor 2 (FGF2) and E-cadherin in these subclusters. We identified a similar heterogeneous expression of STC markers in the single-cell dataset as observed on protein level for ANKRD2, vimentin, c-JUN and FGF2 (Figure 5A-C, E).

Next, we performed pseudotime analysis, a measure of how far a cell is in a biological process (Figure 5F, Table S5). Nearly all STC markers are significantly associated with pseudotime and are highly expressed in especially STC subclusters 2 and 3 (Supplemental figure 6A)⁴. Also, several transporters,

including sodium/glucose co-transporter 2 (SGLT2), organic anion transporter 1 (OAT1) and cubilin (CUBN) that enable normal PTEC function are associated with pseudotime and are transiently lower expressed in STC subclusters (Supplemental figure 6A). However, STC cluster 4 has increased expression of transporters and decreased expression of STC markers compared to cluster 3, which might reflect a redifferentiation cluster towards PTECs.

By comparing the PT and STC subclusters from the single-cell dataset with our bulk RNA samples, we could indicate their resemblance and confirm cell characteristics. PT cells showed similarly overrepresented gene sets (bile acid, heme and fatty acid metabolism) as sorted PTECs, whereas STC subclusters had overrepresented gene sets (TNF α signalling *via* NF κ B, apical junction, hypoxia and epithelial mesenchymal transition), similarly to our sorted STCs (Figures 3B and 5G). We also observed a switch in PFK and PK isoforms between PT and STC clusters (Supplemental figure 6B). Using PROGENy, we found that similar pathways (NF κ B, TNF α , TGF β) are active in the STC subclusters compared to our sorted STCs (Figure 5H).

We observed heterogeneity in pathway activity, with clusters 1 and 2 being more active in repair pathways (PI3K and EGFR), and clusters 3 and 4 being more active in profibrotic and stress pathways (Wnt and hypoxia) (Figure 5H)²⁹. We also observed heterogeneity in the ligand-receptor interactions; for instance, STC subclusters 2 and 3 are the only clusters that signal with the proximal tubule cells (Figure 5I). Also, the majority of ligand-receptor interactions between STC subclusters and PTs are *via* EGFR-receptor family ligands and receptors (Table S6), suggesting an important role for EGFR signalling in STC and PTEC communication.

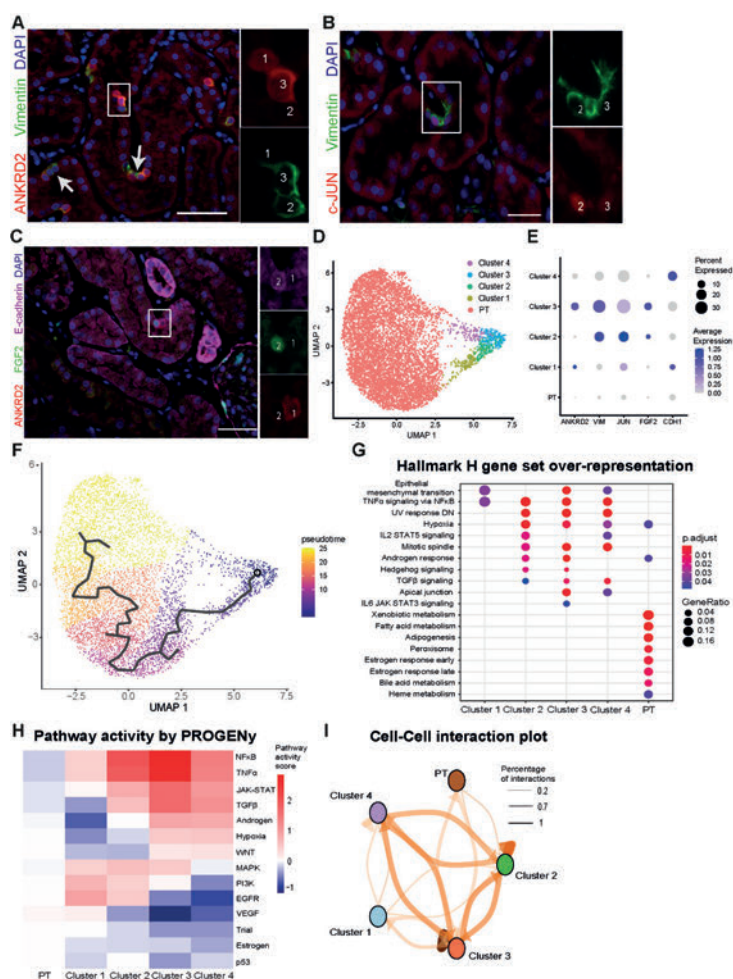


Figure 5. Single-cell sequencing identifies multiple cell clusters that resemble STCs in normal human kidney (A) Immunofluorescence staining for ANKRD2 (red) and vimentin (green). Cell 1 shows expression of ANKRD2 but not vimentin, whereas vimentin is expressed and not ANKRD2 in Cell 2. Both ANKRD2 and vimentin are detected in Cell 3. (B) Similar heterogeneity was observed for c-JUN (red) and vimentin (green) and for (C) ANKRD2 (red), FGF2 (green) and E-cadherin (magenta). (D) Isolated proximal tubule cluster with 4 subclusters of proximal tubule cells expressing VCAM1 from an online dataset. (E) Dot plot of gene expression of the immunofluorescent markers in (A-C). The clusters to which the cells belong are indicated in the immunofluorescent images and except for E-cadherin all other heterogeneous expression patterns are also observed in the single cell RNA sequencing dataset. (F) Pseudotime trajectory in isolated proximal tubules and VCAM1⁺ proximal tubule clusters as determined by Monocle. (G) Comparative over-representation analysis using the Hallmark H gene sets from MSigDB for the proximal tubule cells and PT_VCAM1 clusters. (H) Pathway activity per cell cluster using PROGENY. (I) Cell-cell interaction plot to visualize the amount of ligand-receptor interactions between clusters. Line thickness reflects the amount of ligand-receptor interactions. Scale bars: (A-C) 50 μ m. ANKRD2, ankyrin repeat domain 2; FGF2, fibroblast growth factor 2; MSigDB, molecular signature database; STC, scattered tubular cell.

Discussion

In this study, we aimed to characterize the origin and role of the human STC population. We showed that STCs are a dedifferentiated PTEC population since STCs are rarely present in young children and increase in number with age and injury. STCs are not senescent, which is a hallmark of cells contributing to maladaptive repair, and are therefore not likely contributing to maladaptive repair. Transcriptome analysis using bulk RNA sequencing revealed an upregulation of inflammatory pathways in STCs, whereas metabolism and especially the TCA cycle and oxidative phosphorylation were downregulated, indicating that STCs show a metabolic switch towards glycolysis. Furthermore, we could indicate that STCs represent a heterogeneous population.

Since the origin and function of STCs are still debated, we analysed the number of STCs in human kidney tissue in relation to age and kidney injury^{7,8,14,30,31}. Progenitor and stem cells are known to be present in high numbers in younger tissue. Our results showed that STCs were hardly ever present in kidney tissue of patients 0-2 years of age, but their numbers increased with age. In line with our findings, the cells described by Hansson et al. showed a similar morphology using electron microscopy, have the same STC marker expression (vimentin, CD24 and CD133) and also show an absence of mitochondria³². In contrast, that study showed no correlation between age and STC number, which is likely attributable to the relatively small tissue cohort used.

Furthermore, we showed that STCs are not located in a protective niche environment, but are primarily located at the inner turns of the proximal tubule. We hypothesize that STCs are more likely to be exposed to stressors in the inner bends, such as shear stress, because fluid flow velocity is highest in the inner bend of a tubing system³³. This localization has previously been mentioned, as STCs were described to be generally seen at the tubular plicae, where the proximal tubule makes hairpin turns^{4,32,34}. Additionally, STC number correlated with increased kidney injury and tubulointerstitial fibrosis, supposedly due to an increased number of injured PTECs stimulating dedifferentiation to STCs. Altogether, these results support the hypothesis that STCs are dedifferentiated PTECs rather than being a progenitor or stem cell population.

A second question regarding human STCs is whether they contribute to tubular regeneration or if the morphological and functional changes are irreversible, leading to cellular senescence contributing to interstitial fibrosis and tubular

atrophy³⁵⁻³⁸. Although cellular senescence is linked to aging^{39,40}, our results indicate that STCs are not cell cycle arrested or senescent, as no expression of senescence-associated markers was detected in STCs. These findings are in line with earlier studies, which showed that STCs express markers associated with cell survival and proliferation, suggesting that STCs are able to proliferate and contribute to tubular repair^{4,6,7}. However, a recent study indicates that endocycle may play a role in which cells expand in size to restore the integrity of the epithelium and also express proliferation markers⁴¹. Therefore, it is still debated whether the observation of proliferation markers indicates dividing cells or cells in endocycle.

The RNA sequencing results, the electron microscopy and immunofluorescence staining indicate that STCs are glycolytic and do not rely on mitochondrial respiration to obtain energy. STCs showed expression of both the PFKP and PKM2 isoform, instead of PKL and PFKL. The PKM2 dimer stimulates the conversion of pyruvate into lactate, whereas the tetramer of PKM2 stimulates normal entry of the TCA cycle after glycolysis and thus oxidative phosphorylation^{42,43}. Furthermore, these results do not support the suggestion that STCs actively proliferate to replace lost cells as metabolism is downregulated in such a way that the formation of new essential cellular components needed for cell division is absent. It was shown earlier in mice that STC-like cells were the main proliferating cell population contributing to regeneration after ischemic-reperfusion (IR) injury⁵. However, in human, it was found that the few proliferating cells are STCs rather than PTECs but most human STCs are not proliferating⁴. The observation that STCs show a heterogeneous phenotype, even within one cluster of cells, suggests a transient process of PTEC dedifferentiation and metabolic reprogramming, which could facilitate cell survival. Possibly, STCs are able to survive the hypoxic environment that is associated with AKI due to their glycolytic switch and are then able to proliferate and redifferentiate into functional PTECs again.

Our human STC samples show similar STC marker expression as the human STC clusters we identified in the Muto et al. database and therefore we consider these cells as the same²⁸. Muto et al. showed that their human STC (PT-VCAM1) cluster resembles the failed-to-repair proximal tubule cell cluster (FR-PT) identified in mice²⁸. Indeed, both the two human STC populations and the murine FR-PTs show increased pro-inflammatory and pro-fibrotic pathway activity^{15,28}. However, the authors compared the entire human STC population to these mouse FR-PTs, whereas we now identify several subclusters of human STCs with heterogeneous

marker expression, on both gene and protein level, and pathway activity profiles. The heterogeneity of human STCs is crucial to unravel, since some human STC subclusters have a different pathway activity profile and/or marker expression compared to mouse FR-PTs. For example, STC cluster 1 shows lower pro-inflammatory and pro-fibrotic pathway activity, STC cluster 4 shows higher transporter gene expression (i.e. LRP2 and CUBN) important for normal PTEC function, and both STC cluster 1 and 2 show higher pro-survival EGFR activity²⁴. Altogether, some human STCs might be more similar to FR-PTs, whereas other STCs might still be able to recover or are in the process of recovering.

One of the heterogeneously active pathways that might be involved in STC formation and PTEC survival is the EGFR pathway, which was also highly active in the bulk RNA sequencing results of our STCs compared to PTECs. Since subcluster 1 resembles the proximal tubule cluster most and has EGFR activation that was not seen in subclusters 3 and 4, EGFR might activate a downstream cascade leading to the STC phenotype. A previous study in mice identified FOXM1 as an early induced transcription factor in injured PTECs, which was dependent on EGFR stimulation²⁴. EGFR pathway activity may lead to KRAS signalling activation, which in turn can activate both MAPK and PI3K pathways⁴⁴. This might lead to PFKF phosphorylation, which leads to β -catenin stabilization and PKM2 binding to β -catenin to activate Wnt signaling⁴⁵. The PI3K pathway can then lead to NF κ B activation and subsequent TNF α signalling. EGFR signalling can increase the expression of PKM2, which is extensively reviewed in cancer cells to promote tumour growth and might also contribute to the proliferative phenotype of injured PTECs^{26,46}. This possible role for EGFR questions anti-EGFR therapy, which is indeed associated with many renal adverse events, such as electrolyte disturbances, and might be a "second hit" for the development of AKI^{47,48}. Hence, the use of anti-EGFR agents could interfere with STC pathways important for proximal tubular regeneration⁴⁹.

In conclusion, our results indicate that STCs are not a fixed progenitor cell population but are dedifferentiated PTECs. Even though STC number is related to age and renal injury, STCs are not senescent. Furthermore, STCs show a metabolic switch towards glycolysis, which could be facilitated by EGFR signalling and PKM2 expression and might be important for tubular cell survival. Importantly, STCs are a heterogeneous cell population. However, whether human STCs actively contribute to adaptive repair of the proximal tubule and whether these cells are able to redifferentiate into functional PTECs needs further investigation.

Material and Methods

Ethical statement and renal tissue

All experimental protocols were approved by the local ethical committee of the Radboud University Medical Center, Nijmegen, The Netherlands (CMO 2017-3652) within the remit of the Medical Research Involving Human Subjects Act (WMO). All experiments were performed in accordance with the relevant guidelines and regulations. For marker analysis and isolation of PTECs as well as STCs, the unaffected pole of normal human renal cortex tissue from tumour nephrectomies was used. For distribution analysis of STCs, normal human kidney tissue was obtained from tumour nephrectomies using the unaffected pole of 37 patients (48-85 years). Additionally, 29 kidney tissue samples were obtained from kidney transplant biopsies from donor kidneys prior to transplantation (age 23-76). Samples of 41 kidney tissues were obtained from autopsy (age 0-61 years). The paraffin samples consisted of postmortem autopsy kidney samples and surgical adult kidney samples from the Erasmus MC Tissue Bank, Rotterdam, The Netherlands. Tissues, which were selected for having no renal abnormalities in pathology and primary diagnosis, were procured at the time of autopsy within 48 hours after death. In addition, all autopsy tissue was evaluated for autolysis and only included when the material had no or only mild autolysis. In total, autopsy tissue from 7 patients was excluded, whereas 41 had no or only mild autolysis and were included. The Erasmus MC Research Ethics Board waived the need for formal ethics approval according to the Dutch Law on Medical Research in Humans. Paediatric tissue was collected when parental written informed consent for both autopsy and the explicit use of the tissue for research was present, adult tissue was collected within a non-objection clause.

Immunofluorescent and immunohistochemical staining

Procedures were performed according to Jansen et al.⁵⁰; see supplementary materials for details. For formalin-fixed, paraffin-embedded (FFPE) tissue, STC markers have previously been validated by co-staining with CD24 and/or CD133⁴.

Slide registration and 3D tissue analysis

After immunohistochemical staining of consecutive slides of renal cortex tissue, light microscopic images of the whole tissue slides were made and digitized using the Panoramic P250 Flash II tissue scanner (3DHistech, camera CIS VCC/FC60FR19CL, software version 1.22.0.67865, Budapest, Hungary). The digitized slides were registered using the non-commercially available software HistokatFusion (Fraunhofer MEVIS lab, Bremen, Germany). The

registration consisted of a pre-alignment, a parametric registration computed on coarse resolution, and a nonlinear, precise registration⁵¹. In all three steps the Normalized gradient Fields distance, which measures the alignment of image gradients, is minimized⁵². The nonlinear registration adds curvature regularizer to the distance term to favour smooth deformations without foldings⁵³. After registration of the slides, ten regions of interest were randomly selected and serial images were created using an automated slide analysis platform (ASAP, version 1.9, open-source software). The number of STCs present as singlets or present in groups, as well as the number of STCs present at the inner bends of the proximal tubule were counted. 3D reconstructions were further created using the ImageJ 3D viewer plugin.

Image analysis

For STC distribution analysis, eight random images (20x, fluorescent microscope) were taken of each patient tissue section stained for AQP1, PFKP and nuclear counterstaining. The number of STCs was manually scored by two independent researchers. The number of PTECs was automatically counted using ImageJ (v.1.51) as outlined in the supplementary material (supplementary methods, Supplemental figure 7). As a result, the percentage STCs (PFKP+) inside the PTECs (AQP1+) was calculated. For α -SMA analysis, five random images were taken of each patient tissue section (10x). The percentage of α -SMA was calculated using the tissue sample surface area and the surface area of α -SMA expression inside this area. This was automatically determined using ImageJ (v.1.51) as outlined in the supplementary methods.

Tissue preparation for FACS

Human renal cortex tissue from the unaffected pole of kidneys after tumour nephrectomy was cut into small pieces (≤ 1 mm) and further processed using enzymatic digestion and manual dissociation (supplementary materials). The isolated cell pellet was resuspended in 1 ml PBS containing 1% BSA (v/v, Sigma-Aldrich, Zwijndrecht, the Netherlands) and antibodies against CD133, CD24 and CD13 (Table S1) were used to stain PTECs and STCs. Antibody incubation was performed for 1 hour at 4 °C in the dark. The pellet was washed three times with PBS and centrifuged at 1,500xg for 5 minutes, supernatant was removed and the pellet resuspended in PBS. This cell suspension was rinsed through a cell strainer (70 μ m) and collected for cell sorting using the strategy described in supplementary materials.

Bulk RNA sequencing

RNA isolation

RNA was isolated according to the PicoPure RNA isolation Kit (Thermo Fisher Scientific, Breda, the Netherlands) manufacturer's protocols. Samples were eluted in 11 μ l elution buffer, the purification column was incubated for 1 minute at room temperature and centrifuged for 1 minute at 1,000xg followed by 1 minute at 16,000xg. The RNA sample was stored at -80°C .

Bulk RNA-sequencing sample preparation

Total RNA was used for library preparation using the RNA HyperPrep Kit with RiboErase (KAPA Biosystems, Wilmington, MA). A fragmentation step was carried out for 6.5 min and NextFlex DNA barcodes (Bioo Scientific, Austin, TX) were used for adapter ligation. Libraries were amplified using 15 amplification cycles. Library concentration was measured using the dsDNA Fluorescence Quantification Assays (DeNovix, Wilmington, DE), and library size was determined using the BioAnalyzer High Sensitivity DNA Kit (Agilent, Amstelveen, the Netherlands). Sequencing was performed using an Illumina NextSeq 500, and 50-bp paired-end reads were generated.

RNA sequencing bioinformatic analysis

A similar workflow as described in Jansen et al.⁵⁰ was used, please see the supplementary methods and Supplemental figure 8 for a detailed description.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 8.1.2, San Diego, CA). Two-tailed unpaired t-tests were performed in the 3D analysis of STC. A linear regression analysis was performed to analyse the number of STCs in relation to age and α -SMA expression. A two-tailed unpaired t-test (Mann-Whitney U test) was performed to determine the association between KIM-1 expression and the number of STCs. A one-way ANOVA with Bonferroni's Multiple Comparison Test was used to analyse the number of STCs in relation to age clusters. To determine the statistical difference between males and females, an unpaired t-test (two-tailed) was applied.

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Author contribution statement

JE and MB performed data acquisition, analysis, interpretation, literature search, generation of figures and drafted the first version of the manuscript and completed the final version of the manuscript. LM, VVM, BB, FM, BW, VLV, JD and RW carried out experiments and analysed/interpreted data. MH and PB carried out computational experiments and data analysis. SW and SF provided crucial kidney samples and revised the manuscript. ES, RK, MM, MV, MFS and JW designed experiments and revised the manuscript. JV designed experiments, interpreted the data and revised the manuscript. JJ and BS conceived the study and experiments, interpreted data and aided in writing the second version of the manuscript and generation of figures. All authors had final approval of the submitted version.

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Declaration of interest

The authors declare not conflict of interest

Data availability

For single-cell sequencing analysis, we used a dataset that is deposited on GEO with accession number GSE151302. The bulk RNA sequencing data are deposited on GEO with accession number GSE202824 (accessible at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE202824>). All scripts used for RNA sequencing analysis in RStudio are deposited on Zenodo and accessible using the following link: <https://doi.org/10.5281/zenodo.6168927>.

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Supplementary Files and Data

The full supplementary information (including supplementary tables S3-S6 and supplemental video S1) is accessible online via:

<https://pathsocjournals.onlinelibrary.wiley.com/doi/10.1002/path.6029>

Supplementary methods

Immunofluorescence staining

Formalin-fixed, paraffin-embedded tissue sections (4 μm) were deparaffinized using xylene and rehydrated. Antigen retrieval was performed using citrate buffer (pH 6, ScyTek Laboratories, Logan, UT). Prior to primary antibody incubation, sections were incubated with normal goat serum (Vector Laboratories, Peterborough, United Kingdom) in PBS for 10 minutes. Incubation with the primary antibodies in 1% BSA (v/v, Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS was performed overnight at 4 °C (Table S1). The secondary antibodies were incubated for 2 hours at room temperature in the dark (Table S2). Slides were mounted with DAPI fluoromount G (Southern Biotech, Birmingham, Alabama) and covered with a cover slip. Sections were examined using high content fluorescent microscopy (Leica DMI6000B).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded human renal tissue sections were deparaffinized using xylene and rehydrated. Antigen retrieval was performed in Tris/EDTA buffer pH 9 (VWR Chemicals, Amsterdam, The Netherlands). Endogenous peroxidase blocking was performed for 10 minutes in 0.3% hydrogen peroxide (v/v, Boom B.V., Drenthe, The Netherlands). Avidin and biotin blocking kit (Vector Laboratories) was used following the manufacturer's instructions. Prior to primary antibody incubation, sections were incubated with normal horse serum (Vector laboratories) in PBS for 10 minutes. Primary antibody incubation was done for 2 hours at room temperature (Table S1). Hereafter, sections were incubated with biotinylated horse anti-mouse antibody (Table S2) for 30 minutes at room temperature, followed by incubation with ABC*AP solution (Vector Laboratories), following the manufacturer's instructions. After washing, tissue sections were incubated with stay blue AP developing solution (Abcam, Cambridge, United Kingdom) for 5 minutes. Incubation with a second primary antibody was performed overnight at 4 °C (Table S1), followed by incubation with HRP-conjugated

secondary antibody (Table S2) for 30 minutes at room temperature. Colour development was done using AEC substrate (Abcam). Tissue sections were mounted with quick D mounting medium (Klinipath B.V., Duiven, The Netherlands) and covered with a cover slip. Slides were analysed using light microscopy (Leica DMLB S/N:178295).

FACS labelling

Human renal cortex tissue from the unaffected pole of kidneys after tumour nephrectomy was cut into small pieces (≤ 1 mm) and further processed using enzymatic digestion and manual dissociation. Briefly, small pieces of tissue were incubated in incubation medium consisting of DMEM/F12 (Gibco/Thermo Fisher Scientific, Breda, the Netherlands) supplemented with 200 U/mL collagenase IV (Thermo Fisher Scientific), 2 U/mL dispase II (Sigma-Aldrich) and 50 U/mL DNase I (Sigma-Aldrich) for 15 minutes at 37°C. This was pressed through a 150 μ m sieve using the plunger of a syringe and washed with incubation medium. After this, the tissue solution was incubated again for 10 minutes in the incubation medium at 37°C. This solution was rinsed through a 70 μ m and a 32 μ m sieve and washed using a saline solution. The flow-through was collected and centrifuged for 5 minutes at 1500 $\times$ g, the supernatant was removed and the pellet was resuspended in NaCl solution. The isolated cell pellet was resuspended in 1 ml PBS containing 1% BSA (v/v, Sigma-Aldrich) and antibodies against CD133, CD24 and CD13 (Table S1) were used to stain proximal tubule cells (PTECs) and scattered tubular cells (STCs). Antibody incubation was performed for 1 hour at 4°C in the dark. The pellet was washed three times with PBS and centrifuged at 1,500 $\times$ g for 5 minutes, the supernatant was removed and the pellet resuspended in PBS. This cell suspension was rinsed through a cell strainer (70 μ m) and collected in a FACS tube for cell sorting.

FACS

Cell sorting was performed to isolate PTECs and STCs for RNA sequencing using BD FACSAria instrument and software (BD Bioscience, Vianen, the Netherlands). PTECs were detected based on CD13 (PECy5) positive staining, without CD24 (FITC) and CD133 (PE) staining. STCs were detected based on CD13 (PECy5) CD24 (FITC) and/or CD13 (PECy5) CD133 (PE) positive staining. For bulk RNA sequencing (100,000 cells per sample), cells were collected in Eppendorf tubes in PBS and RNA isolation was performed.

Bulk RNA sequencing analysis

Reads were trimmed, using TrimGalore! v0.4.5, and mapped to a human reference genome (GRCh38.95, Ensembl) with Star v2.6.0a. MultiQC was used to combine results and perform quality checks. Counts were produced with HTSeq v0.11.0. Differential gene expression analysis was carried out with DESeq2 (v1.32.0) in R (v4.1.0), with internal statistical and normalization methods (i.e. multiple testing correction with Benjamini–Hochberg)⁵⁴. The average mRNA expression of PTEC samples was compared to the average mRNA expression of STC samples. All heatmaps were created using the pheatmap package (v1.0.12). Significantly (adj p-value < 0.05) up- or downregulated genes between PTEC samples and STC samples were used as input for the overrepresentation analysis using ClusterProfiler (v.4.0.2) and the ReactomePA package (v.1.36.0)^{55,56}. Pathway activity was analysed using the PROGENy package (v.1.14.0) using the top 100 pathway "footprint genes"⁵⁷. Next, transcription factor activity was analysed using the DoRothEA package (v.1.4.1) using regulons with confidence levels: A, B and C⁵⁸.

Single-cell RNA sequencing analysis

The publicly available human kidney dataset from a study by Muto et al. with accession number GSE151302 at the gene expression omnibus (GEO) was downloaded²⁸. Using Seurat (v.4.0.6) in R, this dataset was processed. First, cells were filtered to keep only cells containing 500–4000 features with less than 20% of mitochondrial gene expression. Next, all datasets were merged using Seurat and the following functions were performed with standard parameters: "NormalizeData", "FindVariableFeatures", "ScaleData" and "RunPCA". Next, harmony (v.0.1.0) integration of the datasets was performed, followed by the next steps in the Seurat workflow: "FindNeighbors", "FindClusters" and "RunUMAP". Twenty-four principal components (PCs) were used to find neighbours and to run the UMAP, whereas clusters were found with a resolution of 0.7. We identified 20 clusters and the VCAM1 positive and negative proximal tubule cells were identified by SLC34A1 and HAVCR1 expression based on the results in the study by Muto et al. (see supplemental figure 8A–B). After isolation of the PT and PT_VCAM1 clusters, we excluded cells that were part of other clusters, which were named possible doublets and shown in supplemental figure 8C. Next, we performed a new umap projection using the "RunUMAP" function (supplemental figure 8D) Similar as for the bulk RNA dataset, we performed overrepresentation analysis using the significant cluster markers with a positive log fold change found by "FindAllMarkers" as input for the ClusterProfiler package. We also performed pathway analysis

using PROGENy (using the top 500 "footprint genes") and transcription factor activity analysis using DoRothEA (confidence levels A, B and C)⁵⁹. Additionally, we performed pseudotime analysis using Monocle3 (v. 1.0.0)⁶⁰. Before pseudotime analysis using Monocle3, the Seurat object was first converted to a cell dataset object and subsequently, we performed the standard workflow as provided by the Cole-Trapnell lab. Finally, ligand-receptor analysis was performed using CellphoneDB to calculate ligand-receptor interaction scores between clusters⁶¹. Counts and metadata were extracted from the Seurat object and saved as text files. The text files were used as input for the CellphoneDB (v.1) analysis in Ubuntu on Windows. After analysis, the output was processed and cell-cell interaction (CCI) plots and Sankey plots were created using the R-package CrossTalkR (v. 1.2.0)⁶².

Workflow ImagJ quantification

1. Workflow PTEC analysis

ImageJ, calculation of the number of proximal tubule epithelial cells:

C0: DAPI

1. Image - Adjust - Threshold: B&W, Huang, Dark background - apply
2. Process - Binary - convert to mask
3. Process - binary - watershed

C1: AQP1

1. Image - adjust - threshold: B&W, huang, dark background - apply
2. Remove gloms by circle them with freehand polygon selection - backspace
3. Process - binary - fill holes
4. Edit - Selection - create selection

Click on C0:

1. Shift + E
2. Analyse - Analyse particles: size (10-500), show outlines, display results, clear results, summarize

2. Workflow α -SMA analysis in Image J

ImageJ, Analysis α SMA expression surface area:

1. Open lif file: view stack with hyperstack, colour mode: default, split channels, autoscale.

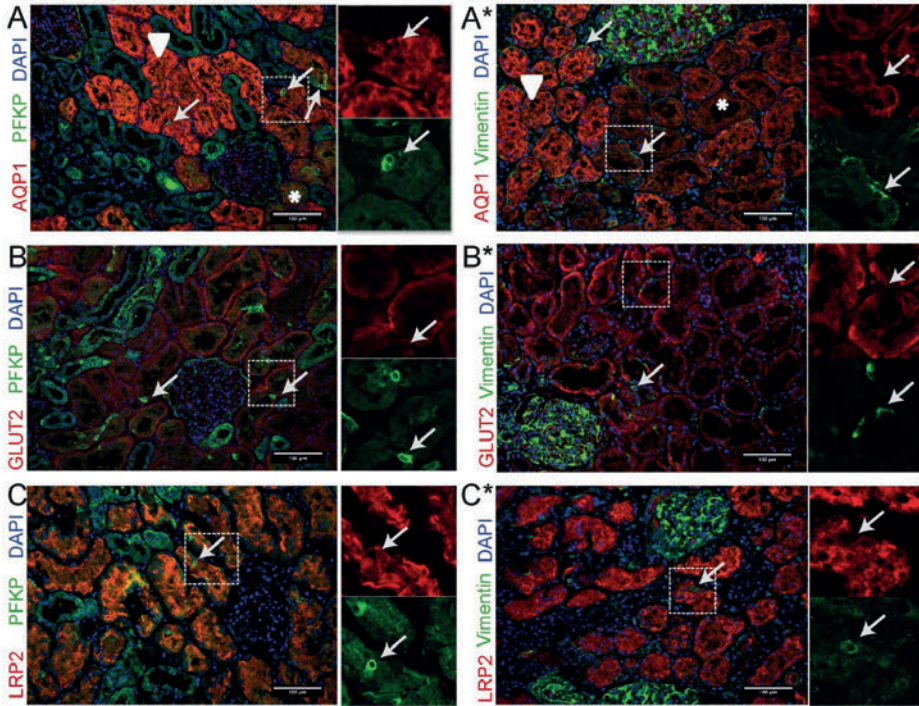
2. Make 8bit image
3. Image – adjust – threshold – isodata, red, dark background - apply
4. Analyse – set measurements – area, area fraction, limit to threshold, display label, mean gray value – ok
5. Draw polygon selection
6. Analyse – measure

Table S1. Primary antibodies used for immunostaining

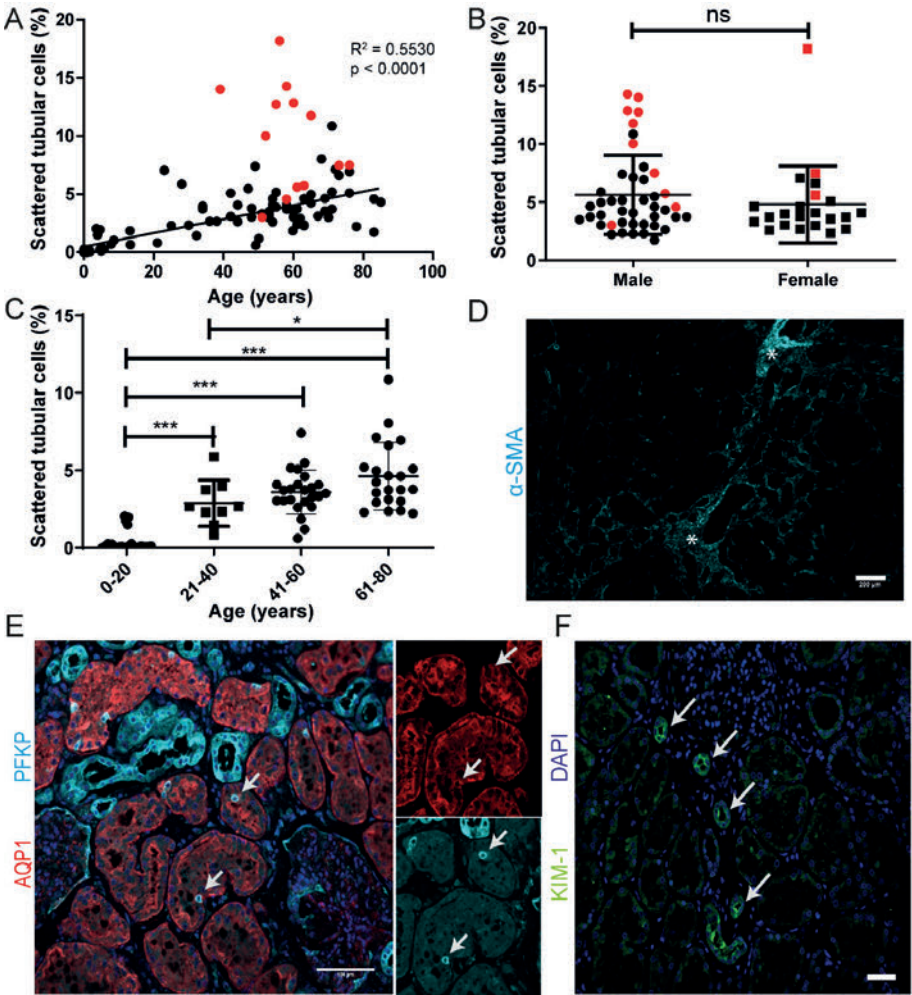
Primary antibody	Species	Dilution	Manufacturer
α -smooth-muscle-actin (α -SMA)	Mouse	1:500	ab7817, Abcam, Cambridge, United Kingdom
Annexin A3 (ANXA3)	Rabbit	1:100	HPA013398, Sigma Aldrich, Zwijndrecht, The Netherlands
Annexin A2 (ANXA2)	Mouse	1:200	sc-28385, Santa Cruz Biotechnology, Dallas, TX
Aquaporin 1 (AQP1)	Rabbit	1:200	#800765777, Alpha Diagnostics, San Antonio, TX
Megaline/LRP2	Rabbit	1:100	HPA064792, Sigma Aldrich
Glucose transporter 2 GLUT2/SLC2A2	Rabbit	1:100	HPA028997, Sigma Aldrich
Phosphofructokinase, platelet, PFKP	Mouse	1:100	TA503983, Origene, Herford, Germany
P16, cyclin-dependent kinase inhibitor 2A, CDKN2A	Mouse	1:100	ILM0632-C01, Immunologic, Duiven, The Netherlands
Tumour suppressor p53	Mouse	1:100	ILM27011-C01, Immunologic
Vimentin/V9	Mouse	1:100	MA5-11883, Invitrogen/Thermo Fisher Scientific, Breda, The Netherlands
Vimentin	Rabbit	1:100	RM-9120-S1, Thermo Fisher Scientific, Breda, The Netherlands
Lamin B1, LMNB1	Rabbit	1:100	HPA050524, Sigma Aldrich
A-kinase anchor protein 12, AKAP12	Rabbit	1:100	TA322670, Origene
Pyruvate kinase PKL	Mouse	1:100	sc-1332222, Santa Cruz Biotechnology
Pyruvate kinase PKM2	Rabbit	1:100	#3198, Cell signalling, Leiden, The Netherlands
Lotus Tetragonolobus lectin, LTA-FITC	Directly labeled	1:200	FL1321, Vector laboratories, Peterborough, United Kingdom
aminopeptidase N, CD13-PECy5	Directly labeled	1:25	A07763, Beckman Coulter, Woerden, The Netherlands
Prominin-1, CD133-PE	Directly labeled	1:25	#130-113-108, Miltenyi Biotec, Leiden, The Netherlands
cluster of differentiation 24, CD24-FITC	Directly labeled	1:25	MHCD2401, Thermo Fisher Scientific

Table S2. Secondary antibodies used for immunostaining

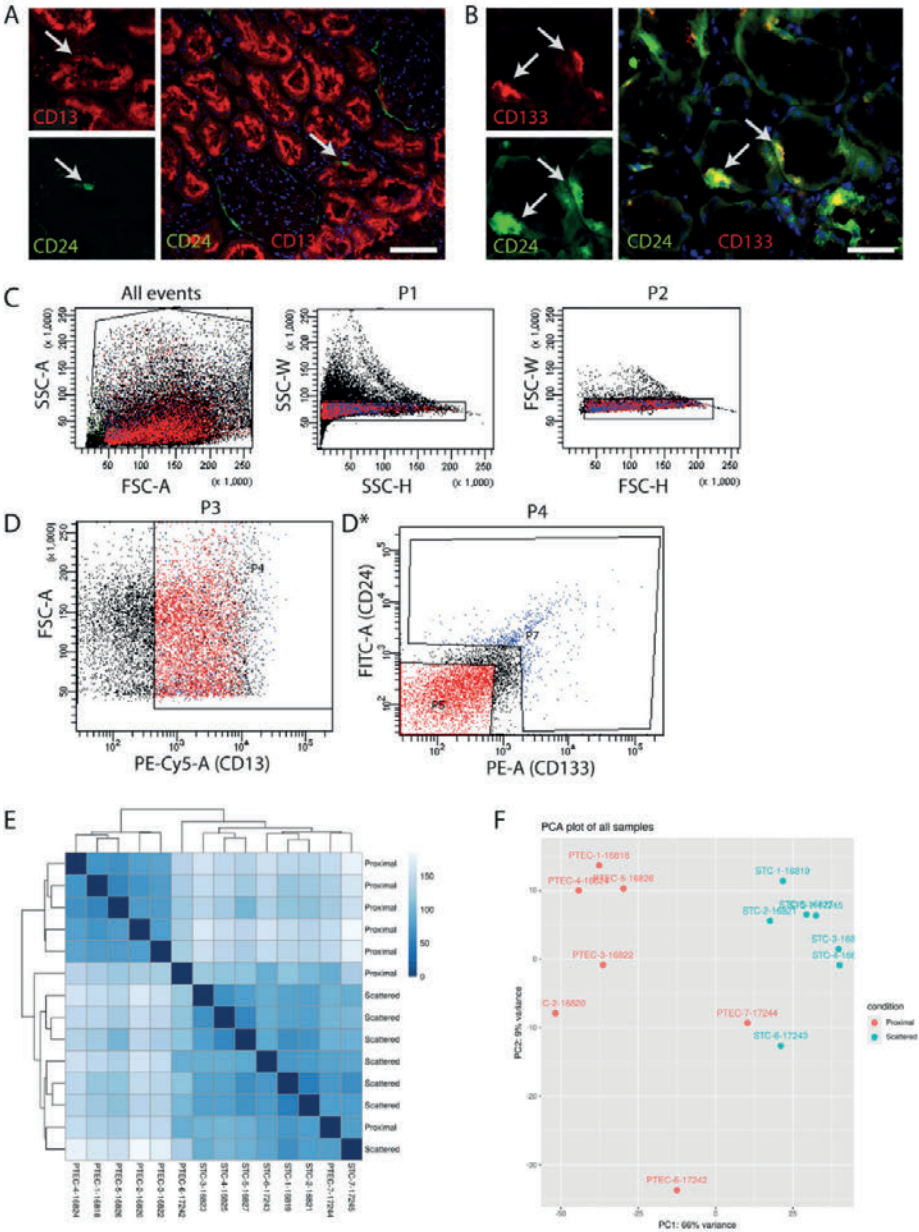
Secondary antibody	Species	Dilution	Manufacturer
Alexa fluor 647	Donkey-anti-mouse	1:200	ab150107, Abcam, Cambridge, United Kingdom
Alexa fluor 647	Goat-anti-rabbit	1:200	A-21245, Invitrogen/Thermo Fisher Scientific, Breda, The Netherlands
Alexa fluor 568	Goat-anti-rabbit	1:200	A-11036, Invitrogen
Alexa fluor 569	Donkey-anti-rabbit	1:200	A-10042, Invitrogen
Biotinylated	Horse-anti-mouse	1:200	BA2000, Vector laboratories, Peterborough, United Kingdom
Alexa fluor 488	Goat-anti mouse	1:200	A-11029, Invitrogen
Alexa fluor 488	Donkey-anti-rabbit	1:200	A-21206, Invitrogen
Brightvision+ Poly-HRP	Goat-anti-mouse		DPVM110HRP, Immunologic, Duiven, The Netherlands
Brightvision+ Poly-HRP	Goat-anti-rabbit		DPVR55HRP, Immunologic



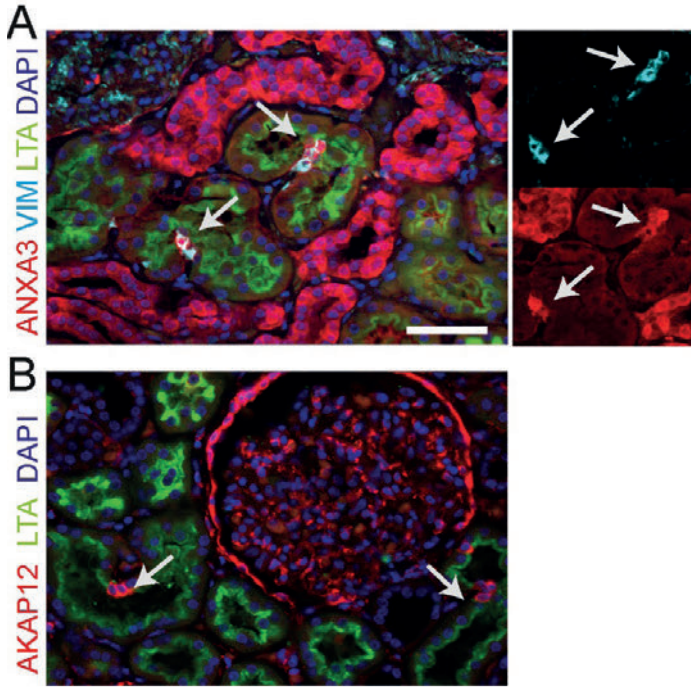
Supplemental figure 1. Scattered tubular cells are present in all segments of the proximal tubule. (A, A*). Immunofluorescence staining of AQP-1 (red) can be detected inside the proximal tubule, showing staining of different intensity with strong expression in the proximal straight tubules (arrowheads) and less intense staining in the convoluted part of the proximal tubule (asterisk). In both parts of the proximal tubule, STCs (white arrows) can be detected, indicated by the expression of PFKP (green, A) or (vimentin (green, A*). (B, B*) Immunofluorescence staining of GLUT2 (red) as proximal tubule marker, together with STC marker PFKP (green, B) or vimentin (green, B*) showing the presence of STCs (white arrows) inside the proximal tubule. (C, C*) Staining of megalin (LRP2) (red) which is expressed in the proximal tubule segments S1 and S2. In the megalin positive proximal tubules, STCs (white arrows) can be detected indicated by the expression of PFKP (green, C) and vimentin (green, C*). Scale bars: (A-C*) 100 μ m. Abbreviations: AQP-1, aquaporin-1; GLUT2, glucose transporter 2; PFKP, phosphofructokinase-platelet; STC, scattered tubular cell.



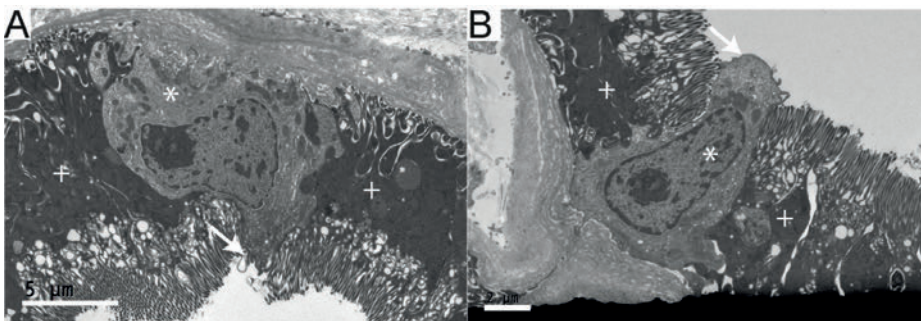
< Supplemental figure 2. Scattered tubular cell numbers increase with age. (A) Regression analysis of STC number versus age was performed for normal human kidney tissue obtained from tumour nephrectomies using the unaffected pole of 37 patients (48-85 years), 29 renal tissue samples obtained from renal transplant biopsies from donor kidneys prior to transplantation (age 23-76) and 41 young kidney tissues obtained from autopsy (age 0-61 years). The number of STCs significantly increases with age. The red points show patients with α -SMA expression of more than 5 % of the whole tissue slide surface area. (significant deviation from zero excluding all tissues with more than 5% α -SMA expression (red points), $R^2 = 0.5530$, $P < 0.0001$). (B) Analysis of the number of STCs in relation to gender showed no significant difference between male and female in normal human kidneys derived from the unaffected pole after tumour nephrectomy. Kidneys with a high expression of α -SMA are indicated in red. (C) In the clustering of age groups (child -young adults, adults, middle aged and elderly), the samples with a high α -SMA expression of more than 5 % of the whole tissue slide surface area are excluded. One-way analysis of variance shows significant differences between the age groups. (D) Representative microscopic image of immunofluorescence staining for α -SMA (cyan) as marker for interstitial fibrosis (asterisk). Surface area stained positive for α -SMA was calculated automatically in ImageJ. (E) Representative image of AQP1 staining as PTEC marker (red) together with STC marker PFKP (cyan) showing STCs (arrows) located inside the proximal tubule. With this staining, the percentage of PTECs which showed PFKP expression was calculated as percentage STCs present in the kidneys. Analysis was performed on 8 images per kidney (20x). (F) Representative image of immunofluorescence staining for KIM-1 (green) as marker for acute tubular injury shows expression of KIM-1 in proximal tubules at the luminal side (arrows). * = $p < 0.05$, *** = $p < 0.001$. Abbreviations: α -SMA, alpha-smooth muscle actin; AQP1, aquaporin-1; KIM-1, kidney injury molecule 1; PFKP, phosphofructokinase-platelet; PTEC, proximal tubular epithelial cell; STC, scattered tubular cell.



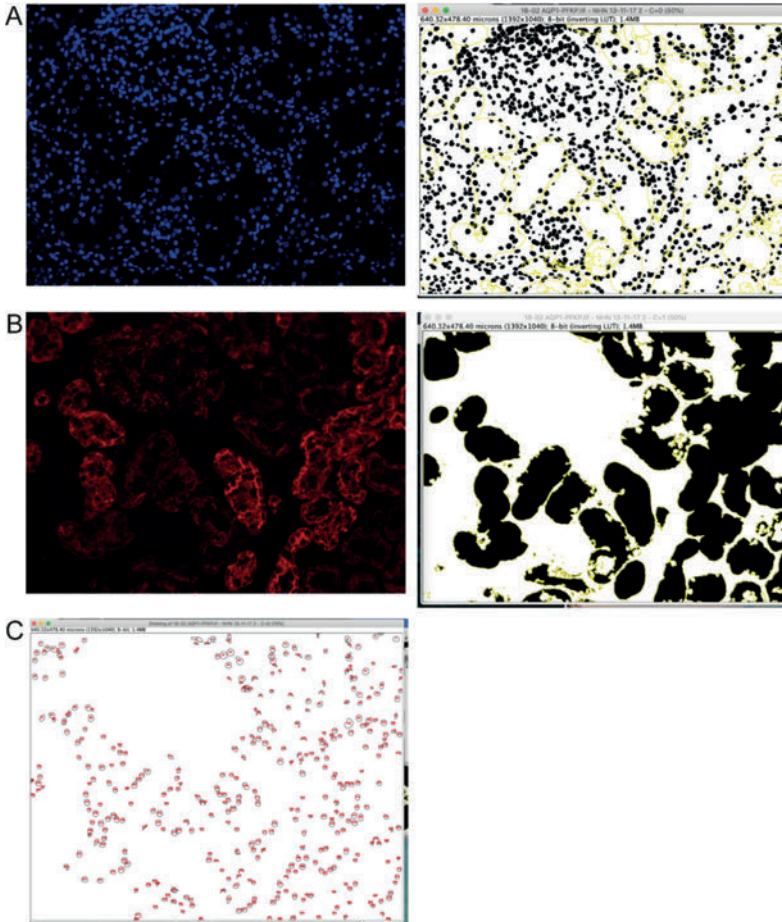
Supplemental figure 3. Fluorescence-activated cell sorting strategy and exploratory bulk RNA sequencing analysis. (A) Immunofluorescence staining of CD13 (red) as a marker for the proximal tubule and CD24 (green) as a marker for STCs shows a STC expressing CD13 and CD24 (white arrow) inside the proximal tubule. (B) Representative image of immunofluorescence staining against CD24 and CD133 which co-localize in STCs (white arrow) and are not expressed by the other PTECs. (C) Cell sorting using BD FACSARIA to isolate STCs and PTECs, showing the forward and side scatter gating. (D) P3 was selected based on forward and side scatter gating and a new selection of cells was made based on cell size (FSC-A) versus CD13 expression (PE-Cy5). CD13 positive cells were selected in P4. (D*) All CD13 expressing cells were screened for expression of CD24 (FITC) and/or CD133 (PE). CD13 expressing cells negative for CD24 and CD133 were selected and sorted for PTEC analysis (P5). CD13 positive cells expressing CD24 and/or CD133 were selected and sorted for STC analysis (P7). (E) Exploratory data analysis of raw RNA-seq samples was performed using DESeq2 software packages. The Euclidean distances between the samples as calculated from the regularized log transformation are shown in the heatmap of the sample-to-sample distances. (F) PCA generated with the ggplot2 library showing a PCA plot for all samples. Different colours denote the two conditions, orange = PTECs, blue = STCs. Scale bar: (A) 100 μ m, (B) 50 μ m. Abbreviations: PCA, principal component analysis; PTEC, proximal tubular epithelial cell; STC, scattered tubular cell.



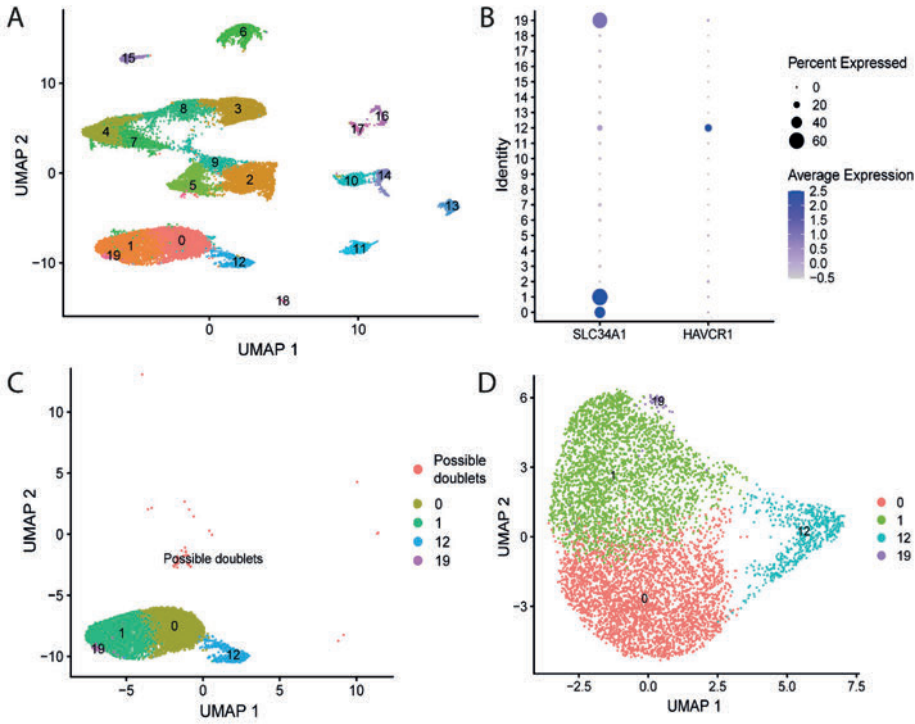
Supplemental figure 4. Immunohistochemical validation of STC markers upregulated in bulk RNA sequencing data. (A) Representative image of STCs (arrows) expressing vimentin (VIM, cyan) and annexin A3 (ANXA3, red), located in the proximal tubule (LTA positive, green). (B) Immunofluorescence staining of AKAP12 (red) expressing STCs (arrows) inside the proximal tubule (stained for LTA (green) which is located at the apical side). Scale bars: (A, B) 50 μ m. Abbreviations: A-kinase anchoring protein 12; LTA, Lotus tetragonolobus lectin; STC, scattered tubular cell.



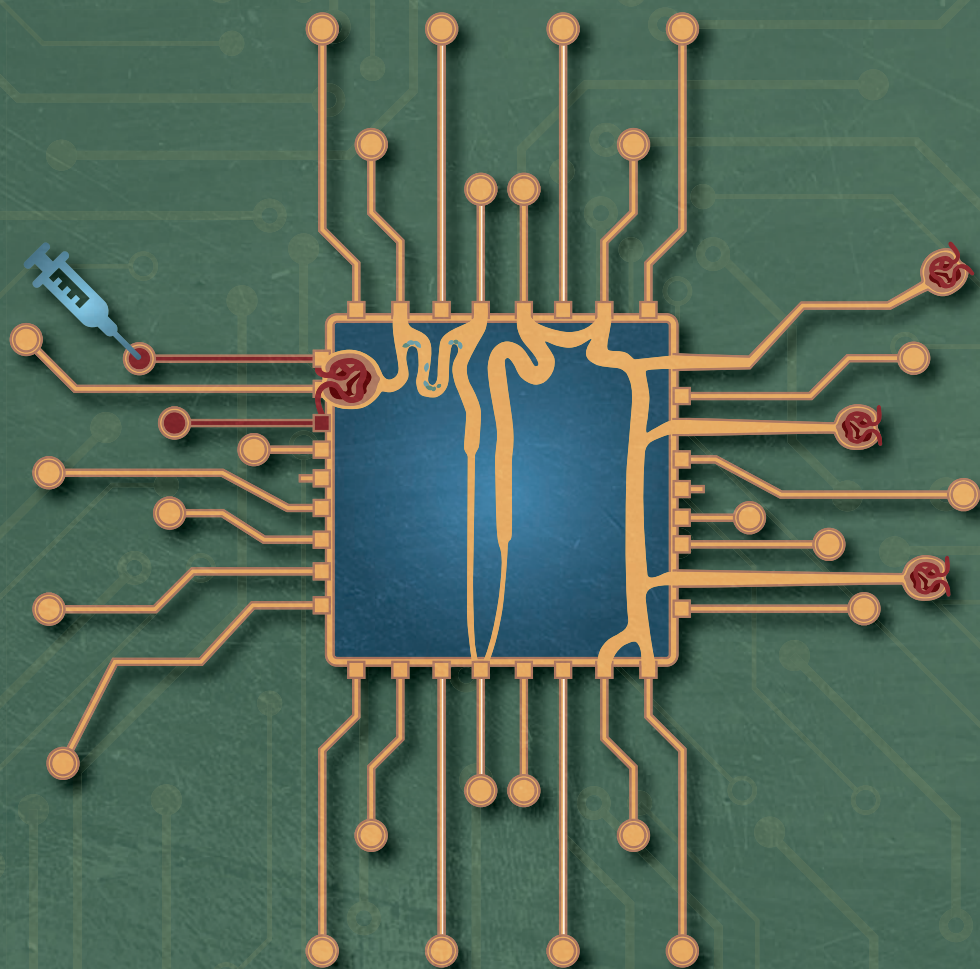
Supplemental figure 5. Scattered tubular cells show less mitochondria and a rudimentary brush border and basal labyrinth. (A, B) Representative images of STCs (asterisks) using transmission electron microscopy. Adjacent PTECs (plus) are characterized by an intact brush border and basal labyrinth and show a high density of mitochondria (dark grey colour), identifying them as PTECs. STCs show loss of brush border (arrow) and are less packed with mitochondria and other cell organelles. Scale bars: (A) 5 μ m, (B) 2 μ m.



Supplemental figure 7. Fiji workflow for automated counting of proximal tubular epithelial cells. (A) DAPI staining for all nuclei (blue). Using a threshold and a binary mask, all nuclei are shown as black dots (see right image) in ImageJ. (B) AQP-1 staining as PTEC marker (red). An automated threshold was used and glomeruli were manually deleted. The tubular lumen was filled and whole tubuli including the tubular lumen are shown in black in ImageJ (see right image). (C) Using this selection, the nuclei only present inside the proximal tubule areas can be automatically counted using the ImageJ analysis tool. The workflow of this analysis per channel is summarized above (1. Workflow PTEC analysis). Abbreviations: AQP-1, aquaporin-1; PTEC, proximal tubular epithelial cell.



Supplemental figure 8. Pre-processing of the single-cell sequencing database and subsequent clustering. (A) UMAP projection of 5 integrated single-cell RNA sequencing datasets of healthy human kidneys, downloaded from the online gene expression omnibus repository (accession number: GSE151302). (B) DotPlot showing the expression of SLC34A1 and HAVCR1 in the clusters identified in the human kidney. IN the original manuscript, the authors identified the proximal tubule cluster and the PT_VCAM1 cluster using these two genes and therefore we used a similar approach to separate the PT and PT_VCAM1 clusters from the entire kidney dataset. (C) We removed cells that belonged to the clusters that we separated from the dataset, but were present in other cell clusters since these might be doublets belonging to two different clusters. (D) New UMAP projection after separating the PT and PT_VCAM1 clusters and removing the possible doublets. Abbreviations: UMAP, uniform manifold approximation and projection.



Chapter 6.

General discussion and conclusion

One of the primary functions of the kidneys is to maintain blood homeostasis by filtering the blood and eliminating metabolic waste products and toxins produced by the body. The functional units of the kidneys, the nephrons, comprise several distinct segments, each with unique functions, structures and biological characteristics. Injuries affecting different parts of the nephron can have various causes and clinical manifestations. For instance, idiopathic nephrotic syndrome (iNS) is caused by injury to the podocytes, leading to a compromised glomerular filtration barrier. Consequently, iNS patients lose proteins in the urine, which need to be kept in the blood. On the other hand, acute kidney injury (AKI) is most often caused by injury to the proximal tubule and leading to disrupted urine processing and possible obstruction. Currently, iNS and AKI are both managed by treating symptoms, since there are no specific therapies targeting the cause of the disease. If not treated properly, these diseases can progress to end-stage kidney disease, and the patient will become dependent on kidney replacement therapy by either dialysis or a kidney transplantation. This chapter will discuss the key findings in this thesis and outline future research opportunities aimed at improving therapeutic strategies for iNS and AKI.

6.1 Nephrotic syndrome pathophysiology and treatment with prednisolone

iNS is caused by injury to the podocytes of the glomerulus and is often treated with prednisolone. Unfortunately, prednisolone is hardly specific, and treatment responses as well as side-effects are variable within patients. In part, this might be because its molecular mechanism of action is still poorly understood, even though prednisolone has been used for over 60 years for the treatment of iNS. It remains elusive which cell type is most affected by prednisolone to achieve remission and, perhaps even more striking, the causative factors of iNS are still to be uncovered. Therefore, targeted therapies are needed for patients with iNS and, in particular, patients with minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) where glucocorticoids are most commonly used.

Immune cells are a major driver of iNS, but not the only cell types involved in prednisolone-induced recovery

Malfunctioning of the immune system has been implicated as a cause of iNS based on several observations: 1) Sensitivity to immunosuppressive treatment

with prednisolone or cyclophosphamide, 2) Association of iNS with T-cell lymphoma and 3) Spontaneous remission of iNS after measles infection, which suppresses T-cell function¹. After these initial observations, several studies have reported changes in subsets of T cells; however, these results have not been conclusive yet^{2,3}. Shalhoub proposed the existence of a circulating permeability factor released by T-cells in 1974, which has been the topic of extensive research ever since. Given the vast number of studies identifying circulating permeability factors, it is likely that multiple exist and that the expression in iNS patients is heterogeneous^{2,4}.

The involvement of B cells in MCD or FSGS pathology has gained a lot of attention, among others by the discovery of anti-nephrin antibodies in iNS⁵⁻⁹. In line with these findings, recent studies that performed immune cell profiling by single-cell RNA (scRNA) sequencing and flow cytometry have shown that the major changes in childhood iNS are found in the B-cell population rather than in the T-cell population^{10,11}. In the most recent study, anti-nephrin antibodies were found in a large percentage of steroid-sensitive iNS patients, in which glucocorticoids induce remission, but in a much smaller proportion of patients with steroid-resistant iNS, indicating a link between glucocorticoid treatment efficacy and anti-nephrin autoantibody presence¹². However, the methods used to detect anti-nephrin antibodies in patient plasma may not yet be optimal and might lead to false-positive results¹³. Although the methods used to detect anti-nephrin antibodies might be suboptimal, the pathogenicity of anti-nephrin antibodies is also supported by the successful use of rituximab, an antibody targeting CD20 that is expressed by proliferating and antibody producing B cells, in the treatment of iNS. However, also for rituximab, direct effects on the podocyte have been shown, which opens up the possibility that, similar to glucocorticoids, effects on the immune system do not represent the full mechanism of action^{14,15}. When anti-nephrin antibodies are involved in iNS pathogenesis, glucocorticoids may aid in the resolution of the disease by stopping anti-nephrin production in B cells. However, some patients respond very rapidly to treatment, sometimes even within 1-3 days. This rapid response suggests another mechanism of action since depletion of B cells by prednisolone is likely to be slower than 1-3 days¹⁶⁻²¹. Altogether, the immune system may be involved in iNS and prednisolone could partly help in recovery by dampening immune responses. However, since prednisolone can induce remission within three days, effects on other cell types must be considered to be at least partly involved in the mechanism of action of prednisolone in iNS. Unravelling the specific effects on other cell types may provide novel targets

for the development of more effective and more specific treatment options for patients with iNS.

Prednisolone effects on parietal epithelial cells, mesangial cells, glomerular endothelial cells and podocytes

Other cell types that have been mentioned in the literature to be affected by prednisolone are parietal epithelial cells (PEC), glomerular endothelial cells, mesangial cells and podocytes, and will be discussed next.

We observed PEC activation in chapter 4, characterized by CD44 expression on PECs that decreased with prednisolone treatment, which poses the question: does prednisolone directly target PECs to decrease CD44 or is decreased PEC activation an indirect effect? *In vitro* studies have shown that prednisolone can decrease PEC activation directly^{22,23}. Prednisolone has also been suggested to stimulate Wilms tumor 1 (WT1) expression in PECs and the potential conversion of PECs to podocytes to restore podocyte number²⁴. However, it has also been shown that PEC activation was decreased because glucocorticoids reduce epidermal growth factor receptor (EGFR) signalling in podocytes, leading to lower Notch signalling in PECs, which normally stimulates PEC activation²⁵. In our study, we did not observe increased EGFR or Notch signalling in the ligand-receptor interaction analysis, which could be because of the scarcity of the single-nuclei dataset. However, this may also indicate more direct effects of prednisolone on the PECs. Besides PECs, there might also be an effect of prednisolone on glomerular endothelial or mesangial cells. For instance, increased reactive oxygen species (ROS) induced injury was found in a model of iNS and prednisolone was found to increase antioxidant enzyme activity that protects from ROS injury^{26,27}. More specifically, prednisolone induced antioxidant enzyme activity in glomerular endothelial and mesangial cells, in line with a decrease in symptoms of iNS like proteinuria and podocyte foot process effacement.

Overall, glucocorticoids may exert effects on multiple glomerular cell types, such as PECs, mesangial cells and glomerular endothelial cells. However, the epigenetic and transcriptional changes that we observed in **chapter 4** primarily affected podocytes and not PECs, mesangial cells or glomerular endothelial cells. If we also consider that treatment of glomerular cell types might not target the disease cause, it would be good to focus on the main injured cell type, i.e. the podocyte, for which we know recovery can lead to direct relief of clinical symptoms.

In the following sections, several molecular mechanisms will be discussed that were found in **chapter 4** to be regulated by prednisolone. These mechanisms include the stimulatory effects of prednisolone on the circadian rhythm, the dysregulation of lipid metabolism in iNS, as well as the mechanism of action for peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1 α), carnitine palmitoyltransferase 1b (Cpt1b) and pyruvate dehydrogenase kinase 4 (Pdk4) (Figure 1).

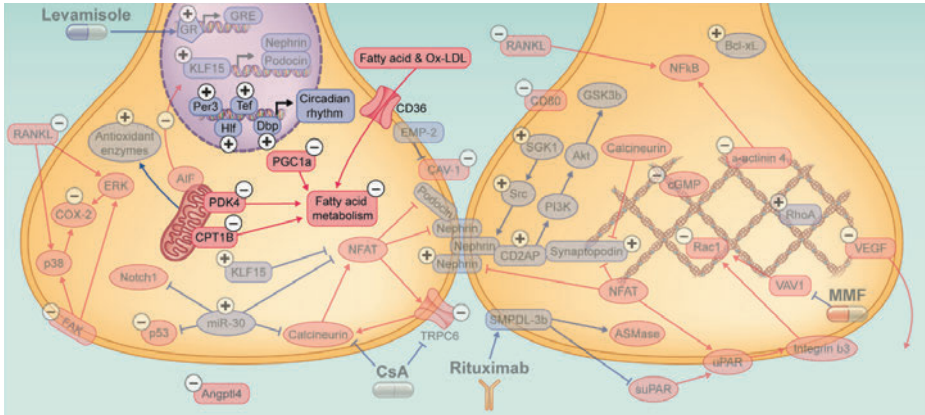


Figure 1, adapted from Broek, M et al.¹⁴¹ Schematic overview of proposed molecules affected by glucocorticoid treatment, with a focus on our results from chapter 4. The mechanism of podocyte injury and recovery by prednisolone that were described in chapter 4 are added to the figure that was adapted from chapter 3. Blue arrows show a mechanism that favours podocyte survival, while red arrows show mechanisms that favour podocyte apoptosis. Molecules in green are beneficial for podocytes when expressed, whereas molecules in red are detrimental for podocyte health when highly expressed. An arrowhead line represents a stimulatory reaction, meaning that the interaction yields an increased expression or activity. A bar-headed line shows an inhibitory reaction, meaning that the interaction yields a decreased expression or activity. A circle with a minus means this molecule is inhibited by glucocorticoids, whereas a circle with a plus means this molecule is stimulated by glucocorticoids. The mechanisms of action of other iNS therapeutic agents: rituximab, mycophenolate mofetil, cyclosporin A and levamisole are also indicated. Image created with Biorender. Abbreviations: AIF, apoptosis inducing factor; Akt, protein kinase B; Angpt14, angiopoietin-like 4; ASMase, acid sphingomyelinase; Bcl-xL, B-cell lymphoma-extra large; CAV-1, caveolin-1; CD2AP, CD2- associated protein; CD80, cluster of differentiation 80; COX-2, cyclooxygenase 2; CPT1B, carnitine palmitoyltransferase 1b; cGMP, cyclic guanosine monophosphate; CsA, cyclosporine A; Dbp, D-Box binding PAR bZIP transcription factor; EMP-2, epithelial membrane protein 2; ERK, extra- cellular regulated kinase; FAK, focal adhesion kinase; GR, glucocorticoid receptor; GRE, glucocorticoid responsive elements; GSK3 β , glycogen synthase kinase 3 β ; Hlf, HLF transcription factor, PAR bZIP family member; KLF15, krüppel-like factor 15; MMF, mycophenolate mofetil; miR30, microRNA family member 30; NFAT, nuclear factor of activated T- cell; NF κ B, nuclear factor κ B; p38, p38 mitogen-activated kinase; p53, cellular tumour antigen p53; PDK4, pyruvate dehydrogenase kinase 4; Per3, period circadian regulator 3; PGC1 α , peroxisome proliferator-activated receptor γ coactivator 1 α ; PI3K, phosphoinositol-3 kinase; Rac1, ras-related C3 botulinum toxin substrate 1; RANKL, receptor activator of nuclear factor kappa B ligand; RhoA, ras homology family member A;

SGK1, serum and glucocorticoid-regulated kinase 1; SMPDL-3b, sphingomyelin phosphodiesterase acid-like 3b receptor; suPAR, soluble urokinase-type plasminogen activator receptor; Tef, TEF transcription factor, PAR bZIP family member; TRPC6, transient receptor potential channel 6; uPAR, urokinase-type plasminogen activator receptor; VAV1, vav guanine nucleotide exchange factor 1; VEGF, vascular endothelial growth factor.

The involvement of the circadian rhythm and its stimulation in podocytes by prednisolone

The circadian rhythm is involved in regulating day and night processes in our body and is also important for healthy kidney physiology. For instance, circadian rhythm regulates the expression of glomerular basement membrane components and autophagy, which is important for healthy podocyte function²⁸⁻³⁰. In **chapter 4**, we describe that prednisolone induces the expression of several circadian rhythm genes, which might lead to restored healthy podocytes. These findings are in line with previous studies that have also found that treatment with glucocorticoids increases circadian rhythm-related genes in podocytes³⁰⁻³². In one study, glucocorticoids were even shown to stimulate the expression of 928 rhythmic podocyte genes, of which 19 are known podocyte disease-associated genes, showing a link between glucocorticoid treatment and the regulation of podocyte genes associated with injury³⁰.

In tubular cells, knockout of a circadian mediator leads to metabolic changes, demonstrating a link between circadian rhythm and metabolism in kidney tubular cells^{33,34}. This is interesting since we observe increased circadian rhythm gene expression in prednisolone-treated podocytes and observe differences in metabolism between healthy and injured podocytes, indicating that the link between circadian rhythm and metabolism might also be present in podocytes. And although the use of circadian modulators may be of interest in a more chronic setting, the timing of treatment with prednisolone could be considered, and perhaps variations in timing might lead to more effective induction of remission^{35,36}. Especially since previous studies have shown that the timing of glucocorticoid administration can be very important for the observed effects on hepatic and muscle cells^{37,38}.

However, the stimulation of circadian rhythm by prednisolone in podocytes brings up an important caveat in the kidney field. In almost all studies mentioned above, including our own study, only male mice or rats are used. The rationale for using male animals is that kidney injury is usually more pronounced in male animals (unpublished data from the animal study described in **chapter 4**). The incidence of iNS, which is modelled and investigated, is also higher in men versus women, which could be caused by sex hormones, sex chromosomes or

gut microbiota/immunology³⁹. But we also have to investigate the differential regulation of the circadian rhythm between men and women to explain these differences. In an elegant study by Talamanca et al., a higher rhythmicity is described in women compared to men⁴⁰. If the increased expression of circadian rhythm genes by prednisolone reflects a restoration of the circadian rhythm and desynchronization of the circadian rhythm contributes to podocyte injury, woman might have a lower incidence of iNS because they have a higher rhythmicity and are more difficult to desynchronize. However, future studies should prove that male versus female differences in rhythmicity also exist in podocytes and that desynchronization of circadian rhythm is causative for the development of podocyte injury.

Lipids cause toxicity in iNS and prednisolone decreases lipid metabolism

As previously mentioned, the circadian rhythm is described to regulate cellular metabolism. In **chapter 4** we observed increased fatty acid metabolism in injured podocytes, whereas prednisolone decreased fatty acid metabolism and increased glycolysis, an important metabolic pathway for foot process homeostasis⁴⁰⁻⁴². For instance, knockdown of the glycolytic enzyme pyruvate kinase type M2 (PKM2) can lead to podocyte injury, and restoration of PKM2 expression can induce recovery⁴³. Therefore, specific stimulation of glycolysis in the podocytes may present an effective treatment to restore foot process morphology and function.

The effects of increased lipid levels in kidney diseases have been extensively investigated, confirming the toxicity of lipid accumulation in podocytes^{44,45}. Many of the identified lipid-mediated toxic pathways are now targeted, largely in diabetic nephropathy^{44,46}. However, it is increasingly recognized that lipotoxicity might also be an important player in iNS. For instance, an upregulation of CD36 (a scavenger receptor for oxidized-low density lipoprotein (LDL) and fatty acids) was observed in patients with MCD, which decreased after treatment with glucocorticoids, indicating that an increased influx of oxidized-LDL and fatty acids in podocytes is harmful and is decreased by glucocorticoids⁴⁷. Also, in primary FSGS lipid droplet formation has been found, which may act as protection against injury by lipids by safely packaging the lipid in droplets⁴⁵. Lipid toxicity could be an important contributor to podocyte injury since dyslipidaemia is often seen in iNS, and free fatty acids bound to albumin have been found to stimulate macropinocytosis (the internalization of extracellular fluid) in podocytes⁴⁸. Albumin bound by free fatty acids was a more potent stimulator of macropinocytosis in podocytes compared to albumin without free fatty acids.

Thus, although lipid toxicity may not be causative, increased free fatty acid concentrations would lead to more uptake of albumin with free fatty acids, which would lead to podocyte injury and could therefore present a mechanism that maintains podocyte injury after an initial insult. In line with this, altered plasma profiles of free fatty acids have been found in small cohorts of patients with iNS, and one has even observed a change in profile upon glucocorticoid treatment⁴⁹⁻⁵¹. In a cohort of steroid-dependent children in remission, persistent abnormalities in free fatty acid profiles were observed⁵². Steroid-dependent iNS was defined by at least 2 consecutive relapses during tapering or within 14 days after stopping glucocorticoid treatment. If the changed free fatty acid profile stimulates podocytes to take up albumin with free fatty acids that leads to podocyte injury, this could explain the relapses after stopping glucocorticoids. Glucocorticoids may induce remission by direct stabilization/recovery of podocytes or other cell types, but as soon as treatment is stopped, free fatty acid uptake would lead to podocyte injury and proteinuria. Interestingly, exposure of *in vitro* podocytes to the plasma of patients with FSGS induced lipid droplets in these podocytes, indicating that something in the plasma of FSGS patients induces lipid deregulation⁴⁵. In this context, it would be interesting to uncover whether different fatty acids have different effects on the podocyte, which could indicate that treatment during remission should also focus on lowering certain fatty acids levels, perhaps in part by dietary interventions.

PDK4, PGC1 α and CPT1B

PDK4 is a metabolic regulator that drives the shift from glycolysis to fatty acids as the major energy source. In several studies, upregulation of PDK4 is associated with podocyte injury, and downregulation of PDK4 can restore injury^{53,54}. The inhibition of PDK4 was done by delivery of extracellular vesicles containing miRNA that suppresses the expression of PDK4 leading to decreased podocyte injury. In **chapter 4**, we performed pilot experiments using several inhibitors, including a PDK4 inhibitor. However, high concentrations of PDK4 inhibitor were toxic to the conditionally immortalized podocytes (ciPODs), whereas lower concentrations were not effective in reversing PAN-induced podocyte injury. In the future, it would be interesting to test the extracellular vesicles containing miRNA or identify different PDK4 inhibitors that may result in podocyte protection or recovery.

The increased expression of PDK4 is regulated by increased expression of PGC1 α ⁵⁵. Overexpression of PGC1 α has also been shown to lead to collapsing glomerulopathy, which is in line with our observations in **chapter 4** that

podocyte injury is accompanied by PDK4 and PGC1 α overexpression. However, conflicting results have been found in the literature, showing decreased PGC1 α levels after injury in kidney cortex samples^{56,57}. These results highlight the importance of single-cell analysis, since expression levels in a mixture of kidney cortex cells do not reflect the podocyte expression that we investigated. However, it should be noted that these studies indicate that expression of PDK4 and PGC1 α may be different among the several cell types in the kidney. Similarly, CPT1 has an important function in healthy proximal tubule cells. For instance, in contributing to fatty acid oxidation, the primary energy source for proximal tubule cells and in kidney organoid models, more mature proximal tubule cells are characterized by high CPT1A expression^{58,59}. However, in **chapter 4**, we discussed that upregulation of CPT1B in podocytes is associated with injury and human ciPODs are protected from PAN-induced injury by treatment with etomoxir, a CPT1 inhibitor. These results argue for more targeted treatment strategies, since downregulation of one of these targets may present with adverse effects in proximal tubule cells, where expression is essential for healthy physiology.

In several studies, the effects of podocyte injury on the expression of the three CPT1 isoforms: CPT1A, CPT1B or CPT1C were described, with some conflicting results. For instance, in microdissected glomeruli of diabetic nephropathy patients, one study showed upregulation of all three isoforms of CPT1, whereas another study showed downregulation of A and upregulation of C^{60,61}. In a study by Bhayana et al., a non-significant increased transcriptomic expression for CPT1B was seen in diabetic kidneys and MCD/FSGS kidneys²⁹. However, all these studies performed bulk RNA sequencing on microdissected glomeruli, which contain a mixture of all the cell types. In **chapter 4**, we found an increased CPT1B expression in the podocytes, but did not observe changed expression in other cell types of the glomerulus. This may explain the absence of a significant effect in the bulk RNA sequencing samples, which may not show subtle cell-specific differences. Regarding functional assays, CPT1A expression was shown to decrease in diabetic mice, and CPT1A upregulation was protective in human ciPOD and diabetic mice^{61,62}. This could indicate that the different isoforms of CPT1 have different functions, and while CPT1B upregulation may be detrimental, upregulated CPT1A may be beneficial. Keeping this in mind, the next steps should focus on investigating the roles of the different isoforms in podocyte injury and optimizing pharmacological inhibition since etomoxir is a broad CPT1 inhibitor, and finding more specific CPT1B inhibitors may prove more effective as treatment. On the other hand, CPT1 expression was found

to increase in mouse ciPODs in another model of injury, which could mean that different mechanisms of podocyte injury also have a differential regulation of CPT1, either up- or downregulated⁶³. Hence, studying the homogeneity of CPT1 regulation in different forms of iNS would be interesting to further elucidate using single-cell and spatial omics techniques and more physiologically relevant human *in vitro* models.

Targeted delivery of compounds to the podocyte

In the previous sections, several molecular mechanisms have been discussed that contribute to prednisolone-induced podocyte recovery. Developing therapies specifically targeting these mechanisms in podocytes would be key to developing effective therapies to halt iNS.

The charge and size of the drug determine its ability to cross the negatively charged and size-selective glomerular filtration barrier to reach the podocyte. However, in iNS the glomerular filtration barrier is affected, making the barrier less selective than under healthy conditions⁶⁴. Specific targeting of the podocyte is challenging, and some attempts using albumin, temperature-sensitive liposomes and polymeric nanomaterials did target the podocytes, but could not prevent non-specific targeting of other organs as well⁶⁵⁻⁶⁷. And even though various studies reported non-specific targeting, there are a handful of studies that show successful targeting of the podocyte, inducing recovery of the podocyte in disease.

Nanolipogels decorated with anti-nephrin and anti-podocin antibodies were used to restore the podocytes after injury⁶⁸. Similarly, mesoporous silica nanoparticles and nanoliposomes covered with a podocyte-expressed melanocortin-1 receptor (MC-1R) agonist were used to target podocytes^{69,70}. Interestingly, the authors show that targeted treatment with dexamethasone resulted in decreased podocyte injury with significantly fewer side effects as measured by serum IgG levels and white blood cell count in an experimental model of membranous nephropathy⁷⁰. Although dexamethasone is a much more potent stimulator of the glucocorticoid receptor than prednisolone, this study shows that targeted glucocorticoid delivery can have beneficial effects on the podocyte while diminishing the off-target effects of glucocorticoids. More specific targeting of the podocyte was demonstrated by Guo et al., who used tacrolimus crosslinked to the polypeptide Arg-Gly-Asp (RGD) and human serum albumin⁷¹. Human serum albumin functions as a carrier in the blood, whereas the RGD peptides can specifically bind to alpha-V-beta-3

integrin on podocytes. But also using a polymeric nanoparticle delivery system that accumulates in the GBM because of its size (approximately 100nm) and cationic components that interact with the negatively charged GBM⁷².

All in all, since the podocyte is the main injured cell type in iNS, it is the prime cell type for therapeutic targeting. The studies discussed above show that nano-targeting of podocytes is feasible and could provide an effective drug delivery strategy. Potentially, when combining a nanoparticle delivery system loaded with specific drugs (i.e. small molecule inhibitors) perturbing crucial processes in podocyte recovery, such as lipid metabolism, accompanied with optimal timing, an effective therapy without harmful side effects could be developed. Meanwhile, the search towards the pathogenic mechanism of iNS development can continue to provide definitive treatment for patients, which cures not only the symptoms, but tackles the root of the injury.

In summary, the effect of prednisolone on the podocyte might especially be important in the early response and quick resolution of proteinuria. Besides podocytes, other cell types might also be directly targeted, however, these effects may also be caused by podocyte recovery and decreased injurious signalling between the cell types. Prednisolone is likely to regulate the circadian rhythm in podocytes and decrease fatty acid metabolism, in order to contribute to podocyte recovery. Fatty acid metabolism and circadian rhythm may be promising therapeutic targets in iNS, and we should also consider including specific targeting of the drugs using nano delivery systems. Using this approach, the side effects from treatments currently used could be overcome, while we continue to unravel the pathological mechanism of iNS. However, the causes of iNS may be heterogeneous. In a highly heterogeneous patient population, it would be unlikely to find a treatment that would effectively cure the source of the injury for all iNS patients. Therefore, focusing on treatments that recover the podocyte, induce remission and halt disease progression, with the least amount of side effects possible, should be a key priority in the field.

6.2 Scattered tubular cells and their therapeutic potential in AKI-to-CKD transition

The proximal tubule is sensitive to ischemic and toxic injury, which results in a severe loss of function and is often encountered in patients in the intensive care unit⁷³. This leads to AKI, which in hospitalized patients is associated with

high morbidity and mortality rates. The proximal tubule has some capacity to regenerate after injury. However, kidney function does not always recover to baseline, and this can start the progression to chronic kidney disease (CKD). Management of AKI is primarily supportive, and therefore, more effective or specific treatment options are needed for patients⁷⁴. Unravelling the mechanisms of proximal tubule repair and stimulating this process could vastly improve AKI management and prevent progression to CKD. As discussed in **chapter 5**, several hypotheses have explained the regenerative capacity of the proximal tubule, including the existence of a progenitor population or the formation of scattered tubular cells (STCs), dedifferentiated proximal tubule epithelial cells (PTECs) that can replicate and re-differentiate into healthy proximal tubule cells to restore tubular function. The following section will discuss the origin and fate of STCs, important pathways for STC function that we highlighted in **chapter 5**, such as EGFR signalling and glycolysis. Finally, we will discuss the applicability of STCs in the treatment of AKI and prevention of progression to CKD.

The origin and fate of STCs

The origin of STCs has been a subject of debate for as long as STCs have been described, as discussed in **chapter 5**. So far, we have mainly discussed the existence of a progenitor population or the dedifferentiation of PTECs that give rise to STCs. However, in a lineage tracing study by Hong et al., WT1⁺ PECs were found to differentiate to PTECs⁷⁵. Since the authors show that a large proportion of the PTECs that were injured in their model after ischemia and reperfusion injury (IRI) were not derived from WT1⁺ PECs, these results suggest that most STCs originate from dedifferentiated PTECs. In our study in **chapter 5**, we observed heterogeneous marker expression and pathway activity in the STC population. Heterogeneity in the STC population may be partially caused by STCs originating from different cell types. Ultimately, other technologies may report on the origin of STCs in mouse models of AKI. For instance, using the reporter mouse strain mentioned in the study above, *in vivo* imaging is possible, and may shed more light on the origin of STCs. Intravital imaging is already well-established in the kidney and can even be expanded to three-dimensional imaging by making z-stacks with two-photon microscopy for several hours⁷⁶⁻⁷⁸. Since the appearance of fluorescently labelled WT1⁺ PECs in the proximal tubule occurs between 6 and 12 hours after IRI, it should be feasible, after optimization, to perform intravital imaging tracing the fluorescently labelled WT1⁺ PECs and their migration to the proximal tubules. In other studies, mouse strains have been established that label both PECs and STCs^{79,80}. Combining the mouse

strain described by Hong et al. that labels WT1⁺ PECs with the mouse strain that labels both PECs and all STCs would increase the ability to distinguish and trace newly formed STCs from PECs and PTECs.

Another technique that might be useful to investigate STC formation is SLAM-seq, a technique that allows profiling both old mRNA and newly formed mRNA after administration of nucleoside analogues with a chemical tag⁸¹. This method was optimized for *in vivo* usage by Yin et al., allowing the profiling of gene expression over 5.3 hours, again long enough to capture the appearance of PECs in the proximal tubule⁸². However, this would probably take a lot of refinement and would rely heavily on the ability to identify different cell types based on their transcriptomic profile, which can be troublesome for cell types that are quite similar.

The characteristics of STCs

Although it is currently not possible to investigate the origin of STCs in human kidneys, we (in **chapter 5**) and others have investigated their characteristics. In mouse models of IRI and unilateral urinary obstruction (UUO), subpopulations of PTECs are found after injury that resemble the human STC population. Some of these populations still exist several weeks after injury and hence were coined as failed-repair proximal tubule cells (FR-PTC). The mouse FR-PTC cells have a high similarity to a population of PTECs in normal human kidney tissue that express vascular cell adhesion molecule 1 (VCAM1) which we validated to be much like the human STCs we characterized in **chapter 5**⁸³. In the following section, the characteristics of STCs are discussed, using studies that report on the human STCs, the mouse injured PTECs or STCs, or the mouse FR-PTCs.

EGFR, FOXM1 and SOX9 in driving STC and tubular repair

In **chapter 5**, we observed an upregulation of the EGFR pathway in a subset of human STCs, in line with other studies^{84,85}. EGFR signalling was essential for forkhead box protein M1 (FOXM1) upregulation, which drives tubular proliferation during repair⁸⁶. Next to EGFR and FOXM1 expression, also SRY-box 9 (SOX9) expression was found to contribute to tubular repair by stimulation of Notch and Wnt signalling^{84,87}. Since the aforementioned studies show the expression in mouse models, it is interesting to note that a study by Kha et al. observed SOX9 expression in a scattered distribution throughout the proximal tubule in human tissue⁸⁸. SOX9 co-localized with the STC marker vimentin, was expressed in some PECs and increased with injury, much like we showed for STCs in **chapter 5**. Whereas we showed an increase of STCs with age, Nie

et al. showed a decrease of SOX9⁺ renal cells in the urine with age⁸⁹. However, Kha et al. showed in an *ex vivo* biopsy explant model increased proximal tubule recovery along with increased SOX9 positivity. Therefore, SOX9⁺ STCs may present STCs that are still capable of re-differentiating into functional PTECs. The lower number of urinary SOX9⁺ STCs in older individuals may reflect reduced regenerative capacity, possibly explaining the accumulation of non-functional STCs in the tissue. Similar age-related trends are observed in mice, with more fibrosis after IRI, indicating impaired repair⁸⁵. Therefore, it would be valuable to stain for SOX9 in our samples to assess age-related changes in SOX9⁺ STCs. In summary, EGFR, Notch and Wnt signalling together with expression of FOXM1 and SOX9 transcription factors seem to indicate STC formation and recovery of PTECs.

Increased glycolysis and fatty acid metabolism

Not only podocytes, discussed in the previous sections, are affected by metabolic changes. STCs also show a glycolytic switch and decreased oxidative phosphorylation compared to the PTECs. In **chapter 5**, we described the increased expression of PKM2, which is a key glycolytic enzyme that can even promote a glycolytic switch. Switching to glycolysis might be useful for cells that need to proliferate and survive in an often hypoxic environment. However, prolonged glycolysis is also associated with inflammatory signalling and will be discussed in this section. Similar to the glycolytic enzyme PKM2 discovered in our study, others have found an increase of another glycolytic enzyme, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) in CKD patients and in an IRI mouse model⁹⁰. PTEC-specific knockdown or pharmacological inhibition of PFKFB3 leads to less fibrosis and better clinical parameters by reducing lactate buildup that causes H4K12 lactylation of histones, activating nuclear factor- κ B (NF κ B) signalling⁹⁰. These results indicate that a glycolytic switch might benefit STCs for a short period to allow them to replicate and survive the hypoxic environment, but should be quickly downregulated to prevent lactate buildup, inflammatory processes and fibrosis. Especially since increased lactate production was also observed in a spatial metabolomics study in FR-PTCs, which presents a subpopulation of non-functional STCs⁹¹.

Should glycolysis always be avoided in STCs to prevent them from getting trapped in their pro-inflammatory and pro-fibrotic phenotype? Perhaps not, since a study by Li et al. shows that a higher proportion of STCs fail to repair when glycolysis is decreased⁹². These data suggest that glycolysis is

required for STC to PTEC differentiation, however, glycolysis also needs to be downregulated since prolonged activation of glycolysis leads to lactate buildup and pro-inflammatory and fibrotic signalling. Similarly, cyclic AMP-responsive element-binding protein 5 (CREB5) was found to be upregulated in the FR-PTC population and CREB5 siRNA downregulated FR-PTC markers and upregulated healthy PTEC markers^{93,94}. However, knockout of CREB5 downregulated, amongst others FOXM1 expression, which is beneficial for early PTEC repair. Apparently, CREB5 expression is needed at the beginning of PTEC injury to stimulate the switch to STC phenotype, however, it also needs to be downregulated to stimulate redifferentiation to healthy PTECs.

In summary, injured PTECs are likely adopting an STC phenotype, which is stimulated by increased glycolysis, SOX9, FOXM1, EGFR signalling and CREB5 expression. In optimal circumstances, the injured PTEC/STC will re-differentiate into a PTEC again and will help to restore the proximal tubule. If CREB5 and glycolysis are not downregulated, the STC gets trapped in its phenotype, in which it maintains glycolytic, leading to lactate build-up and inflammation. What is needed for this process to stimulate PTEC redifferentiation is still under investigation; potentially, the correct functioning of SOX9 may be involved. Investigating the markers mentioned before in normal kidney tissue of different ages, AKI and CKD tissues could provide valuable insights into the heterogeneity of these markers in the STC population. This might allow identification of markers that may be more associated with temporary injury and repair (AKI) or more chronic failed-repair (CKD) or that predispose for lower repair efficiency (old age).

To further dissect the human STC population, the extensive single-cell atlas that is currently available from the lab of Prof. Benjamin Humphreys could be used. Since in **chapter 5** we show that the STC population is heterogeneous, we must use single-cell methods to characterize STCs. Ultimately, spatial (transcript) omics at a single-cell resolution would aid in the characterization of STCs. Currently, techniques like Stereo-seq are reaching the (sub)cellular level and whole-genome RNA sequencing is performed, but the number of UMIs observed per "spot" is still pretty low for the highest resolutions⁹⁵. Better resolution is obtained with techniques like MERFISH and Xenium, however, these techniques are only profiling panels of genes (5,000 – 10,000) with no ability to capture the entire transcriptome^{96,97}. To overcome these hurdles, current bioinformatic approaches are aimed at combining the spatial transcriptomic data with more deeply sequenced single-cell RNA sequencing data, which may be

integrated with single-cell epigenomic, proteomic or metabolomic datasets⁹⁸. One example of such an approach would be to perform exploratory analysis using single-cell RNA sequencing, and using this information to choose a panel of 480 genes to explore the spatial expression using Xenium. High-resolution transcriptomic and epigenomic single-cell atlases of the kidney, with a focus on tubular cells, are already available to integrate with new spatial transcriptomic datasets. Given the role of lipid metabolism, glycolysis and lactate production in STC formation and differentiation, spatial metabolomics and lipidomics would be valuable to perform on human tissue to characterize the STC population in more depth^{91,99,100}.

The fate of STCs

In the previously mentioned paper by Buse et al., the ability of STCs to form new healthy PTECs was shown using a system in which cells that adopted the STC phenotype after injury were reversibly labelled with GFP and irreversibly expressed the LacZ gene⁷⁹. Therefore, the expression of LacZ was present in cells that had adopted the STC phenotype after injury, irrespective of their later phenotype. The authors showed that 6 days after injury, the LacZ gene is expressed in cells that have a fully mature and healthy PTEC phenotype in their scRNA sequencing dataset. This strategy has also been used by others who also show large reductions in the population of STCs after 2-4 weeks^{86,101}. However, in contrast to the study from Buse et al. in which recovered PTECs 6 days after injury are transcriptomically healthy, the study by Gerhardt et al. shows that, after 4 weeks, the phenotype of STCs has recovered but their transcriptomic profile is still a little different, whereas transcriptomic recovery was found after 6 months. In summary, these studies show that most STCs form healthy PTECs and although phenotypic recovery is quick (within 2-4 weeks), transcriptomic recovery may be slower and take up to 6 months. A slower transcriptional recovery of STCs may hamper cluster classification in transcriptomic studies and should be kept in mind.

The FR-PTCs are usually regarded as a driver of progression to CKD since they are thought to be unable to form healthy PTECs again and are drivers of pro-inflammatory and pro-fibrotic signalling. However, FR-PTC that were labelled 4 weeks after injury still decreased in numbers by 90% after 5 months, indicating that these FR-PTC either died or they can recover and form healthy PTECs¹⁰¹. And although FR-PTC are harmful for kidney recovery after injury, in Pax2 and Pax8 double knockout mice, a new PTEC cluster was observed using single-cell transcriptomics¹⁰². This new cluster resembled FR-PTC

with increased tumor necrosis factor- α (TNF α), NF κ B and EGFR expression. The authors showed increased SOX9 expression, previously mentioned to be important for healthy PTEC recovery. Interestingly, double knockout mice were more resistant to injury. Therefore, these studies suggest that FR-PTC may still contribute to healthy PTEC formation and, although their numbers should not become too significant, small numbers may be less injurious than sometimes suggested. Altogether, this suggests that the pro-inflammatory and pro-fibrotic FR-PTCs may also still be able to form functional PTECs and, when expressing the correct combination of markers, may even be beneficial for recovery. However, it is important to perform research into the exact molecular signature that could lead to successful recovery.

STCs: Possibilities for treatment of AKI and CKD

So far, it is likely that having part of the STC population is beneficial and should be stimulated or even supplied, whereas another part of the STC population may be trapped in their phenotype and may be targeted for removal. To stimulate proximal tubule recovery, numerous studies report successful treatment of mice with AKI with either CD133-single positive or CD24 and CD133-double positive renal cells¹⁰³⁻¹¹⁰. One study describes the treatment of podocytes using a similar cell population, making this not only an interesting field for AKI treatment but also for podocyte injury in NS¹¹¹. Of course, by using primary human cells, donor differences and tissue availability would make it hard to establish this treatment option. However, with advancements in the generation of induced pluripotent stem cells (iPSCs) and possibilities to treat patients with autologous cells, these relatively old studies might be re-activated. Recently, for instance, human embryonic stem cells (ESC) were used to differentiate towards CD24⁺ and CD133⁺ renal progenitor cells, and injection of these cells reduced kidney injury in mice after gentamycin-induced AKI¹¹². Comparable attempts have been described with human iPSCs, which should be more easily accessible, have fewer ethical concerns and can provide patient-specific treatment options¹¹³. However, since AKI is very acute, the timeline for production of iPSC-derived renal progenitor cells might be too long, with 14 days to be used for treatment. Storing a pre-differentiated iPSC population of kidney progenitors may circumvent this timeline, if preservation techniques allow for storing these cells. Neonatal kidney stem/progenitor cells (nKSPC) can be non-invasively isolated from the urine of preterm children, expanded *in vitro* and stored for later use¹¹⁴. Infusion of discarded donor kidneys with ischemic injury has shown the potential of nKSPC to stimulate endogenous recovery of the proximal tubules¹¹⁵. However, donor variations need to be

tackled since nKSPC can only be isolated from preterm newborns. Overall, the use of iPSC-derived progenitor cells or nKPSCs may be further investigated as a treatment method aimed at stimulating recovery of the proximal tubules. This treatment option might be combined with a therapy aimed at removing a population of STCs that are trapped in their phenotype and counteract the recovery of the proximal tubule.

The STCs that are trapped in their phenotype are often associated with a senescence-associated secretory phenotype (SASP)¹¹⁶. In order to target senescence, senolytics, a class of drug selectively killing senescent cells, are currently being investigated. However, treatment with senolytics in the acute phase of AKI is detrimental and worsens injury¹¹⁷. Since we showed in **chapter 5** that STCs are not senescent, but might have SASP, senomorphics might be a better choice for treatment of failed-repair STCs, because senomorphics are blocking the senescent secretory phenotype but not killing senescent cells like senolytics do¹¹⁸. Hence, it would be interesting to see whether changing the timing of senomorphic treatment to after the acute phase would be beneficial to get rid of "trapped STCs".

Conclusion

Taken altogether, the human STC population likely comprises several subpopulations. Some of them may be beneficial for the recovery of the proximal tubule, whereas a proportion of the STCs might be trapped or failed-to-repair. SOX9 could be a marker for functional STCs and was found to decrease with age. Without SOX9 expression, STCs may be more prone to becoming failed-repair STCs, which would be in line with increased amounts of (failed repair) STCs found in kidney tissue with increasing age. Specific combinations of marker expression may determine whether an STC can still form a PTEC or whether it will remain trapped in the STC phenotype, stimulating inflammation and fibrosis. Treatment of AKI to prevent progression to CKD may focus on stimulating recovery by supplying iPSC-derived kidney progenitors or nKSPC. After the initial injury, AKI can transition to CKD when recovery is insufficient, which is worsened by pro-inflammatory and pro-fibrotic failed-repair STCs. Targeting of these harmful STCs with senolytics or senomorphics could be a second therapeutic strategy to halt AKI-to-CKD progression and restore kidney function.

6.3 Improving *in vitro* models to model the complex nephrotic syndrome, cellular interactions and responses to treatment

In **chapter 2**, we characterized the podocytes in human kidney organoids, compared them to the traditionally used ciPODs and explored the feasibility of NS disease modelling in human kidney organoids. We showed superior podocyte marker expression, transcriptomic and morphological characteristics of the organoid podocytes compared to ciPODs. Since the publication of our study, human kidney organoids have been a focus of research and many improvements have been made. In the other research chapters of this thesis, the effects of prednisolone in iNS and the characteristics of STCs have been studied, both of which remain challenging to model accurately *in vitro*. The following section will explore recent advancements in kidney organoid technology and discuss the modifications needed to better support studies of iNS and STCs.

Kidney organoids to model iNS and study pathophysiology and treatment effectivity

Human iPSC derived kidney organoids were used to model iNS using plasma of patients with primary FSGS or recurrent FSGS after transplantation^{119,120}. Podocyte injury was observed upon exposure, allowing for more functional studies in the future. As a start, it would be interesting to investigate changes in circadian rhythm genes and fatty acid metabolism genes, like PDK4, CPT1B and PPARGC1A, in this model. iPSC lines with conditional overexpression of circadian rhythm genes, knockdown of fatty acid metabolism genes or using pharmacological stimulators/inhibitors could be used to validate these mechanisms. However, we should keep in mind that van den Berge et al. recently showed donor and patient specific characteristics¹²¹. Therefore, what works with one iPSC line may not be effective in another iPSC line. So if eventually the aim is to prove effectiveness for a more generalized population, we should be cautious in relying on the results of studies using single iPSC clones. The inclusion of multiple iPSC lines is already done in other fields, and drug effects have been shown to vary among iPSC lines¹²². Besides testing multiple iPSC lines, using iPSC villages for drug testing might also help to increase the scalability of using many iPSC lines¹²³. In iPSC villages, multiple iPSC lines are combined in one culture or "village". After drug treatment, sequencing techniques can deconvolute individual iPSC lines and analyse their responses.

Improving maturation by extending culture time

Modelling iNS and recovery of podocytes is hampered by the many questions that still surround the aetiology of iNS. Modelling of iNS in animal models has focused on replicating the phenotype observed in kidney biopsies. For iNS, animal studies use proteinuria as their main readout, and, depending on the form of iNS that is modelled, the occurrence of podocyte foot process effacement or the formation of sclerosis as a secondary readout. Kidney organoids are superior to other *in vitro* systems in recapitulating the *in vivo* characteristics. When transplanted under the kidney capsule of mice, it is possible to measure dextran and albumin size selectivity in kidney organoids, which would be comparable to measuring proteinuria in animal models¹²⁴. However, ultimately human kidney organoids should not rely on animals for maturation, which would hamper their high throughput potential and oppose the current morale to limit animal experiments. Although filtration slit density, as measure of foot process effacement, has been found to be lower in diabetic kidney injury in organoids (unpublished work), it has not been demonstrated as high-throughput screen for podocyte injury yet, since foot process formation is heterogeneous in organoids. Despite the presence of PECs in the organoids, there are no reports on their functionality and ability to activate and form sclerosis. Although fibrosis has already been shown in kidney organoid cultures, glomerulosclerosis, in which activated PECs migrate into the podocyte clusters, has not yet been reported¹²⁵. The maturation of podocytes and/or PECs may need more time and current protocols do not all allow for long-term culture of kidney organoids. There is a protocol from the group of Morizane that allows for prolonged organoid cultures up to 56 days¹²⁶. More recently, these organoids have also been incorporated with a micro physiological platform that generated uniform kidney organoids¹²⁷. In combination with their previous study on automated 3D imaging of their kidney organoids, this platform could prove very useful for more automated high throughput screening of kidney organoids¹²⁶. However, the maturity of the podocytes and PECs, and the possibilities of mimicking iNS injury in these long-term organoid cultures has not yet been explored. In addition, the images presented in the papers do not allow for overt enthusiasm. Probably, other factors like vascularization and the immune system must be included to promote maturation in the kidney organoids.

Induce vascularization to improve organoid maturity and function

The phenotype of podocytes in organoids is already superior to other *in vitro* systems, as we have also shown in **chapter 2**. However, it could be improved to

even better recapitulate *in vivo* podocytes. Besides the previously discussed longer maturation time, another way forward is to induce vascularization in kidney organoids, in order to further mature podocytes, but also other kidney cell types in the organoids. Also, in most current organoid cultures, a necrotic core is formed over time because bigger organoid size over time hampers nutrient diffusion to the core. Improved vascularization in the organoids may counteract necrosis in the organoid's core, thereby also extending the time organoids can be kept in culture. *In vivo*-like vascularisation can be induced in kidney organoids by transplantation of the organoid just under the renal capsule of immune compromised mice or by transplantation of the kidney organoid in the celomic cavity of chicken embryos^{128,129}. Similarly, decellularized kidney scaffolds from rats have also been used to repopulate with progenitor and differentiated cell types to form functional kidneys again¹³⁰. However, although these methods may be used for small-scale experiments, they cannot be used for high throughput *in vitro* experiments since these models do not present an *in vitro* system anymore. Recently, however, two different methods have proven successful in inducing vascularization in kidney organoids *in vitro*.

First, perfusable vasculature was seen in an organoid-on-a-chip model, where a channel with organoids is separated from a channel with endothelial cells by a gelatin-fibrin matrix¹³¹. Endothelial cells from the organoids migrated to the endothelial cell channel and formed a perfusable connection. The model needs further optimization, including maturation, which might be accomplished with longer term organoid cultures. Next to this, a functional connection between the endothelial cells, podocytes and the tubular system would still be needed in order to have a fully functioning kidney system in which blood can be added in one channel and urine-like fluid can be investigated in another channel. Second, Maggiore et al. have produced kidney organoids using a hybrid approach with a normal iPSC line and an iPSC line with inducible ETS translocation variant 2 (ETV2), a transcription factor important for endothelial cell formation and differentiation¹³². After more in-depth characterization, the induced endothelial cells seemed to connect with the podocytes. Similar to our findings in **chapter 2**, a more mature transcriptomic profile was found in the vascularized podocyte population compared to the non-vascularized podocytes. Overall, this study shows the power of genetically altered iPSC lines. If similar effective transcription factors can be identified for other cell types, such as mesangial cells or macula densa cells, the functionality of kidney organoids could be improved by mixing multiple inducer iPSC lines.

Endothelial cells are not the only cell types needed for kidney development *in vivo* that are underrepresented in organoid cultures. For instance, macrophages are also important for development and have been shown to stimulate endothelial cell formation and correct morphogenesis of endothelial cells¹³³. Therefore, it remains important to focus on the supplementation of multiple of these cell types in kidney organoid cultures to provide an environment, as closely related to *in vivo* as possible

Utilising organoid and tubuloid systems to investigate STCs

Another application of edited iPSC lines, as discussed above, is for STC research. Earlier in this discussion, SOX9 and FOXM1 have been discussed to be important for the formation of STCs. So far, unpublished experiments have found proximal tubule cells after hypoxic injury with some STC characteristics. Using SOX9-inducible iPSC lines, the importance of SOX9 signalling for STC formation could be investigated. When combined with an irreversible fluorescent marker, this approach would enable *in vitro* tracking and characterization of STC fate. Such iPSC lines would allow functional studies, for example by visualizing the effect of FOXM1 knockdown on STC formation.

Producing more mature proximal tubules may also aid in improved development of STCs. Recently, it was shown that generating kidney organoids with iPSC crispr-edited to overexpress HNF4a increased the expression of mature proximal tubule markers¹³⁴. And similarly, an enhancement of the kidney organoid protocol led to more mature proximal tubule cells that showed better responsiveness to cisplatin-induced injury, a common model for AKI¹³⁵. Although we have already extensively discussed metabolic changes underlying podocyte injury or STC transformation, the addition of metabolic compounds can also drive proximal tubule cell maturation⁵⁸. Using spatial metabolomics, metabolic changes were observed in developing kidneys and increasing the availability of short-chain fatty acids (acetate and butyrate) leads to more mature proximal tubules *in vitro*. Considering the importance of these metabolic changes, the treatment strategies focusing on metabolism, as discussed in the podocyte section above, may also be very interesting in treating AKI by stimulating or reducing STC formation at certain time points after injury.

Another interesting option to study the STCs is to use CD24-positive cells from kidney tissue, as showed before by Xu et al.¹³⁶. Human renal cortex tissue was used to isolate cells expressing a proximal tubule markers (CD13) and

the STC marker CD24, which were subsequently used to generate tubuloids. These tubuloids proved accurate in modelling autosomal dominant polycystic kidney disease (ADPKD). Injured tubuloids could be treated and showed more functionality in terms of injury and recovery after treatment compared to proximal tubules from iPSC-derived kidney organoids. Exposing these advanced tubuloids to hypoxic or toxic conditions, similar to the ones used in animal models, would be very interesting in the STC context. The tubuloids can withstand long periods of culture of over 100 days, allowing for gene editing prior to experimentation. For STC purposes, culture periods of over 100 days would also allow for studying recovery after injury and even failed-repair phenotypes that are usually reported in animal studies after 4 weeks. Additionally, the ability to perform gene editing prior to experimentation would allow for functional testing of SOX9, FOXM1, EGFR signalling and so on, whereas the model in itself would already allow for pharmacological testing and finding therapeutic targets that could stimulate proper recovery and hamper failed repair mechanisms (if present at all).

Spatial omics techniques, and the future of atlases

The human kidney organoids, as discussed above are an attractive model system to study kidney diseases. However, the presence of multiple cell types which is an advantage of the organoids is also a double-edged sword and complicates analysis. For instance, proteomic analysis has been done in kidney organoids using the kidney organoid culture medium or lysate as input¹³⁷. The protein expression measured in these samples reflects a mixture of proteins expressed or secreted by all kidney cell types in the organoid and only large changes will be detected. For cell type-specific analysis, analysis should be done in the environment or cell types should be isolated prior to analysis. To isolate certain cell types before analysis, FACS can be used. However, we also observed contamination of other cell types in FACS sorted populations (unpublished observations). For instance, we used an anti-nephrin antibody to sort podocytes, but could also detect markers for PECs and PTECs, showing that these cell types are not well separated, presumably because they are in development and have an incomplete separation of marker expression. A second option could be non-specific binding of the antibody.

Another option to analyse the different cell types separately, keeping in mind their biological context, is spatial omics, also described earlier. However, although for normal human kidney, AKI and chronic kidney disease many datasets are already available, we should expand these datasets with MCD,

MN and FSGS data to cover the most common causes of iNS as well^{138,139}. Since metabolism, and more specifically lipids, have been discussed to be important in both podocytes and PTECs/STCs, future studies may look to perform spatial metabolomics and lipidomics using tissue from patients with iNS or AKI/CKD. And even though in the past kidney research has been dominated by using animals of male sex, it is important for future spatial datasets to include both sexes. This is more firmly suggested since a recent spatial transcriptomic study highlighted that there are differences in gene expression between male and female kidneys, with differential distributions or sometimes even complete absence of certain enzymes¹⁴⁰.

In conclusion, both iNS and AKI lack specific treatment options that avoid the progression to kidney replacement therapies. In iNS, the aim should be at supporting the podocytes, for instance by specifically targeting them with circadian modulators or metabolic regulators that reduce free fatty acid metabolism and/or stimulate glycolysis. For AKI, treatment may consist of treatment with senolytics to get rid of harmful STCs in combination with stimulation of SOX9⁺ STC or even supplementation of iPSC-derived progenitors or nKSPCs to restore tubular function. For both diseases, future studies should focus on the combination of single-cell omics techniques with spatially resolved techniques and the inclusion of metabolomics, lipidomics and proteomics techniques.

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English summary & Nederlandse samenvatting

I. English summary of the thesis

The kidneys are responsible for the filtration of our blood, to get rid of waste products and toxins. This thesis investigated kidney biology in the context of kidney diseases. The first part of this thesis focused on the glomerulus and more specifically podocyte injury and recovery, the second part concentrated on the proximal tubule and the regeneration of proximal tubule cells.

Damage to the podocytes, cells important for kidney filtration, leads to nephrotic syndrome (NS), which is characterized by leakage of proteins in the urine that normally need to be retained in the blood. The cause of NS and what molecular mechanisms specifically contribute to podocyte injury or recovery are still largely unknown. In **chapter 2**, this thesis started by exploring a new *in vitro* model to advance NS research: human induced pluripotent stem cell (iPSC)-derived kidney organoids. **Chapter 2** compares the maturity of organoid podocytes compared to *in vivo* podocytes and the traditionally used *in vitro* podocyte cell line (ciPOD). Using RNA sequencing and electron microscopy, we showed increased podocyte marker expression in kidney organoids with a more mature phenotype compared to ciPODs. Single-cell RNA sequencing revealed the presence of three subclusters of podocytes in organoids. One of the subclusters that was close to endothelial cells, expressed more mature podocyte markers, reaffirming that podocyte-endothelial interactions are important for podocyte maturation. To better understand the causes and molecular mechanisms underlying NS, we modelled NS in kidney organoids. NS was successfully modelled using protamine sulphate, puromycin aminonucleoside and patient plasmas, as well as genetic NS using an iPSC line with two mutations in an important podocyte gene: podocin. CRISPR/Cas9 was used to restore one of the podocin mutations in the iPSC line, leading to better podocin expression and localization in the kidney organoid podocytes, indicating rescue of the genetic disease.

NS is commonly treated using glucocorticoids, however, similar as the pathogenesis of NS, the molecular mechanism of glucocorticoid action in NS is still largely unknown. Podocyte-specific effects have been demonstrated and in **chapter 3** an overview of existing literature was provided with different mechanisms of glucocorticoid action on podocytes. In **chapter 4**, we used a rat model of NS that was treated with prednisolone, which is a glucocorticoid commonly used in children with NS, to unravel the effects of prednisolone on podocytes. A single-nuclei RNA and assay for transposase accessible

chromatin (ATAC) dataset was generated and effects on several cell types were seen. For instance, the parietal epithelial cells expressed a known activation marker (CD44) in the injury condition, which was decreased by prednisolone treatment. In the podocytes, increased expression of molecules described in **chapter 3** were seen in injury, which were decreased by prednisolone treatment, confirming some of the literature. Furthermore, prednisolone induced strong expression of circadian rhythm-related genes that could contribute to recovery. We identified increased fatty acid metabolism in podocytes, which decreased with prednisolone treatment. In addition, inhibition of one of the key enzymes involved in fatty acid metabolism, prevented podocyte injury *in vitro*.

Whereas damage to the glomerulus leads to leakage of proteins in the urine, damage to the proximal tubule leads to acute kidney injury (AKI) with massive proximal tubule cell loss and obstruction of the urine. AKI is often encountered in hospitalized patients and, similar to NS, no specific treatment is available to stop AKI from progressing to chronic kidney disease (CKD), a condition where patients are dependent on dialysis or kidney transplantation. The proximal tubule has the ability to recover from injury and the origin of these regenerating cells have been heavily debated over the years. Several studies have already indicated that the scattered tubular cell (STC) population, phenotypically different cells in the proximal tubule, are likely contributing to proximal tubule recovery after injury. In **chapter 5**, the human STC population is characterized, and we show that they are mainly present in groups in the inner bends of proximal tubules. The number of STCs increases with age and with kidney injury. RNA sequencing revealed decreased oxidative phosphorylation in the STC and increased glycolysis, in line with fewer mitochondria present in the STCs. This glycolytic switch was also evident by expression of several glycolytic enzymes in STCs, that were not present in proximal tubule cells and the population was found to be heterogeneous in a single-cell RNA sequencing dataset. Altogether, the study indicated that STCs are likely injured and dedifferentiated proximal tubule cells that contribute to proximal tubule repair, and that several subclusters of STC may have different functions in recovery.

Ultimately, the findings in this thesis may contribute to finding better treatment options for patients with NS and/or with AKI/CKD. For instance, lipid metabolism may be targeted in NS, whereas supplementation or stimulation of certain subclusters of STCs might be used to aid recovery of proximal tubular damage after AKI to prevent progression to CKD.

II. Nederlandse samenvatting van de thesis

De nieren zijn verantwoordelijk voor het filtreren van ons bloed, om afvalstoffen en gifstoffen kwijt te raken. In deze thesis is onderzocht wat er gebeurt in de cellen van de nier tijdens bepaalde nierziekten. In het eerste deel van deze thesis is de nierfilter, oftewel de glomerulus, onderzocht en meer specifiek wat er gebeurt tijdens podocytschade en herstel. Het tweede deel van deze thesis richt zich op de proximale tubulus en hoe de proximale tubuluscellen herstellen na schade.

Podocyten zijn cellen die op het nierfilter zitten en belangrijk zijn voor een goede werking van het nierfilter. Ze zorgen ervoor dat afvalstoffen worden doorgelaten naar de urine, en dat goede stoffen zoals eiwitten worden binnengehouden in het bloed. Schade aan podocyten leidt tot het nefrotisch syndroom (NS). Patiënten met NS lekken eiwitten in de urine, die normaal in het bloed behouden moeten blijven. De oorzaak van NS en welke moleculaire mechanismen aan de podocytschade of het herstel ten grondslag liggen, zijn nog grotendeels onbekend. In **hoofdstuk 2** van deze thesis zijn we gestart met het bestuderen van een nieuw model om het NS-onderzoek verder te helpen, namelijk nierorganoïden (mini-niertjes). Nierorganoïden worden in het laboratorium gemaakt uit stamcellen en bestaan uit meerdere celtypen die in de nier aanwezig zijn, met onder anderen podocyten, endotheelcellen en proximale tubuluscellen. Podocyten uit de nierorganoïden lijken meer op de podocyten in ons lichaam qua morfologie, en hebben een verbeterde expressie van podocyt-markergenen, vergeleken met een veel gebruikte podocytcellijn in het laboratorium (ciPODs). Met behulp van een genanalyse techniek (single-cell RNA sequencing) lieten we zien dat er drie podocytclusters in de nierorganoïden zitten. De podocyten met het beste genprofiel werden gevonden in de buurt van endotheelcellen, waarmee we bevestigden dat podocyt-endotheelinteracties belangrijk zijn voor de differentiatie van podocyten. Om het mechanisme van NS beter te begrijpen, hebben we ook NS nagebootst in de nierorganoïden. Met behulp van chemische stoffen (protamine sulfaat en puromycine aminonucleoside) en patiëntenplasma hebben we dit voor elkaar gekregen. Door het gebruik van stamcellen van een NS-patiënt met twee mutaties in het podocinegen (belangrijk voor podocytfunctie), konden we genetische NS nabootsen. Deze genmutatie zorgt ervoor dat we lage expressie en verkeerde lokalisatie van het podocine-eiwit zagen in de nierorganoïd-podocyten. Met behulp van CRISPR/Cas9 waren

we in staat om de mutatie te herstellen en zagen we een betere expressie en lokalisatie van het podocine-eiwit zien in de podocyten uit de nierorganoïden.

NS wordt meestal behandeld met prednisolon, alhoewel het moleculaire mechanisme hiervan niet helemaal bekend is. In **hoofdstuk 3** wordt een overzicht gegeven van directe effecten van prednisolon op de podocyt die in de literatuur zijn beschreven. In **hoofdstuk 4** wilden we uitzoeken op welke manier prednisolon de podocyten herstelt. We maakten gebruik van een ratmodel, waarbij ratten met NS behandeld werden met prednisolon. Met behulp van de technieken: single-nuclei RNA en assay for transposase accessible chromatin (ATAC) sequencing, konden we effecten op verschillende cellen van de nier ontdekken. We zagen bijvoorbeeld dat pariëtale epitheelcellen een bekende activatiemarker (CD44) tot expressie brachten na schade, wat eerder beschreven is in patiënten met NS, die weer verdween na prednisolon behandeling. In de podocyten zagen we effecten op sommige mechanismen die beschreven zijn in **hoofdstuk 3**, waarmee we (een deel van) de literatuur bevestigen. We ontdekten ook een sterke stijging in de expressie van genen die betrokken zijn bij het circadiane ritme (het dag-nacht ritme) na prednisolon behandeling in podocyten, wat mogelijk bijdraagt aan herstel van de podocyt. Verder ontdekten we een verhoogd vetzuurmetabolisme in beschadigde podocyten, dat werd verminderd na prednisolon behandeling. Een extra indicatie voor het belang van vetzuurmetabolisme gaven we door te laten zien dat podocytschade voorkomen kon worden door podocyten te behandelen met een remmer van een enzym dat betrokken is bij het vetzuurmetabolisme.

In tegenstelling tot schade aan podocyten dat tot NS leidt, leidt schade aan de proximale tubulus tot acute nierschade met een enorm verlies van proximale tubuluscellen en obstructie/verstopping van de tubulus (urinebuis). Acute nierschade wordt vaak gezien bij patiënten op de intensive care, en net als bij NS hebben we momenteel nog geen specifieke behandelmethode om deze nierschade te stoppen. Wanneer acute nierschade niet succesvol wordt behandeld, leidt dit tot chronische nierschade, waarbij patiënten uiteindelijk afhankelijk kunnen worden van dialyse of een niertransplantatie. De proximale tubulus heeft het vermogen om (deels) te herstellen van schade, alhoewel de cellen die daarvoor verantwoordelijk zijn nog ter discussie staan. Meerdere studies hebben laten zien dat er cellen voorkomen in de proximale tubulus die er anders uitzien en waarschijnlijk bijdragen aan herstel. Deze cellen worden scattered tubular cells (STCs) genoemd. In **hoofdstuk 5** karakteriseren we de STC-populatie in menselijk nierweefsel en laten we zien dat STCs voornamelijk

in groepen in de binnenbocht van de proximale tubulus voorkomen. Ook zagen we dat het aantal STCs toenam met de leeftijd en bij nierschade. Met behulp van genanalyse en eiwitkleuringen lieten we zien dat STCs een verminderde oxidatieve fosforylering hebben en een verhoogde glycolyse, wat overeenkomt met de verminderde hoeveelheid mitochondriën die we met elektronenmicroscopie in STCs zagen. Normaal gesproken gebruiken proximale tubuluscellen onder andere oxidatieve fosforylering om aan hun energie te komen. Dit is een “langzame” suikerverbranding, waarbij suiker met behulp van de mitochondriën wordt omgezet in energie. Glycolyse is een “snelle” suikerverbranding, waarbij suiker zonder de mitochondriën wordt omgezet in energie. Het is interessant om deze verandering tussen proximale tubuluscellen en STCs te zien, aangezien deze verandering ook vaak wordt gezien in kankercellen, die snel delen en daar energie voor nodig hebben. Dit zou in lijn liggen met de gedachte dat STCs na beschadiging delen om de verloren proximale tubuluscellen te kunnen vervangen en dus bijdragen aan het herstel. We hebben de STC-populatie ook onderzocht met single-cell RNA sequencing en daaruit concluderen we dat deze populatie heterogeen is. Deze studie laat zien dat de STCs waarschijnlijk bijdragen aan het herstel van de proximale tubulus en dat verschillende populaties van STCs bestaan, die mogelijk verschillende functies hebben met betrekking tot dit herstel.

De bevindingen in deze thesis dragen hopelijk bij aan het vinden van betere behandelopties voor patiënten met NS of acute nierschade. Zo zou voor NS bijvoorbeeld het vetzuurmetabolisme in podocyten aangepakt kunnen worden om de podocytschade te herstellen en daarmee het eiwitlek te stoppen. Bij acute nierschade zouden bepaalde STC-populaties kunnen worden gestimuleerd om het herstel van de proximale tubulus te bevorderen en daarmee de progressie naar chronische nierschade te voorkomen.

Appendices

- A. Research Data Management
 - B. List of publications
 - C. Curriculum vitae
 - D. PhD portfolio
 - E. Dankwoord
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A. Research Data Management

Ethics and privacy

In **Chapter 2** of this thesis, induced pluripotent stem cells (iPSC) were used. Human adult skin fibroblasts from a healthy volunteer and erythroblasts from a nephrotic syndrome patient were used to generate iPSC, after giving informed consent. The study was conducted in accordance with the Helsinki Declaration as revised in 2013 and permission for the use of iPSC was obtained from the local ethical commission for human-related research of the Radboud university medical center, Nijmegen (approval numbers: 2015-1543 and 2006-048). In the same chapter we also used archived human kidney tissue, which was used under approval number 2018-4086. Furthermore, patient and healthy control plasmas were used after getting approval by the regional medical-ethical committee (Arnhem-Nijmegen) under file number 09/073. The material was used after obtaining informed consent from all participants and used fully anonymous to the researchers.

In **Chapter 4** animals were used to answer part of the research question. All the studies were in accordance with local regulations and approved by the Animal Ethics Board of the Radboud University, Nijmegen. We adhered to all the guidelines for reduction of the number of animals used, refinement of procedures to cause the least amount of discomfort and replacement by only using the animal model for the questions that could not be answered using other model systems.

Finally, **Chapter 5** uses archived kidney tissue, which was approved by the local ethical committee of the Radboud university medical center, Nijmegen (CMO 2017-3652). Some of the used kidney tissues originating from the Erasmus MC tissue bank for which the Erasmus MC Research Ethics Board waived the need for formal ethics approval according to the Dutch Law on Medical Research in Humans. Paediatric tissue was only used after explicit parental written informed consent for use for research and adult tissue was collected within a non-objection clause. The data was completely anonymous, since besides age and gender, no other information was stored/shared for each of the kidney sections.

Data collection and storage

Data for **Chapter 2** was collected through laboratory experiments using kidney organoids. All the raw data and rest material from many of the biological

samples are stored at the Department of Pathology from the Radboud university medical center, Nijmegen and are accessible from one of the project members on request. A second part of the data was obtained by analysis of both bulk RNA sequencing and single-cell RNA sequencing datasets that were produced in the laboratory. This data is securely stored on the department server that has back-ups in the cloud and in the Gene Expression Omnibus (GEO) database as described later in this section. For **Chapter 3**, data collection consisted of a literature search using Pubmed. The publications used to write the review are included as references, and thus this data is stored in the original publications that are accessible via Pubmed. The data described in **Chapter 4** are majorly derived from bioinformatic analysis of single-nuclei RNA and ATAC sequencing datasets. This data is stored on the same departmental server as described above and is accessible via GEO (including scripts via Zenodo). Similarly, it involved laboratory experiments involving culture cells and animal studies. Much of the material is still available for re-analysis on request to one of the project members at the department of Pathology, and digital data is all stored on the department server. In **Chapter 5**, RNA sequencing data was used, which is stored both on the department server and GEO. The laboratory data is also stored on the department server. For the single-cell RNA sequencing data, we used a dataset that was previously published by Prof. Benjamin Humphreys after obtaining written consent from him by mail and with a proper reference to the owners of the original data. The data was downloaded from the GEO website, providing a perfect example of the usefulness of storing datasets on GEO for easy re-use.

Data sharing according to the FAIR principles

Chapter 2 and **5** have been published open access under the CC BY 4.0 license. **Chapter 3** has been published under the Oxford University Press (OUP) Journals, Standard Publication Model, which entails an exclusive license and reuse rights are retained and controlled by Oxford University Press. **Chapter 4** will soon be submitted and published as preprint on Biorxiv. All the RNA and ATAC sequencing datasets have been published on GEO for easy reuse of the data to other researchers. The bioinformatic code that was used to produce the figures presented in the papers/manuscripts have been deposited on Zenodo and github (<https://github.com/MartijnLvandenBroek>). Below, a schematic overview of the datasets belonging to the chapters and the data repository including accession numbers:



Chapter	Dataset	GEO accession	Zenodo DOI
2	Bulk RNA sequencing	GSE181954	10.5281/zenodo.5155999
	Single-cell RNA sequencing	GSE181954	10.5281/zenodo.5155999
4	Single-nuclei RNA sequencing	GSE304592	10.5281/zenodo.15715854
	Single-nuclei ATAC sequencing	GSE304586	10.5281/zenodo.15715854
5	Bulk RNA sequencing	GSE202824	10.5281/zenodo.6168927

For **Chapter 5** a previously published single-cell RNA sequencing dataset was used accessible on GEO under accession number: GSE151302



B. List of publications

van den Berge BT, **van den Broek M**, Di Giovanni G, Debiec H, Gloudemans S, Leusink Q, den Braanker D, Wetzels JFM, Ronco P, Smeets B, Jansen J, Maas RJ. Personalized disease recurrence modelling using iPSC-derived podocytes in patients with idiopathic nephrotic syndrome. *Nephrol Dial Transplant*. 2025 Sep;40(9):1736-1745. doi: 10.1093/ndt/gfaf045. Epub 2025 Feb 28. PMID: 40036883; PMCID: PMC12451694.

Eymael J, **van den Broek M**, Miesen L, Monge VV, van den Berge BT, Mooren F, Velez VL, Dijkstra J, Hermesen M, Bándi P, Vermeulen M, de Wildt S, Willemsen B, Florquin S, Wetzels R, Steenbergen E, Kramann R, Moeller M, Schreuder MF, Wetzels JF, van der Vlag J, Jansen J, Smeets B. Human scattered tubular cells represent a heterogeneous population of glycolytic dedifferentiated proximal tubule cells. *J Pathol*. 2023 Feb;259(2):149-162. doi: 10.1002/path.6029. Epub 2022 Dec 19. PMID: 36373978; PMCID: PMC10107692.

Jansen J, van den Berge BT, **van den Broek M**, Maas RJ, Daviran D, Willemsen B, Roverts R, van der Kruit M, Kuppe C, Reimer KC, Di Giovanni G, Mooren F, Nlandu Q, Mudde H, Wetzels R, den Braanker D, Parr N, Nagai JS, Drenic V, Costa IG, Steenbergen E, Nijenhuis T, Dijkman H, Endlich N, van de Kar NCAJ, Schneider RK, Wetzels JFM, Akiva A, van der Vlag J, Kramann R, Schreuder MF, Smeets B. Human pluripotent stem cell-derived kidney organoids for personalized congenital and idiopathic nephrotic syndrome modelling. *Development*. 2022 May 1;149(9):dev200198. doi: 10.1242/dev.200198. Epub 2022 May 6. PMID: 35417019; PMCID: PMC9148570.

Veys K, Berlingerio SP, David D, Bondue T, Held K, Reda A, **van den Broek M**, Theunis K, Janssen M, Cornelissen E, Vriens J, Diomedi-Camassei F, Gijsbers R, Heuvel LVD, Arcolino FO, Levchenko E. Urine-Derived Kidney Progenitor Cells in Cystinosis. *Cells*. 2022 Apr 6;11(7):1245. doi: 10.3390/cells11071245. PMID: 35406807; PMCID: PMC8997687.

van den Broek M, Smeets B, Schreuder MF, Jansen J. The podocyte as a direct target of glucocorticoids in nephrotic syndrome. *Nephrol Dial Transplant*. 2022 Sep 22;37(10):1808-1815. doi: 10.1093/ndt/gfab016. PMID: 33515261.

Jansen J, Reimer KC, Nagai JS, Varghese FS, Overheul GJ, de Beer M, Roverts R, Daviran D, Fermin LAS, Willemsen B, Beukenboom M, Djurdjaj S,

von Stillfried S, van Eijk LE, Mastik M, Bulthuis M, Dunnen WD, van Goor H, Hillebrands JL, Triana SH, Alexandrov T, Timm MC, van den Berge BT, **van den Broek M**, Nlandu Q, Heijnert J, Bindels EMJ, Hoogenboezem RM, Mooren F, Kuppe C, Miesen P, Grünberg K, Ijzermans T, Steenberg EJ, Czogalla J, Schreuder MF, Sommerdijk N, Akiva A, Boor P, Puelles VG, Floege J, Huber TB; COVID Moonshot consortium; van Rij RP, Costa IG, Schneider RK, Smeets B, Kramann R. SARS-CoV-2 infects the human kidney and drives fibrosis in kidney organoids. *Cell Stem Cell*. 2022 Feb 3;29(2):217-231.e8. doi: 10.1016/j.stem.2021.12.010. Epub 2021 Dec 25. PMID: 35032430; PMCID: PMC8709832.

Miesen L, Wetzels R, Eymael J, Mooren F, Villacorta Monge V, van den Berge BT, **van den Broek M**, van der Velden TJAM, van den Heuvel LPWJ, Wetzels JFM, Schreuder MF, van der Vlag J, Jansen J, Smeets B. Establishment and characterization of a novel conditionally immortalized human parietal epithelial cell line. *Exp Cell Res*. 2021 Aug 15;405(2):112712. doi: 10.1016/j.yexcr.2021.112712. Epub 2021 Jun 25. PMID: 34181939.

Hundscheid T, **van den Broek M**, van der Lee R, de Boode WP. Understanding the pathobiology in patent ductus arteriosus in prematurity-beyond prostaglandins and oxygen. *Pediatr Res*. 2019 Jul;86(1):28-38. doi: 10.1038/s41390-019-0387-7. Epub 2019 Apr 9. PMID: 30965358.



C. Curriculum vitae

Martijn van den Broek was born in Oss on the 25th of June, 1994. He completed secondary school at Mondriaan College choosing biology, chemistry, mathematics, informatics, and physics as core courses. Here, his passion for biology bloomed and the idea to become a "medicine maker" was founded.



After completing secondary school, Martijn studied Life Sciences at the HAN university of applied sciences in Nijmegen in September 2012. He enjoyed his days at the lab bench and molecular life sciences. During his second internship, he had his first encounter with the interesting world of nephrology, including the podocytes and nephrotic syndrome. In a collaborative project at the department of Nephrology (Prof. dr. Johan van der Vlag) and the department of Pathology (dr. Bart Smeets), Martijn aimed to look for the presence of a certain circulating factor in the plasma of patients with idiopathic nephrotic syndrome.

After finishing his Life Sciences education with complements in October 2016, he continued with a Master in Medical Biology at the Radboud University in January 2017. Since he wondered what industry was like, he conducted his final master internship at the pre-clinical department of Synthon Biopharmaceuticals (now Byondis). Although very interesting, this internship sparked his motivation to look for a PhD project after completing his master in July 2019.

In April 2020, he started his PhD project at the department of Pathology and Paediatric Nephrology under supervision of dr. Bart Smeets and Prof. dr. Michiel Schreuder, with daily supervision of dr. Jitske Jansen. The results of his PhD project are outlined in this thesis.

After finishing his PhD project, he continued as a postdoc at the department of Molecular Biology at the Radboud University, in the group of dr. Richárd Bártfai. Martijn currently works on unravelling gametocytogenesis in malaria parasites using transcriptomics and epigenomics.

D. PhD portfolio

Department: **Department of Pathology**

PhD period: **01/04/2020 – 30/06/2026**

PhD Supervisor(s): **Dr. B Smeets, Prof. dr. MF Schreuder**

PhD Co-supervisor(s): **Dr. J Jansen**

Training activities	Hours
Courses	
RIMLS - Introduction course "In the lead of my PhD" (2020)	15.00
RIMLS PhD course (2020)	21.00
Advanced Conversation (2020)	42.00
Projectmanagement for PhD candidates (2021)	56.00
Radboudumc - Scientific integrity (2021)	20.00
Workshop Research Professional (2021)	2.00
Seminars	
Meet the Expert online: LinkedIn (2020)	2.80
Meet the expert: FAIR data management (2020)	2.80
Meet the expert: RNA sequencing (2020)	2.80
Seminar stem cell-derived organoids to model human disease (2021)	2.80
RIMLS meet the expert: How to write a rebuttal (2021)	1.00
Radboud research round plus: Renal disorders (2021)	1.50
Lecture Applying single-cell technologies in nephropathology""	
Prof Clatworthy Cambridge (2021)	1.00
Meet the Expert: Adobe Illustrator (2022)	2.00
Seminar stem cell-derived models to study human diseases (2022)	2.00
Pediatric Nephrology research meeting, oral presentation once a year (2022)	2.00
Theme lunch meeting: Renal Disorders, oral presentation once (2022)	6.00
Nephrology central research meeting, oral presentation once a year (2022)	17.00
10x single-cell sequencing seminars: techniques (2022)	1.00
Harmony (2022)	1.00
Lecture Physiology (2022)	1.00
Research Integrity Round (2023)	1.00
Renal disorders Lunch meeting, oral presentation once (2023)	5.00
Nephrology Central Research Meeting, oral presentation once a year (2023)	17.00
Nephrology Central Research meeting, oral presentation once a year (2024)	17.00
Conferences	
X-omics Festival (2020)	5.60
Nederlandse Nefrologiedagen (2020)	14.00
ASN Kidney Week (2020)	28.00
PhD Retreat (2020)	7.00
ENABLE (2021)	28.00
13th International Podocyte Conference (2021)	30.00
NND (2021)	16.00
PhD Retreat 2021, poster presentation (2021)	24.00
Nederlandse Nefrologiedagen (NND), oral presentation (2022)	16.00
Summerschool Dutch Kidney Foundation (2022)	20.00
ASN Kidney week, poster presentation (2022)	28.00
Nederlandse Nefrologiedagen (NND), oral presentation (2023)	16.00
ERCSG, oral presentation (2023)	16.00
PhD Retreat, oral presentation (2023)	24.00
BaCell (2023)	16.00
NFN Fall symposium, poster presentation (2023)	16.00
ASN Kidney week, poster presentation (2023)	40.00

Other	
Member of PhD council (2023)	66.00
Teaching activities	
Supervision	
Supervision of Master student internship (2021)	49.00
Supervision of Bachelor student internship (2021)	49.00
Supervision of final year Bachelor student internship (2022)	49.00
Supervision of final year Bachelor student internship (2022)	49.00
Supervision of Master student internship (2022)	49.00
Supervision of Master student internship (2023)	49.00
Supervision of Master student internship (2023)	49.00
Total	996.30



Dankwoord

Na je in mijn introductie warm te hebben gemaakt, zou ik zeggen: pak een kopje koffie en ga hier maar even lekker voor zitten. Het harde werk zit erop-tijd voor wat lichtere kost! Als je deze verwijzing naar de introductie nu niet begrijpt, dan ben je heus niet de enige die dit boekje niet van voor naar achter, maar van achter naar voor leest. Ook al schrijf je een proefschrift alleen, het wordt samen met anderen gemaakt. En in dit laatste stuk neem ik de ruimte om iedereen te bedanken die me de laatste 4 (lees 6) jaar heeft gemaakt tot wie ik nu ben en me heeft geholpen dit boekje te kunnen maken. Zo was ik hier nooit gekomen zonder mijn promotieteam met daarin dr. Bart Smeets, prof. dr. Michiel Schreuder en dr. Jitske Jansen. Ik wil jullie bedanken voor de enorme vrijheid die jullie me hebben gegeven, hierdoor heb ik kunnen leren om zelfstandig onderzoek te doen. Maar ook bedankt voor de steun, want die is er altijd geweest, of dat nu binnen kantoor tijden was of daarbuiten, ik kon altijd bij jullie terecht. Heel erg bedankt hiervoor.

Bart, toen ik in 2016 mijn HLO-stage bij je liep, had ik nooit gedacht dat ik je tien jaar later in dit dankwoord zou noemen. Na die stage mocht ik terugkomen voor een masterstage, en vergaf je me zelfs dat ik een tijdlang dacht dat de hond mijn USB-stick met stagedata had opgegeten, wat gelukkig niet zo bleek te zijn. Ik heb altijd bewondering gehad voor jouw zichtbare passie voor onderzoek. Vooral bij microscopie en histologie is dat enthousiasme aanstekelijk, en daar heb je mij zeker in meegenomen. Met je scherpe oog heb je meer dan eens dingen in mijn microscopiebeelden gezien die mij nog niet waren opgevallen. Ook jouw vragen over wat een UMAP nu eigenlijk is en hoe bepaalde tools zulke grafieken maken, hebben mij geholpen het zelf beter te begrijpen, al kan ik je het algoritme erachter nog steeds niet helemaal uitleggen. Naast het werk kijk ik met veel plezier terug op onze reis naar de ASN Kidney Week in Orlando. De ochtenden bij iHOP, waar we beiden toch weer voor de “drip coffee” gingen, blijven me bij–best confronterend: een koffieverslaving. Van de “splash zone” tot lederhosen, van de Amsterdamse grachten tot Veldhoven: ik heb ervan genoten. Bart, bedankt voor je passie en gezelligheid!

Michiel, enorm bedankt voor je betrokkenheid. Je hebt vanaf dag één bij elke werkbespreking gezeten, en bent meer dan eens mee het lab op geweest om me bij te scholen of te helpen. En met lab bedoel ik natuurlijk het CDL, en niet het type lab waar met pipetten en chemicaliën gewerkt wordt, want zoals je zelf aangeeft: daar kunnen we je beter weghouden. Door jouw betrokkenheid

was je voor mij vanaf het begin altijd bereikbaar, en dat heb ik enorm gewaardeerd. Tijdens mijn PhD heb je meer dan eens de vinger op de zere plek gelegd, daardoor ben ik dit gaan zien als jouw gave. Je weet uit teksten die ik schrijf precies te halen hoeveel ik begrijp van een onderwerp, en je vult die kennis vervolgens aan met opmerkingen of scherpe vragen. Je tweede gave moet dan wel je positiviteit zijn, na een werkbijeenkomst met jou kreeg ik meer dan eens weer de energie om door te pakken! Ik kijk ernaar uit om binnenkort weer samen te werken en samen iets moois te maken van jouw Vici-project. Michiel, bedankt voor je betrokkenheid en positiviteit!

Jitske, jij was zonder meer een letterlijke dagelijkse begeleider, zeven dagen per week van 6.00 tot 22.00! Na ons kennismakingsgesprek dacht ik: "volgens mij geloofde ze maar de helft van wat ik zei". Daar maakte ik meteen kennis met jouw scherpe vragen en kritische blik. Kritisch en scherp als het om de inhoud gaat, maar ontzettend behulpzaam voor de persoon achter het onderzoek. Vanaf het moment dat ik begon, heb je me onder je vleugels genomen. Het lukt jou altijd om met een eindeloze stroom aan ideeën iets te maken van elke situatie. Zo stelde je meteen voor om te beginnen met een review en het leren analyseren van omics data, omdat we toch nog niet het lab op konden. Ook tijdens mijn PhD: bij elk experiment dat tegenzat, wist jij weer 15 andere experimenten te bedenken om de vraag toch te kunnen beantwoorden. Als ik aan jou denk, hoor ik: *work hard, play hard!* Dat bleek wel tijdens de weekendjes weg met de niergroep, het Oktoberfest, de feestjes na de NND, de Vierdaagsefeesten en het tentfeest bij jou in de buurt. Als ik terugdenk aan al die borrels, feestjes en uitjes, word ik weer vrolijk—wat hebben we gelachen! Je hebt niet alleen indruk op me gemaakt in hoe hard je werkt, maar ook met je gevoel voor humor. Ik zal je 1 april grap nooit meer vergeten, waarin het hele Smeetslab zogenaamd naar Aken verhuisde (ik had mijn koffer al klaarstaan!). Of die keer dat Gigi en ik de beveiligers op ons dak kregen, met een briefje: 1 april! Ik ben ontzettend blij om te zien dat je de Vidi binnen hebt gehaald, heel erg terecht. Ik vind het jammer dat je daarmee naar Maastricht verhuisd, maar ik hoop van harte dat we kunnen blijven samenwerken in de toekomst. Jitske, bedankt voor je bevlogenheid, eindeloze stroom ideeën en humor!

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"All I want is for you to be happy and, take this woman and make you my family and, finally you have someone perfect and, finally you have found... yourself."
– Red Hot Chili Peppers

Ik heb gezegd.



