Cellular and molecular mechanisms of kidney injury and regeneration

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Cover illustration “Heterogeneity of proximal tubular cells in human kidney” by Michelle Kha

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"No research without action, no action without research"
– Kurt Lewin

Abstract

The prevalence of kidney disease is constantly increasing with about 600 million people afflicted globally. Kidney disease may result in end stage renal disease, a condition that requires renal replacement therapy in the form of kidney transplantation or dialysis. However, the mechanisms behind kidney injury and regeneration are still incompletely understood. In this thesis, we aimed to study the phenotype of the scattered tubular cells (STCs), a cell population distributed throughout the proximal tubules (PT) of human kidney involved in injury and regeneration, for better understanding of the underlying mechanisms of tubular repair. In Paper I, we established SOX9 as an STC marker and demonstrated that SOX9 is activated in response to injury in the human kidney. Furthermore, we identified *LBR*, *HMGA2*, and *HIPK3* as potential downstream targets of SOX9 in tubular repair. In Paper II, we observed that the STC phenotype is induced in cultured primary PT cells and that the PT phenotype could be partially restored by re-expression of HNF4A. In Paper III, we showed that the STCs have a general reduction of mitochondrial markers, and we also found another cell population in the PT which displayed selective loss of mitochondrial respiratory chain complex I that increased with age. In Paper IV, we presented ARMH4 and WIPF3 as novel podocyte proteins possibly involved in glomerular injury and disease. Taken together, these findings elucidate potential therapeutic targets during kidney injury and regeneration.

Keywords

kidney, injury, regeneration, repair, scattered tubular cells, proximal tubular cells, podocytes, SOX9, HNF4A

Sammanfattning på svenska

Njurens huvudsakliga funktion är filtration av slaggprodukter samt återupptag av näringsämnen från kroppens blodomlopp. Vid skada tappar njuren sin förmåga till filtration samt återupptag och detta kan slutligen leda till uttalad njursvikt vilket kräver njurtransplantation eller dialys. Trots detta är kunskapen om njurskada och dess läkningsförmåga otillräcklig. I denna avhandling identifierade vi faktorer som är viktiga under njurens skade- och läkningsprocess. Vi fokuserade primärt på de tubulära cellerna som ansvarar för återupptaget. Bland dessa hittas spridda tubulära celler (STC) som aktiveras vid skada och deltar i läkningen. STC skiljer sig från de resterande tubulära cellerna i gen- och proteinuttryck. I delarbete I visade vi att STC uttrycker genen SOX9 och att uttrycket av SOX9 ökar i samband med njurens skaderespons vilket hjälper cellerna att överleva och föröka sig. I delarbete II upptäckte vi att labbodlade tubulära celler omvandlas till STC. Genom att tvinga cellerna att återuttrycka genen HNF4A lyckades vi återfå stora delar av de tubulära cellernas normala genuttryck. Följaktligen föreslår vi att SOX9 krävs vid den tidiga fasen av njurskada som sedan bör efterföljas av HNF4A-uttryck för att cellerna ska återfå sin normala funktion. I delarbete III påvisade vi att STC har en generell avsaknad av mitokondriella proteiner och upptäckte även en ny cellpopulation som endast saknar ett specifikt proteinkomplex i andningskedjan. Denna cellpopulation ökade med åldern och är möjligtvis av betydelse för utvecklingen av njurskada. I delarbete IV fokuserade vi på njurens filtrerande celler (podocyterna) där vi identifierade ARMH4 och WIPF3 som proteiner involverade vid njursjukdom. Sammanfattningsvis har vi i denna avhandling belyst flera möjliga terapeutiska måltavlor vid njurskada som potentiellt kan förbättra utfallet vid läkning.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Kha M, Krawczyk K, Choong OK, De Luca F, Altiparmak G, Källberg E, Nilsson H, Leandersson K, Swärd K, Johansson ME.

The injury-induced transcription factor SOX9 alters the expression of *LBR*, *HMGA2*, and *HIPK3* in the human kidney

Am J Physiol Renal Physiol. 2023;324(1):F75-F90

II. Kha M, Altiparmak G, Lundgren J, Swärd K, Johansson ME.

HNF4A re-expression partially restores a proximal tubular phenotype in cultured primary kidney cells

Manuscript

III. Kha M, Altiparmak G, Elliott K, Xie X, Larsson E, Oldfors A, Swärd K, Johansson ME.

Age and injury-related changes of mitochondrial respiratory complexes in human kidney tubules

Manuscript

IV. De Luca F, Kha M, Swärd K, Johansson ME.

Identification of ARMH4 and WIPF3 as human podocyte proteins with potential roles in immuno-modulation and cytoskeletal dynamics

PLoS One. 2023;18(1):e0280270

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Content

Abbreviations

AKI	acute kidney injury
ARMH4	armadillo-like helical domain-containing protein 4
cDNA	complementary DNA
CKD	chronic kidney disease
CLEM	correlative light and electron microscopy
COX	cytochrome c oxidase
DAB	3,3'-diaminobenzidine
DEGs	differentially expressed genes
ELISA	enzyme-linked immunosorbent assay
ESRD	end stage renal disease
FFPE	formalin-fixed paraffin-embedded
GFR	glomerular filtration rate
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
GWAS	genome-wide association study
H&E	hematoxylin-eosin
HIPK3	homeodomain interacting protein kinase 3
HMGA2	high-mobility group AT-hook 2
HNF4A	hepatocyte nuclear factor 4α
HPA	Human Protein Atlas
HRP	horseradish peroxidase
IFN-γ	interferon-γ
IL	interleukin
IMAC	intermicrovillar adhesion complex
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBR	lamin B receptor
LPS	lipopolysaccharide
LTL	Lotus Tetragonolobus Lectin
MTCO1	mitochondrially encoded cytochrome c oxidase I

mtDNA	mitochondrial DNA
NBT	nitroblue tetrazolium
NDUFB8	NADH dehydrogenase ubiquinone 1 beta subcomplex subunit 8
OCT	optimal cutting temperature
PECs	parietal epithelial cells
PMS	phenazine methosulphate
PT	proximal tubule
PTECs	primary tubular epithelial cells
qPCR	quantitative polymerase chain reaction
RNA-seq	RNA sequencing
SDH	succinate dehydrogenase
siRNA	small interfering RNA
SOX9	SRY box transcription factor 9
STCs	scattered tubular cells
TEM	transmission electron microscopy
TGF-β	transforming growth factor-β
VCAM1	vascular cell adhesion molecule 1
VDAC1	voltage-dependent anion channel 1
VIM	vimentin
WIPF3	WAS/WASL-interacting protein family member 3

Introduction

The kidneys are two bean-shaped organs responsible for filtration of the blood and removal of waste and excess fluid by production of urine. The primary filtrate contains nutrients which are reabsorbed, resulting in secondary urine with the remaining metabolic waste. Other functions of the kidneys include production of hormones and maintenance of homeostasis by regulating electrolyte content, pH balance, and blood pressure. In order to perform all these functions, the kidneys are anatomically and physiologically complex.

Kidney anatomy and structure

The nephron is the functional unit of the kidney. Each human kidney contains around 1 million nephrons. The nephron consists of the glomerulus, a blood-filtering unit, which is followed by the renal tubules that are responsible for reabsorption of electrolytes and nutrients and excretion of toxins and metabolic end products. The renal tubules are composed of a convoluted tube of epithelial cells divided into several distinct segments. They begin with the proximal tubule which is followed by the loop of Henle, the distal tubule, and end in the collecting duct. The structure of the nephron is illustrated in **Figure 1**.

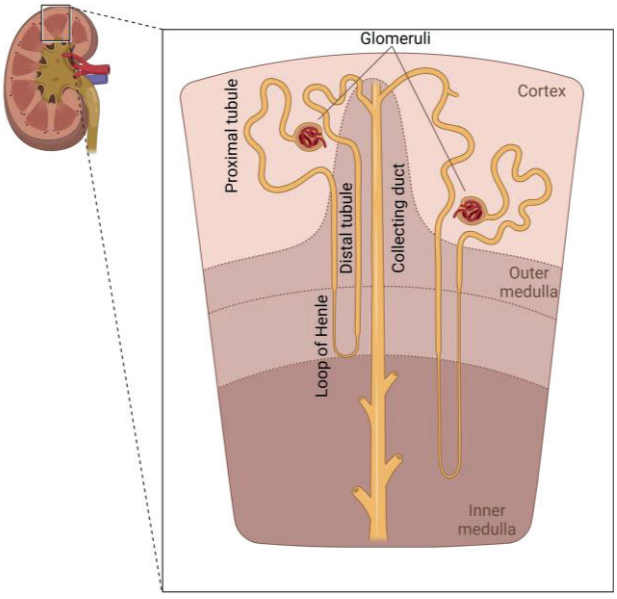

Figure 1. Structure of the kidney and the nephron. The cortex is the outer part of the kidney surrounding the medulla. The nephron is the functional unit of the kidney composed of the glomeruli, proximal tubule, loop of Henle, distal tubule, and collecting duct. *Created with BioRender.com.*

Glomeruli

The glomeruli are capillary tufts, situated between an afferent and efferent arteriole, in the cortex of the kidney. Apart from the endothelial cells that make up the capillaries of the glomerulus, mesangial cells reside between the capillaries to control blood flow and remove debris from the glomerular basement membrane, also known as the glomerular basal lamina. Furthermore, the glomerulus is enclosed by Bowman's capsule. The visceral layer of the capsule contains podocytes, terminally differentiated epithelial cells with pedicels, foot processes that wrap around the capillaries, illustrated in **Figure 2**. The primary processes emerge from the cell body of the podocyte which in turn divide into secondary processes and continue branching into tertiary processes. The outer layer of the capsule is composed of parietal epithelial cells and the area between these two layers is called Bowman's space. Together, the glomerulus and Bowman's capsule constitute the renal corpuscle.

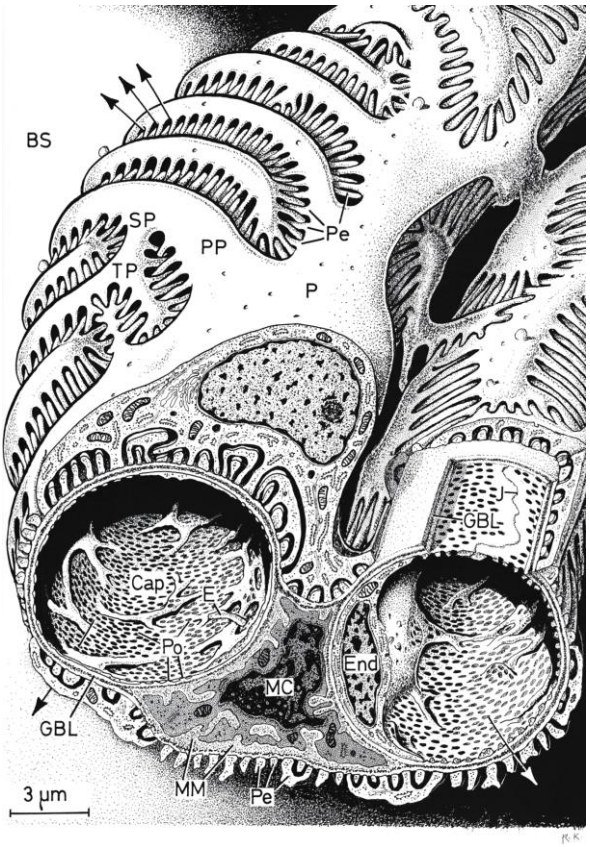

Figure 2. Structure of the podocyte. **BS**, Bowman's space; **Cap**, capillaries; **E**, extensions of mesangial cells; **End**, endothelial cells; **GBL**, glomerular basal lamina; **J**, junctional line; **MC**, mesangial cells; **MM**, mesangial matrix; **P**, podocytes; **Pe**, pedicels; **Po**, pores; **PP**, primary processes; **SP**, secondary processes; **TP**, tertiary processes.
Illustration from Krstić RV. Human Microscopic Anatomy. p. 309. Heidelberg: Springer-Verlag; 1991. *Reproduced with permission from Springer Nature.*

Blood filtration occurs through the glomerular filtration barrier which is composed of endothelial cell fenestrations, a layer of glycocalyx, the glomerular basement membrane, and podocyte slit diaphragms (1), as depicted in **Figure 3**. The endothelial cell fenestrations allow for the permeability of smaller molecules, while blood cells are retained in the blood vessels. The endothelial cells produce a glycocalyx layer which is followed by the glomerular basement membrane. Both are negatively charged and are responsible for charge selectivity in the glomerular filtration barrier, causing electrostatic repulsion to plasma proteins that mostly have a negative charge. The final barrier for glomerular filtration consists of the podocyte slit diaphragms, specialized cell-to-cell junctions, which allow macromolecules of certain size and shape to pass into the tubules.

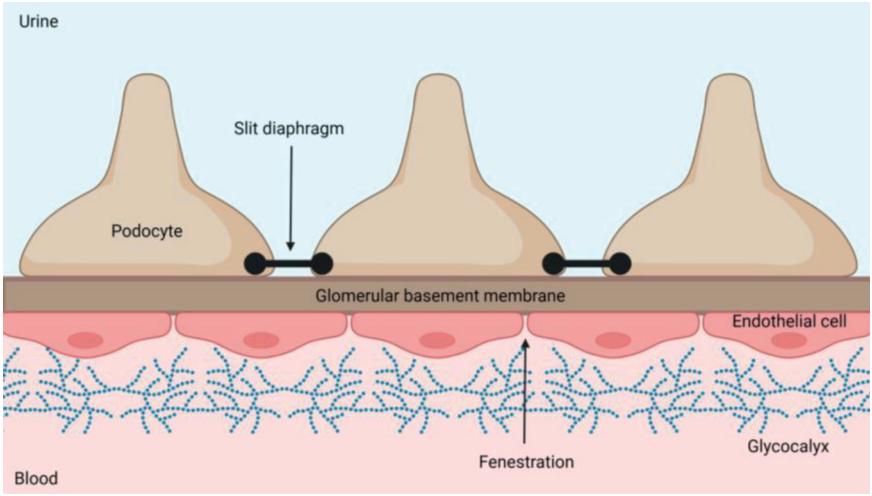

Figure 3. The composition of the glomerular filtration barrier. The glomerular filtration barrier includes endothelial cell fenestrations, glycocalyx, glomerular basement membrane, and podocyte slit diaphragms. *Created with BioRender.com.*

Renal tubules

The proximal tubules (PT) are the longest tubular segments and begin at the outlet of the glomeruli. The PT are divided into three segments where the S_1 segment includes the major part of the proximal convoluted tubule, the S_2 segment constitutes the end of the proximal convoluted tubule and beginning of the proximal straight tubule, and the S_3 segment comprises the remaining part of the proximal straight tubule (2). The ultrastructure of these segments differ, with the S_1 segment containing a larger number of mitochondria and microvilli, explaining the higher reabsorptive capacity of this convoluted segment compared to the straight part of the S_2 and S_3 segments (2).

Around 70% of the reabsorption is performed by the PT, including electrolytes, glucose, amino acids, and water. The resulting filtrate is passed to the U-shaped loop of Henle, beginning in the cortex, extending deep into the medulla, and returning to the cortex. Its main function is to allow the concentration of urine. The loop of Henle is

followed by the distal tubules where further reabsorption of electrolytes occurs. Lastly, the renal tubules end with the collecting ducts where the nephrons converge as the collecting ducts travel deep into the medulla to the renal pelvis. The collecting ducts perform the final regulation of urine concentration by adjusting the water content via the aquaporin channels. The active transport and reabsorption carried out by the renal tubular cells require extensive ATP supply, provided by a high density of mitochondria, and brush border which increases the absorption area remarkably.

Mitochondria

Mitochondria are the energy-producing organelles of the cell. A small fraction of cellular ATP is generated outside of the mitochondria, via anaerobic glycolysis where glucose is converted to pyruvate. However, 15 times more ATP is produced when pyruvate is transported into the mitochondria where it is further metabolized.

The mitochondrion contains an outer membrane and an inner membrane, creating an internal matrix within the inner membrane and an intermembrane space. Pyruvate is metabolized into acetyl CoA in the matrix which in turn is oxidized to NADH and CO_2 in the citric acid cycle. Next, electrons from NADH are transported across the respiratory chain, a series of enzyme complexes embedded in the inner membrane, resulting in a proton gradient that drives ATP generation in a process called oxidative phosphorylation.

Brush border

The tubular epithelial cells have brush border made of microvilli which are protrusions of the apical membrane of the cell. The uniformly lengthened microvilli form a highly organized and packed network, resulting in a hexagonal pattern and maximization of the surface area for

absorptive function (3). The microvilli are supported by actin filaments which are bundled by villin, espin, and fimbrin and connected to the terminal web, a cytoskeletal network that anchors and stabilizes the brush border (3, 4). Furthermore, brush border assembly and organization require intermicrovillar adhesion in the form of the protocadherins, CDHR2 and CDHR5, connecting at the tip of microvilli (5). The protocadherins interact with MYO7B which in turn links them and the scaffolding proteins USH1C and ANKS4B to the actin bundles (6, 7). Moreover, CALM4 further stabilizes the intermicrovillar adhesion complex (8).

Kidney diseases

Kidney diseases can be categorized based on histological grounds from which they are divided into glomerular and tubulointerstitial diseases. The glomerular diseases may be further divided into diseases that present clinically as a nephrotic or nephritic syndrome. Kidney diseases can also be categorized according to duration, where acute kidney injury (AKI) describes rapid loss of kidney function and chronic kidney disease (CKD) is defined as kidney injury that has lasted over three months (9).

The nephrotic syndrome

Kidney diseases that primarily cause disruption of the glomerular filtration barrier result in the nephrotic syndrome. Due to protein leakage, the nephrotic syndrome is characterized by massive proteinuria, previously defined as >3.5 g protein per day (10). This definition has been replaced by an albumin-creatinine-ratio exceeding 300 g/mol as a more precise measurement of albuminuria. The protein loss is compensated by protein synthesis from the liver, but when this is overwhelmed, other symptoms appear such as hypoalbuminemia, edema, and hyperlipidemia. Hypoalbuminemia is usually mirrored by

albuminuria. However, occasionally, reduced albumin is only observed in the serum while remaining constant in the urine, which makes the serum level a more accurate measurement (11). Edema develops gradually and is believed to be caused by decreased colloid osmotic pressure as a consequence of hypoalbuminemia. Furthermore, a combination of increased lipoprotein synthesis in the liver and reduced clearance of large proteins results in hyperlipidemia.

The nephritic syndrome

In some kidney diseases, nephrotic syndrome is observed together with a nephritic syndrome, defined by concurrent proteinuria, hematuria, hypertension, and reduced glomerular filtration rate (GFR). Epithelial injury that targets the podocytes of the glomerular filtration barrier mainly results in the nephrotic syndrome. However, injury to the endothelial or mesangial cells by immune complex deposition leads to recruitment of inflammatory cells, i.e. a nephritic syndrome, resulting in proliferative patterns. Damage to the endothelial layer of the glomerular filtration barrier is characterized by endocapillary proliferation and swelling, accumulation of neutrophils and lymphocytes in the capillaries, and instant reduction of GFR. Damage to the mesangial cells results in mesangial proliferation, phagocytosis of immune complexes, and a slower reduction of the GFR. The glomerulonephritides comprise a group of kidney diseases that lead to a nephritic syndrome and they are usually caused by a mix of endocapillary and mesangial proliferation. Untreated glomerulonephritides may result in chronic kidney disease or end stage renal disease (12). **Table 1** lists common kidney diseases, including different types of glomerulonephritides, together with the presented syndrome.

Table 1. Kidney diseases with presented syndrome.

KIDNEY DISEASE	SYNDROME
Membranous nephropathy	Nephrotic
Minimal change nephropathy	Nephrotic
Focal segmental sclerosis	Nephrotic
IgA nephritis	Nephritic/nephrotic
Endocapillary glomerulonephritis	Nephritic
Membranoproliferative glomerulonephritis	Nephritic
ANCA glomerulonephritis	Nephritic
Lupus nephritis	Nephritic

Tubulointerstitial diseases

The PT cells perform the majority of the tubular reabsorption which results in high energy requirements and makes them more susceptible to inadequate supply of oxygen and nutrients (13). Moreover, since the PT is the initial tubular segment following glomerular filtration, these cells are more exposed in the case of endogenous or exogenous toxins. Hence, the PT is almost always affected during kidney injury and disease, even during glomerular diseases. The tubulointerstitial diseases are a group of disorders that directly targets the renal tubules and interstitium. The most common tubulointerstitial disease is interstitial nephritis which can be categorized as acute or chronic. Acute interstitial nephritis is mainly drug-induced (14), whereas chronic interstitial nephritis normally occurs secondary to pyelonephritis (kidney infection) or obstructive uropathy (obstructed urinary flow) (15). Interstitial nephritis is characterized by inflammatory infiltration in the renal interstitium. This causes gradual reduction of tubular function while the glomeruli initially remain unaffected, mirrored by proteins of lower molecular weight, glucose, and amino acids in the urine. By contrast, proteinuria in the nephrotic syndrome is reflected by the presence of albumin and other larger proteins in the urine.

Acute kidney injury

Acute kidney injury is a syndrome characterized by sudden loss of kidney function for seven days or less (16). AKI is defined by increased serum creatinine levels and decreased urine output (9, 16). However, the diagnosis of AKI is challenging since these markers reflect the function of the kidney rather than injury (16). Urinary output as a diagnostic marker has low specificity since it can be affected by various parameters, whereas serum creatinine has low sensitivity. Even in the absence of injury, some patients may fulfill the criteria and get misdiagnosed. The most accurate indicator of kidney function is the GFR, but it remains difficult to measure. Therefore, serum creatinine level is often utilized as an estimate of the GFR (16).

About 20% of hospitalized patients suffer from AKI (17). AKI is induced by sepsis, major surgery, and numerous other causes (9). Approximately two-thirds of AKI cases recover within seven days (18). However, when kidney injury has exceeded three months, it is defined as CKD (9). Notably, AKI patients suffer a nearly nine-fold increase in risk for CKD (19), whereas pre-existing CKD increases the risk for development of AKI by a factor of ten-fold (20).

Chronic kidney disease

Chronic kidney disease is an irreversible state of kidney injury which often leads to end stage renal disease (ESRD). The prevalence of CKD is around 10% in the general population and the main causes are diabetes and hypertension (21, 22). CKD is determined based on a GFR less than 60 mL/min/1.73 m^2, corresponding to a reduction of half of the normal renal function, or other markers of kidney injury such as albuminuria, that has persisted over three months (23). Depending on the level of GFR, CKD is classified into five stages. GFR less than 15 mL/min/1.73 m^2 indicates the progress of ESRD (21). Once ESRD is reached, renal replacement therapy, in the form of kidney

transplantation or dialysis, is required. However, for a CKD patient, the risk of death is five to ten times higher than the progress to ESRD (24).

Kidney repair

During normal conditions, proximal tubular cells rarely proliferate (25). However, in response to injury, the kidney has the capacity to repair and regenerate, where surviving tubular epithelial cells are reported to undergo dedifferentiation and proliferation to repopulate the tubules (26, 27). This has been challenged by studies that found a subpopulation of parietal epithelial cells (PECs) in Bowman's capsule in adult human kidney with expression of the stem cell markers CD24 and CD133, displaying proliferative multilineage differentiation potential, including tubular regeneration (28). Furthermore, they are suggested to originate from renal embryonic progenitors during the early stages of nephrogenesis since they share marker composition and properties (29). During glomerular disease, these multipotent progenitors are believed to be involved in the uncontrolled PEC proliferation resulting in crescent formation (30) and they also showed the potential to regenerate podocytes (31). Interestingly, the $CD24^{+}CD133^{+}$ renal progenitors demonstrated a hierarchical distribution where the cells closest to the urinary pole of Bowman's capsule exhibited regenerative capacity towards both tubular cells and podocytes (31). However, as they localize closer towards the vascular pole, the cells begin to show podocyte commitment, by expression of the podocyte marker PDX, and eventually lose expression of CD24 and CD133 as well as their regenerative capacity (31). The mechanism behind podocyte regeneration is still debated, although PECs are believed to be involved during this process (32, 33). Subsequent studies using lineage tracing after ischemia-reperfusion injury in rodents demonstrated that tubular regeneration was driven by self-duplication of randomly surviving tubular cells, arguing

against the involvement of a resident stem or progenitor cell population (34).

Scattered tubular cells (STCs) were identified in the proximal tubules of healthy adult human kidney. These cells were positive for CD24 and CD133 as well as numerous other markers, including vimentin (VIM) and cytokeratins 7 and 19 (KRT7 and KRT19), that were shared with the PEC progenitors but not detected in the rest of the tubular cells (35). The STCs frequently localized to the tubular curvatures and demonstrated stem cell characteristics as well as activation during tubular regeneration (35). CD106, also known as vascular cell adhesion molecule 1 (VCAM1), was presented as a marker that could differentiate between the PEC progenitors and STCs (36). Furthermore, $CD24^{+}CD133^{+}CD106^{-}$ progenitors were shown to only be capable of tubular regeneration, hence referred to as tubular-committed progenitors (36), whereas podocyte-committed $CD24^{+}CD133^{+}PDX^{+}$ progenitors were restricted to regeneration of podocytes (31, 36). The third multipotent $CD24^{+}CD133^{+}CD106^{+}PDX^{-}$ progenitor population demonstrated regenerative potential of both podocytes and tubular cells (36). Importantly, $CD24^{+}CD133^{+}CD106^{+}$ cells were found in the tubules but at a substantially lower frequency and displayed higher proliferative rate compared to $CD24^{+}CD133^{+}CD106^{-}$ cells (36).

Further studies of the STCs revealed a distinct morphology with diminished brush border and fewer mitochondria, suggesting a dedifferentiated phenotype and resistance to injury (37, 38). While STCs were found in healthy human kidney and increased in frequency upon injury, they were rarely detected in healthy murine kidney (37). Instead, cells expressed STC markers *de novo* in response to injury, arguing against the hypothesis that STCs represent a progenitor cell population (37, 39). Additional studies using lineage tracing and cell fate tracking in mice further strengthened the hypothesis that kidney regeneration occurs from terminally differentiated epithelial cells that obtain an injury-

induced stem cell-like phenotype (39, 40). However, Rinkevich *et al.* interpreted their lineage tracing results differently, proposing that the kidney epithelium repair occurred via fate-restricted and segment-specific unipotent progenitors (41). Different mechanisms of kidney repair or varying frequencies of spontaneous injury across species have also been suggested which is in agreement with studies that showed presence of STCs in higher mammals such as human, primates, and pig, while being harder to detect in rodents (37, 39, 42). Another possibility is that both mechanisms, illustrated in **Figure 4**, occur and complement each other depending on the severity of injury (43).

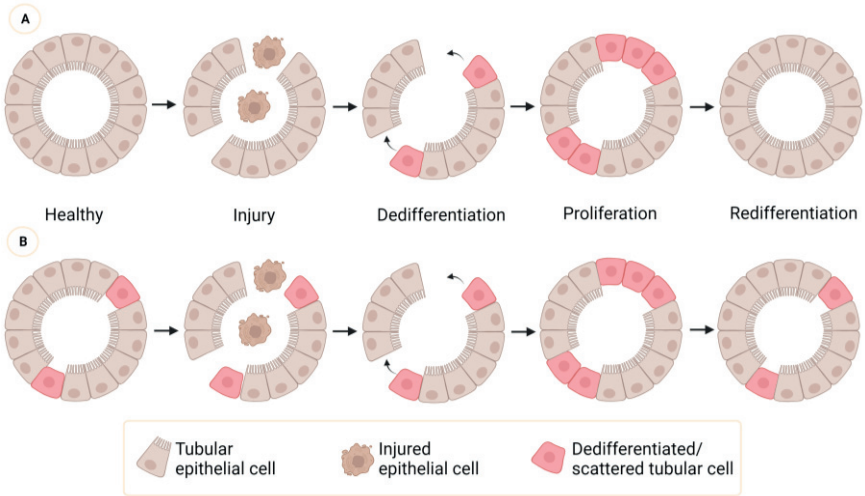

Figure 4. Two mechanisms for kidney regeneration following injury. (A) The traditional view is that tubular regeneration occurs from stochastically surviving cells that dedifferentiate, proliferate, and redifferentiate. (B) The existence of a progenitor or stem cell population has been proposed more recently, which activates in response to injury and divides to repopulate the tubules. *Created with BioRender.com.*

Over the years, additional markers have been presented for the STCs in humans (37, 38) or have demonstrated activation upon kidney injury in rodents, including transcription factors such as Sox9 (44, 45) and Foxm1 (46). Further investigations regarding these transcription factors might reveal the mechanisms of regeneration which was why we focused on establishing the role of SOX9 in human kidney and

identifying downstream targets in Paper I (47). In Paper II, we continued our investigation of transcription factors with a role in kidney injury and regeneration but approached it from the opposite perspective by studying the proximal tubular transcription factor hepatocyte nuclear factor 4α (HNF4A) which is downregulated in kidney injury (48, 49).

Kidney injury may result in maladaptive repair, characterized by incomplete tubular repair, fibrosis, and inflammation (25). The maladaptive cells get arrested in the G_2/M phase which promotes secretion of cytokines, such as TGF-β and proinflammatory interleukins, which is termed senescence-associated secretory phenotype (50, 51). The secretion of cytokines results in activation of signaling pathways that initially are beneficial during repair. However, sustained expression is suggested to promote fibrosis and lead to chronic kidney disease (32). Maladaptive repair results in an altered kidney structure as well as reduced function (19).

Adaptive and maladaptive repair are interconnected and therefore their mechanisms are essential to delineate and understand. Single-cell RNA sequencing (RNA-seq) studies of kidney have revealed a proinflammatory and profibrotic failed-repair proximal tubular cell population in mice (52). Furthermore, a cell population, characterized by expression of VCAM1, was observed in healthy adult human kidney and showed a similar molecular signature to the failed-repair PT cell population in mice (53). Studies by Gerhardt *et al.* found that VCAM1-positive cells displayed a senescence-associated secretory phenotype without G_2/M cell cycle arrest (48). These failed-repair PT cells were most abundant four weeks after AKI and showed substantial decrease six months post-AKI which was partly explained by cell death (49). Moreover, Muto *et al.* identified coexpression of STC markers by VCAM1-positive cells (53). Further analyses of the single-cell RNA-seq data from Muto *et al.* indicated that the STCs represent a heterogenous population and

that the molecular signature of VCAM1-positive cells partially overlaps with the STCs (53, 54). However, Eymael *et al.* also demonstrated that the STCs were not senescent, proposing that they do not contribute to maladaptive repair (54).

The role and origin of the STCs remain unsettled, although more recent reports argue for the traditional view where the STCs represent a dedifferentiated phenotype induced by injury (47, 49, 53-55). Interestingly, a recently published study based on single-cell RNA-seq data of mouse kidney identified five subpopulations of the PECs, including a population of tubular progenitors, as well as their transition relationships (56). The findings were in line with the reported heterogeneity and hierarchical distribution of the PECs observed in adult human kidney (36) and further strengthened the hypothesis of a bipotent progenitor pool among the PECs that may transition to both podocyte and tubular progenitors. Of note, one of the subpopulations was found to give rise to crescent formation during glomerular disease (56). This subpopulation was derived mainly from the tubular progenitors (56). Moreover, another study connected the two hypotheses regarding tubular regeneration by identifying a subpopulation of PECs positive for WT1 which is mainly expressed by the podocytes in healthy kidney (57). Following AKI, the WT1-positive PECs were proposed to migrate to the tubules, transition to the STC phenotype, and develop into PT cells (57). This was based on data from lineage tracing and single-cell RNA-seq of mouse kidney (57). Future studies are required to further elucidate the cellular responses following kidney injury and the resulting regenerative mechanisms.

Aims

The overall aim of this thesis was to identify the mechanisms underlying human kidney injury and regeneration, mainly focusing on the scattered tubular cells found in the proximal tubules of human kidney.

The specific aims for each paper are stated below.

Paper I

To establish the role of the transcription factor SOX9 in human kidney injury and regeneration and identify downstream targets of SOX9.

Paper II

To test if viral overexpression of HNF4A in cultured primary tubular epithelial cells can restore the differentiated phenotype and to define how the expression of HNF4A relates to induction of the phenotype of scattered tubular cells.

Paper III

To determine the distribution of mitochondrial respiratory chain complexes in the proximal tubules of human kidney in relation to injury and aging.

Paper IV

To identify novel markers for podocytes of the glomeruli in kidney with potential roles in kidney injury and disease.

Materials and Methods

This is a brief description of the key methods used in this thesis. More details can be found in the papers and manuscripts.

Tissue procurement

The work in this thesis was based on human patient material in the form of kidney tissue. Normal kidney tissue was obtained from resected kidneys due to renal cell carcinoma following written informed consent from the patients and with permit from the Swedish Ethical Review Authority (LU680-08, 2020-06242). Material for experimental purposes was collected from kidney tissue localized farthest from the tumor. Diagnostic kidney biopsies were acquired during work up of kidney disease.

After tissue procurement, the collected sample must be evaluated to confirm that the normal tissue obtained from kidneys from renal cell carcinoma patients are healthy and to diagnose kidneys from kidney disease patients. This is performed on formalin-fixed paraffin-embedded (FFPE) tissue following deparaffinization, rehydration, and hematoxylin-eosin (H&E) staining. H&E staining is the principal staining utilized for histopathological evaluation in which hematoxylin stains the nuclei blue and eosin stains the extracellular matrix and cytoplasm pink (58). Histopathological evaluation via H&E staining of FFPE sections was performed on all patient material used in this thesis.

An explant model previously established in our lab (38) was further developed in Paper I. The explant model consisted of biopsies obtained from resected kidneys due to renal cell carcinoma. The

intraoperative clamping of vessels lasted for approximately 2 h and acted as warm ischemia in the explant model. Then, the biopsies were placed in cell culture plates with full medium and reoxygenated in a cell culture incubator.

Glomerular and primary cell isolation and cell culture

In Paper IV, kidney tissue was used to isolate glomeruli via mechanical disaggregation by passing the tissue through sieves of gradually reducing mesh sizes. In Paper I, II, and IV, primary tubular epithelial cells (PTECs) were derived from kidney tissue using enzyme digestion and filtration to obtain a single cell suspension. The procedures for glomerular and primary cell isolation are depicted in **Figure 5**.

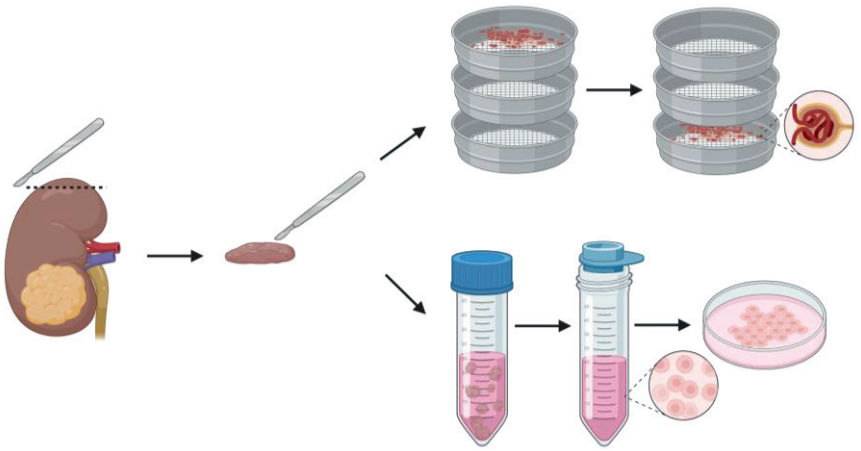

Figure 5. Isolation of glomeruli and primary tubular epithelial cells from kidney tissue. Upper row illustrates glomerular isolation by mechanical disaggregation using sieving. Lower row visualizes primary cell isolation via enzymatic digestion, filtering, and seeding of cells. *Created with BioRender.com.*

Cells derived from primary culture often maintain characteristics of the cells *in vivo* and allows the application of biological replicates from different patients in experimental setups which strengthens the credibility of the obtained results. However, primary cells can only be passaged a limited number of times before they reach senescence, in contrast to

cell lines which can be propagated indefinitely. The drawback of cell lines is that there is a lower resemblance of the cells *in vivo*. The kidney-derived cell line Human Embryonic Kidney 293 (HEK293) was utilized in Paper I. In Paper IV, a conditionally immortalized human podocyte cell line was used which proliferates at 33°C and differentiates at 37°C (59).

Cellular treatments and assays

Molecular biological techniques have been developed to investigate the impact of gene and protein alterations in cultured cells. The forced introduction of foreign nucleic acids into eukaryotic cells can be categorized into transduction and transfection. Transduction describes the process where foreign DNA or RNA is introduced via a viral vector whereas transfection includes non-viral chemical or physical methods, such as liposome-mediated transfection and electroporation. For liposome-mediated transfection, free nucleic acids are encapsulated in liposomes which get delivered to the cell via endocytosis and subsequently released into the cytoplasm. Electroporation creates transient pores in the cell membrane using electricity where nucleic acids can enter the cell. In this thesis, transient transduction and transfection have been applied, resulting in temporary effects. For long-term studies, stable transduction and transfection can be performed where the foreign nucleic acids are integrated in the host cell genome. Gene overexpression was obtained by plasmid DNA transfection and adenoviral transduction in Paper I, II, and IV. In Paper I and IV, small interfering RNA (siRNA) transfection was used for gene knockdown. In Paper I, cells were also transfected with luciferase reporter plasmids to study the binding of the transcription factor SOX9 to promoter regions of potential target genes via measurement of luciferase activity.

The effect of gene and protein alterations can be studied using various cellular assays. One common method to study cell migration is the

wound healing assay. It is a simple and inexpensive assay where a scratch is created in a cell monolayer to mimic a wound. Thereafter, the rate of gap closure is followed by capturing images at regular time intervals or using a time-lapse microscope. The wound healing assay was applied in Paper I and IV to study the effect of SOX9 siRNA transfection in PTECs and WIPF3 transduction in the podocyte cell line, respectively. During the interpretation of the results from a wound healing assay, it is important to exclude any proliferative effects during gap closure. This can be avoided by culturing the cells in serum-reduced medium and performing the assay during a short timeframe. In turn, many commercial kits are available from which cell proliferation can be assessed. These usually include fluorescence measurement of dye bound to the DNA of the cells or a fluorescent product following the addition of a substrate relating to the metabolic activity of the cells.

Gene expression analysis

The investigation of gene expression in cells following treatment is essential for understanding the effect and underlying mechanisms. Quantitative polymerase chain reaction (qPCR), also known as real-time PCR, is a highly sensitive and specific technique that allows quantification of nucleic acids. Prior to qPCR, RNA must be reverse transcribed to complementary DNA (cDNA). This step is essential for accurate quantification since the amount of cDNA should reflect the input RNA. The qPCR reaction requires cDNA as template, primer pairs which target the genes of interest, DNA polymerase, and a fluorescent reporter that binds to the formed product. During the reaction, the DNA sequence of interest is amplified by the polymerase, resulting in a fluorescence signal, where the number of amplification cycles required to reach a specific threshold signal corresponds to the amount of template (60). qPCR is a useful method when a limited number of known genes is studied and was applied in Paper I, II, and IV.

To investigate the full transcriptome, RNA sequencing (RNA-seq) can be performed which was applied in Paper I and II to explore differentially expressed genes (DEGs) following *SOX9* knockdown and *HNF4A* overexpression, respectively. After RNA extraction, RNA is converted to cDNA using reverse transcription and a sequencing library is created from amplification of adapter-ligated cDNA fragments. RNA-seq produces millions of reads that are aligned to a reference genome and quantified. In contrast to qPCR, RNA-seq can be applied to identify both known and novel targets following gene alterations or treatments. However, it remains costly and produces large datasets which require bioinformatical skills for analysis.

Protein analysis and histological methods

Multiple methods can be utilized to detect and quantify the protein expression in tissue and cells. Western blotting is a widely used technique where proteins are separated on the basis of molecular weights using gel electrophoresis and transferred to a membrane. The membrane is incubated with primary antibodies for the protein of interest followed by incubation with secondary antibodies, usually labelled with the enzyme horseradish peroxidase (HRP). By the addition of substrate, a signal is generated for the protein of interest which is detected via chemiluminescence. Although Western blotting is a simple and specific method, it may require optimization of conditions and antibodies. Furthermore, Western blot analysis should be regarded as a semi-quantitative method since it only provides relative protein levels due to variations in loading, transfer, and signal between different blots (61). Western blotting was used in Paper I, II, and IV.

Another quantitative method for protein analysis is enzyme-linked immunosorbent assay (ELISA). In an ELISA, antigens are immobilized on a solid surface and allowed to bind to enzyme-labeled antibodies. After addition of substrate, the produced signal is measured relating to

the quantity of antigen. ELISA is a highly sensitive method, allowing detection of rare or low-abundance proteins, and was applied in Paper IV to quantify interleukin 8 (IL-8) levels in cell culture supernatants following *ARMH4* transduction and lipopolysaccharide (LPS) treatment.

Immunohistochemistry is a commonly applied method to detect protein expression in sections of FFPE tissue. Prior to staining, sections undergo deparaffinization, rehydration, and antigen retrieval by exposure to heat in an aqueous solution of either low or high pH. Thereafter, the primary antibody is added, followed by a linker to enhance the signal. Finally, an enzyme-labeled polymer, usually HRP-conjugated, is added followed by a chromogen acting as substrate. The most broadly used chromogen is 3,3'-diaminobenzidine (DAB), producing a brown-colored product, which was applied in Paper I, II, and IV. In Paper I and II, Magenta was also used as chromogen, resulting in a pink-colored product, in an immunohistochemical double staining together with DAB. Although immunohistochemistry is a non-quantitative method, it is advantageous since it allows identification of the tissue localization of the protein of interest.

The procedure of immunofluorescence is similar to the one for immunohistochemistry. However, instead of the addition of linker, enzyme, and substrate, a secondary antibody conjugated with a fluorophore is added. Immunofluorescence was applied in Paper III and IV. In Paper IV, immunofluorescence was performed on cryosections of fresh-frozen tissue. These were obtained by placing kidney tissue in a drop of Optimal Cutting Temperature (OCT) Compound on a piece of cork and freezing in isopentane cooled with liquid nitrogen, followed by sectioning using a cryostat.

Fresh-frozen tissue is also used for enzyme histochemistry. It visualizes the activity of an enzyme by metabolization of a substrate to a colored

product. In Paper III, two enzyme histochemical assays were applied to evaluate the activity of mitochondrial respiratory chain complexes. In the COX/SDH assay, a solution containing DAB, cytochrome c, and catalase is added, resulting in oxidation of DAB via COX, demonstrated by a brown product, indicating the activity of COX (complex IV). Then, a solution containing nitroblue tetrazolium (NBT) and sodium succinate is added. SDH oxidizes sodium succinate and reduces NBT to formazan, resulting in a blue product, visualizing the activity of SDH (complex II). Cells with functional activity of both complexes will be colored brown-blue, whereas cells with loss of COX activity will be colored blue (62). The NBTx assay allows a more sensitive detection of COX deficiency, where cryosections are exposed to a solution containing NBT, phenazine methosulphate (PMS), and sodium succinate. Cells with functional COX activity oxidize reduced PMS and remain colorless, while cells with COX deficiency reduce NBT, resulting in formation of formazan, a blue product (63). The reactions of the COX/SDH assay and NBTx assay are summarized in **Figure 6**.

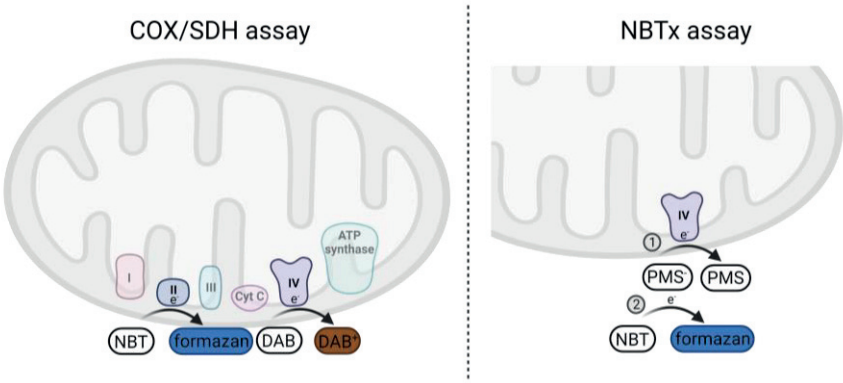

Figure 6. Enzyme histochemistry of the COX/SDH assay and NBTx assay. In the COX/SDH assay, complex IV (COX) oxidizes DAB into a brown product, while complex II (SDH) reduces NBT to formazan, a blue product. In the NBTx assay, PMS gets oxidized by complex IV (COX) resulting in a colorless product (1). However, if there is loss of COX activity, PMS will reduce NBT to formazan, a blue product (2). *Created with BioRender.com.*

Microscopical methods

Laser microdissection allows the visualization of a stained tissue section where a region of interest can be microdissected using a laser and catapulted by a laser pulse into a collection tube. The collected material can be used for downstream analysis of DNA, RNA, or protein. In Paper III, cells with the most intense NBTx staining were subjected to laser microdissection and DNA extraction. Whole genome sequencing followed by bioinformatic analysis via the MitoSAlt pipeline (64) was performed to identify mutations in the mitochondrial DNA (mtDNA).

The previously described histological methods require light microscopy or fluorescence microscopy to visualize the identified proteins and morphology of tissue and cells. By contrast, electron microscopy allows visualization of the ultrastructure of cells. In Paper II, a method was developed for correlative light and electron microscopy (CLEM), where cells of interest can be located by their protein expression using immunohistochemistry and light microscopy. The same sample can then be applied to electron microscopy to study the ultrastructure of the identified cells. However, the sample preparation for CLEM is challenging and requires optimization in terms of fixative and resin to preserve antigenicity as well as morphology. Furthermore, the coating substrate on slides needs optimization to be compatible for both light microscopy and electron microscopy. Transmission electron microscopy (TEM) was also utilized in Paper II to study the brush border of cultured HNF4A-transduced PTECs.

Online resources

With the emerging field of omics technologies, many projects have been initiated that provide open access to the collected data and the following resources have been utilized during the work in this thesis. The Human Protein Atlas (HPA; proteinatlas.org) catalogs human proteins and provides data including antibody-based imaging, proteomics,

and transcriptomics (65). JASPAR (jaspar.genereg.net) is a database storing manually curated transcription factor binding profiles (66). The Molecular Signatures Database (gsea-msigdb.org/gsea/msigdb) is a resource containing annotated gene sets for use during Gene Set Enrichment Analysis (GSEA) (67, 68). The Gene Ontology (GO) Resource (geneontology.org) (69, 70) and Kyoto Encyclopedia of Genes and Genomes (KEGG; genome.jp/keg) are databases of gene functions and for use during GSEA. The Genotype-Tissue Expression (GTEx) portal (gtexportal.org) provides data on tissue-specific gene expression and regulation across 54 non-diseased tissues. The NephroSeq database (nephroseq.org) collects renal gene expression profiles including diseased phenotypes.

Results and Discussion

The results in this thesis will be presented for each paper along with related discussion.

Paper I: The injury-induced transcription factor SOX9 alters the expression of *LBR*, *HMGA2*, and *HIPK3* in the human kidney

The transcription factor SOX9 is involved in organogenesis and development and is mainly known for its key role in chondrogenesis and male sex determination (71). During kidney development, SOX9 controls epithelial branching and maintenance of ureteric tip identity (72). Furthermore, SOX9 is activated as a response to kidney injury in murine models (44, 45, 73, 74). Following AKI, SOX9-positive cells repair the proximal tubules (44) as well as other nephron segments including loop of Henle, distal tubules, collecting ducts, and the parietal layer of the glomerulus (45, 73). Therefore, we aimed to establish the role of SOX9 in human kidney during injury and regeneration in Paper I (47).

SOX9 is expressed in scattered tubular cells and further induced by injury in human kidney

Using immunohistochemistry, we found that SOX9-positive cells were distributed in a scattered pattern throughout the proximal tubules in healthy human kidney. Immunohistochemical double staining for SOX9 and VIM, one of the original STC markers (35), showed colocalization of the SOX9-positive and VIM-positive cells. However, only about 77% of the VIM-positive cells were positive for SOX9, indicating that the STCs represent a heterogeneous population. This is in line with recent reports (54) which also identified four subclusters within

the STC population based on human kidney single-cell RNA-seq data from Muto *et al.* (53). Some of these subclusters showed higher activity in reparative pathways, while others were active in stress and profibrotic pathways (54). Muto *et al.* highlighted a VCAM1-positive population in their study and showed that it was related to the failed-repair proximal tubule cell population identified in mice (52). Furthermore, they found that the VCAM1-positive population shared marker composition with the STCs (53). However, SOX9 was not present among the differentially expressed genes in the VCAM1-positive population compared to the remaining PT cells, further strengthening the hypothesis that the STCs represent a heterogenous population and suggesting that SOX9 and VCAM1 are expressed in different subclusters of STCs.

We observed expanded SOX9 positivity from immunostainings of representative cases of kidneys undergoing AKI and CKD compared to healthy kidney. Findings from a recent publication are in agreement with this, where SOX9-positive cells were quantified to about 4% in healthy kidney and around 23% in AKI (75) based on human kidney single-cell RNA-seq data from Xu *et al.* (76). We also observed increased SOX9 expression in areas in proximity to immune cell infiltrates in otherwise healthy kidney tissue, and cytokine treatment of cultured PTECs displayed altered SOX9 expression. Data from qPCR showed that SOX9 was negatively regulated by IFN-γ and positively regulated by TGF-β. This finding was supported from results obtained both by treatment with TGF-β and an inhibitor of TGF-β, where SOX9 RNA expression responded accordingly in both cases. Previous studies have proposed TGF-β as a regulator of SOX9 in renal and atrial fibrosis (77, 78). Despite reports regarding TGF-β signaling in the promotion of fibrosis, TGF-β signaling was suggested to have protective effects during kidney injury in a recent publication (79). Indeed, excessive TGF-β signaling resulted in fibrosis, but inhibition worsened the outcome of tubular injury and fibrosis (79). Similarly, SOX9 was reported to be required for normal kidney repair in mice (44, 45), while

excessive and sustained SOX9 expression resulted in fibrosis (74). Taken together, this implies that TGF-β signaling and SOX9 expression play key roles during the early injury and repair response in kidney but should eventually attenuate to prevent fibrosis.

SOX9-positive cells contribute to kidney regeneration

Isolated and subcultured PTECs revealed induced SOX9 expression, as well as expression of other previously identified STC markers such as VIM and cytokeratin-7 (35, 38, 42). Based on this observation, we knocked down SOX9 using siRNA transfection of PTECs and performed a wound healing assay. The siSOX9-transfected cells displayed significantly slower migration rate compared to control PTECs, indicating that SOX9 is required for cell migration. Furthermore, RNA-seq followed by GSEA from siSOX9 transfection displayed activation of the terms "cell adhesion molecules" and "focal adhesion". Hence, these pathways are suppressed in SOX9-expressing cells which we connected to the promotion of cell migration.

Our explant model of AKI, composed of reoxygenated biopsies from resected human kidneys that had undergone ischemia due to intraoperative clamping, displayed survival of SOX9-positive cells. Over time, the SOX9-positive cells proliferated, as visualized by Ki-67 staining, and regenerated the proximal tubules of the kidney. This observation based on a human model is consistent with the findings from murine models of AKI, where SOX9-positive cells repaired the proximal tubular epithelium following injury (44, 45, 73, 74). Some of these studies also showed multilineage differentiation capacity of SOX9-positive cells by regeneration of loop of Henle, distal tubules, collecting ducts, and the parietal layer of the glomerulus, in addition to the proximal tubules (45, 73). Moreover, engraftment of urine-derived SOX9-positive cells in mouse models of unilateral ischemia-reperfusion injury reduced injured areas as well as serum creatinine levels (75). Nie *et al.*

also tested treatment with conditioned medium from cultured SOX9-positive cells in the same model and saw similar effects (75).

SOX9 alters the expression of *LBR*, *HMGA2*, and *HIPK3*

Given the induced SOX9 expression observed in cultured PTECs, we performed *SOX9* knockdown using siRNA transfection to study potential target genes and further understand the repair mechanism driven by SOX9. RNA from PTECs transfected with *SOX9* siRNA were sequenced followed by DEG analysis. All 70 significant DEGs were uniformly downregulated and were subjected to our downstream evaluation, summarized in **Figure 7**. The DEGs were examined by their histological expression via HPA where proteins expressed in a scattered pattern throughout the PT in kidney were of interest. Then, their promoter regions were subjected to SOX9 binding site prediction analysis via JASPAR. *LBR*, *HMGA2*, and *HIPK3* possessed these

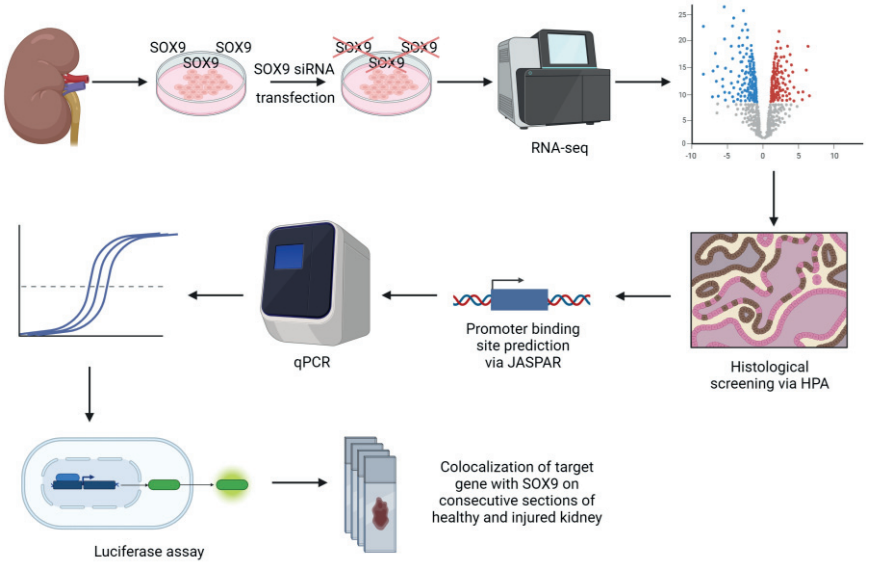

Figure 7. Workflow for identification of SOX9 target genes. SOX9 siRNA transfection was followed by RNA-seq, DEG analysis, histological screening via the Human Protein Atlas (HPA), promoter binding site prediction analysis via JASPAR, qPCR analysis, luciferase assay, and immunostaining of healthy and injured kidney to confirm colocalization. *Created with BioRender.com.*

features, and their RNA expression was significantly downregulated following siSOX9 transfection. However, *HIPK3* was the only one of these genes that was significantly upregulated following SOX9 overexpression via both plasmid DNA transfection and adenoviral transduction. We believe that lack of effect on *LBR* and *HMGA2* following SOX9 overexpression was due to saturated expression levels of SOX9 in the PTECs. Next, we constructed luciferase reporter plasmids with promoter regions of the examined genes which were used to transfect HEK293 cells with low endogenous SOX9 expression. With this approach, we observed increased luciferase activity in cells co-transfected with luciferase plasmids with promoter regions of *LBR* or *HIPK3* and *SOX9* plasmid DNA, respectively, which strengthens the notion of SOX9 binding and alteration of expression via these regions. Moreover, LBR, HMGA2, and HIPK3 displayed colocalization with SOX9 via immunostaining of consecutive FFPE sections of healthy and injured kidney. While SOX9 showed widespread positivity in representative cases of both AKI and CKD, LBR and HMGA2 were mainly expressed in the more severely injured tubules. By contrast, HIPK3 localized to the less injured tubules, suggesting different mechanisms for these genes. Collectively, we propose that the expression of *LBR*, *HMGA2*, and *HIPK3* is altered by SOX9 during kidney injury.

Other studies in mouse models of AKI presented *Wnp2*, *Sema3e*, *Myof*, *Gadd45a*, and *Vgf* as downstream targets of SOX9 (80-82). However, none of these genes were found among the DEGs in our data from *SOX9* siRNA transfection. This could be explained by multiple factors. In the present study, we identified potential target genes from PTECs derived from healthy human kidney with an induced injury profile given the expression of STC markers. In the studies by Kim *et al.*, the findings were based on murine models of AKI. Furthermore, the mechanism of SOX9 could potentially differ between human and rodents.

Paper II: HNF4A re-expression partially restores a proximal tubular phenotype in cultured primary kidney cells

Given the observations from Paper I (47), where we saw an induced STC phenotype in cultured PTECs, we were encouraged to study this transition process further. The transcription factor HNF4A controls key aspects of the differentiated PT including expression of genes associated with metabolism, transport, and brush border, a structure crucial for the absorptive function of PT cells (83, 84). On this basis, the aim of Paper II was to overexpress HNF4A in cultured PTECs and evaluate if this could restore the PT phenotype and suppress the STC phenotype.

The expression of HNF4A is reduced in scattered tubular cells

Immunohistochemical double staining of human kidney tissue for HNF4A and VIM demonstrated that most STCs, indicated by VIM positivity, expressed HNF4A. However, we also observed a subpopulation of VIM-positive cells with reduced expression of HNF4A. We hypothesized that the double-positive cells represented an intermediate state in the transition between STC and PT phenotype which led us to develop a method for CLEM to study the ultrastructural features of these cells. Interestingly, we found that the majority of VIM-positive cells had reduced brush border and fewer mitochondria, whereas a subpopulation displayed complete absence of brush border and mitochondria. This finding corresponded to our hypothesis, suggesting that cells lacking HNF4A display a more dedifferentiated STC phenotype, illustrated in **Figure 8**. In line with this, human kidney single-cell RNA-seq data from Muto *et al.* showed reduced HNF4A expression in VCAM1-positive cells (53). Furthermore, reduced activity of HNF4A was detected as part of the injury response in mouse models of AKI (48, 49). Notably, the VCAM1-positive maladaptive PT cells had lower expression of HNF4A compared to VCAM1-negative adaptive PT cells (49).

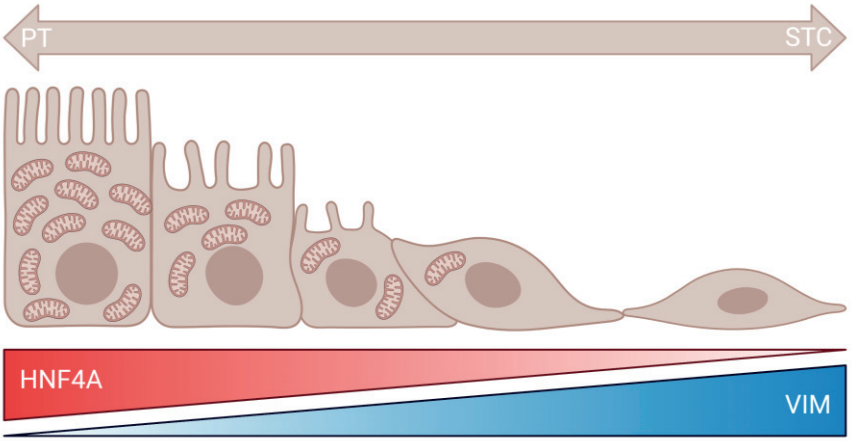

Figure 8. The transition from proximal tubular (PT) to scattered tubular cell (STC) phenotype. PT cells express HNF4A, display an organized brush border, and contain numerous mitochondria. In the transition to the STC phenotype, all these characteristic features are lost. Instead, the cells start to express VIM and other STC markers. *Created with BioRender.com.*

We previously identified an induced STC marker profile in cultured PTECs, including expression of SOX9, VIM, and cytokeratin-7 (47). To follow the transition process during primary cell isolation, we fixed cells directly after tissue dissociation, pre-seeding, and in the following passages. Immunostaining showed that HNF4A expression was instantly reduced after tissue dissociation, while cells from subsequent passages were HNF4A-negative. The expression of another marker for differentiated PT, CD10, was also reduced over time, but not as rapidly as HNF4A. By contrast, the induction of VIM expression was observed immediately after tissue dissociation.

The proximal tubular phenotype is re-established by HNF4A overexpression in cultured primary tubular epithelial cells

HNF4A overexpression via adenoviral transduction, in cultured PTECs followed by RNA-seq analysis, revealed 2033 significant DEGs. Further filtering ($P_{adj} < 10^{-8}$, $|\log_2$ fold change$| > 2$), literature search, and histological screening via HPA led us to focus on eight

genes (*AGMAT*, *ANKS4B*, *CDHR2*, *CDHR5*, *HPD*, *LRP2*, *MYO7B*, and *RARRES1*). The RNA levels of these genes were all confirmed to be upregulated following HNF4A overexpression via qPCR. Using Western blotting, six of the genes (*AGMAT*, *ANKS4B*, *CDHR2*, *HPD*, *LRP2*, and *RARRES1*) demonstrated increased protein levels in the HNF4A-transduced samples compared to non-detectable levels in the control samples. We did not identify any STC markers in the filtered list of DEGs after HNF4A transduction. After examining ten previously validated STC markers (*AKAP12*, *BCL2*, *CAV1*, *CD24*, *KRT7*, *PIGR*, *PROM1*, *SOX9*, *VCAM1*, and *VIM*) in our RNA-seq data, we determined that the expression of these markers was moderately altered by HNF4A transduction. *CD24* and *PROM1* (encoding CD133) were significantly downregulated following HNF4A overexpression, whereas *AKAP12* and *VCAM1* were upregulated, suggesting that HNF4A depletion alone is not sufficient to induce the STC phenotype.

GSEA of DEGs from HNF4A transduction highlighted terms associated with brush border where *ANKS4B*, *CDHR2*, *CDHR5*, *MYO7B,* and *LRP2* were included. This is in agreement with observations in rodents, where *Hnf4* was shown to drive the formation of brush border across epithelial tissues such as kidney and intestine (84). Other studies of the intestinal epithelium have presented the intermicrovillar adhesion complex (IMAC), situated at the distal tips of microvilli, as essential for brush border assembly and organization. Notably, ANKS4B, CDHR2, CDHR5, and MYO7B (5-7) are reported to be components of the IMAC, together with USH1C, CALM4, and MYO5B (8, 85, 86) which also were found to be upregulated in HNF4A-transduced cells from our RNA-seq data. Another IMAC located at the proximal base region of microvilli in intestinal epithelium, consisting of TMIGD1, EBP50, and E3KARP, has also been presented (87). However, these genes were not found among the DEGs in our data. Taken together, these findings suggest that the distal IMAC is driven by HNF4A in

kidney epithelium, but the proximal IMAC is probably regulated by other factors.

GSEA further revealed enrichment of gene sets related to absorption and transport. *LRP2*, which encodes the endocytic receptor megalin, was included in these gene sets as well as gene sets associated with brush border. In accordance with this, *LRP2* has been presented as a target gene of HNF4A (88) as well as a key regulator for ion transport, metabolism, and endocytosis (89). Long *et al.* also presented CUBN and DAB2 as additional components in the endocytic pathway and a recent publication showed that *CUBN* is a target gene of HNF4A (90). Furthermore, Yoshimura *et al.* generated HNF4A-knockout kidney organoids where RNA-seq and GSEA of these demonstrated results that mirrored ours, including gene sets related to lipid metabolism, transport, and brush border (90). However, *CUBN* was not found among the DEGs in our RNA-seq data. This suggests that *CUBN* might require activation of additional transcription factors or a specific niche, in consideration of the kidney organoids used by Yoshimura *et al.* versus two-dimensional culture of PTECs in our study.

Another gene set that was enriched from GSEA was amino acid metabolic processes which included both *AGMAT* and *HPD*. These genes encode enzymes involved in arginine metabolism and tyrosine catabolism, respectively, and are highly abundant in kidney. However, studies regarding their roles are lacking, although normal renal function seems to be disrupted upon loss of these enzymes. Decreased expression of *AGMAT* was observed in renal cell carcinoma (91, 92) and mice with a double deletion of *Fad* and *Hpd* that were given the intermediate metabolite between these two enzymes developed Fanconi syndrome, causing malfunction of PT reabsorption (93). Notably, Fanconi syndrome was also reported to occur following HNF4A deletion in mouse kidney (94).

RARRES1 (retinoic acid receptor responder 1) has previously been presented as a podocyte protein which gets upregulated during glomerular diseases (95, 96). However, retinoic acid signaling has shown beneficial effects during kidney injury and has proven to protect against tubular cell death, but side effects have also been described which may be caused by RARRES1 induction (97). We found that RARRES1 was upregulated by HNF4A and immunostainings from HPA demonstrated localization to the PT brush border, while no staining was detected in the podocytes, as shown in **Figure 9**. Thus, we propose that RARRES1 is indeed expressed in the PT of healthy kidney and that its expression in podocytes might be induced by injury.

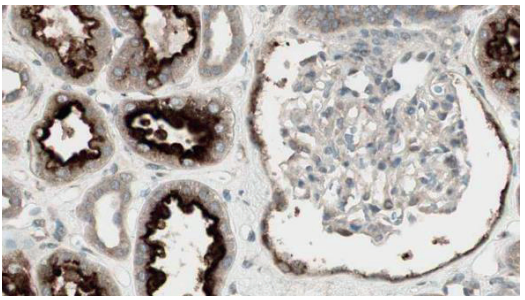

Figure 9. RARRES1 staining in healthy human kidney from the Human Protein Atlas. RARRES1 displays high positivity in the brush border of proximal tubules, while being negative in the podocytes of the glomerulus. Available from v23.proteinatlas.org/ENSG00000118849-RARRES1/tissue/kidney.

The ultrastructure of HNF4A-transduced PTECs was studied using TEM. From TEM imaging, a relatively developed brush border could be observed for some cells, while others were lacking a brush border completely, as shown in **Figure 10**. However, quantification of microvilli did not show any significant difference between cells transduced with Null or HNF4A adenovirus. Of note, the transduction efficiency was moderate. In our lab, we have detected 30-50% transduction efficiency and we could not differentiate between HNF4A-positive and negative cells in the TEM images. Immuno EM would allow us to account for HNF4A expression which could potentially demonstrate effects from HNF4A transduction in the development of brush border in two-dimensional cultured PTECs.

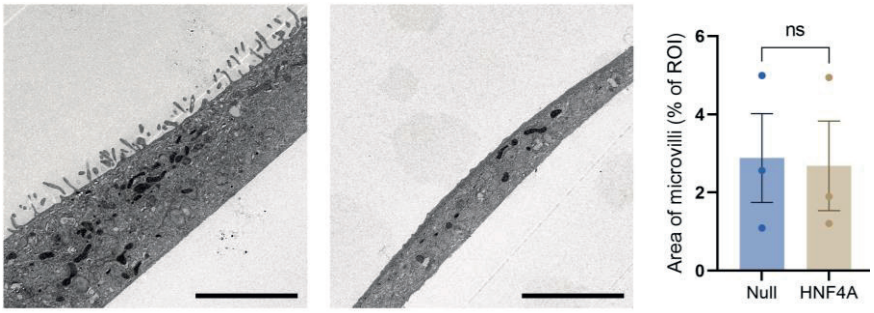

Figure 10. Transmission electron microscopy (TEM) of transduced cultured primary tubular epithelial cells (PTECs). Representative TEM images of cultured PTECs transduced with Null or HNF4A adenovirus. Some cells with developed brush border (left) were observed while others had completely diminished brush border (right). No difference between Null and HNF4A-transduced cells was detected from quantification of the area of microvilli in the region of interest (ROI) from ten randomly acquired images per sample (n = 3). Data are presented as means ± SEM. Statistical significance was determined by paired Student's t test. SEM, standard error of mean; ns, non-significant. Scale bars = 5 µm.

Paper III: Age and injury-related changes of mitochondrial respiratory complexes in human kidney tubules

The scattered tubular cells differ from the proximal tubular cells by loss of typical PT properties, including the organized brush border and the high density of mitochondria (35, 37). Loss of mitochondrial respiratory chain complexes has been reported in various organs including heart, brain, liver, and muscle. The frequency of loss in these organs was found to increase upon aging (98-105). In some cases, this was shown to be caused by mtDNA mutations (100, 104-106). In Paper III, we aimed to study the loss of mitochondria in STCs further and to examine if it could be connected to the deficiency of respiratory chain complexes observed in other organs.

Mitochondrial markers are reduced in scattered tubular cells

Using enzyme histochemistry in fresh-frozen kidney tissue, we evaluated the activity of respiratory chain complexes II and IV. The COX/SDH assay stained most cells brown-blue, indicating functional activity of both COX (complex IV) and SDH (complex II). However, some scattered cells were only stained blue, visualizing functional complex II but deficiency of complex IV. The observed pattern was consistent with the one reported in liver and colon, where the detected complex IV deficiency was explained by mtDNA mutations (104, 106).

Based on these observations, we subjected kidney cryosections to the NBTx assay, where the level of complex IV deficiency was displayed by different shades of blue. The results from the NBTx assay mirrored the ones from the COX/SDH assay but were visualized much more clearly. Next, the most intensely stained cells from the NBTx assay were laser microdissected to detect potential mutations in the mtDNA. Following DNA extraction and whole genome sequencing, the data was subjected to the MitoSAlt pipeline (64). However, preliminary data from two patients showed absence of mtDNA mutations in the

analyzed samples, suggesting that the complex IV deficiency observed in these scattered cells in the kidney is dissimilar from that described in liver, colon, and muscle (104-106). Interestingly, the laser microdissected cells showed higher mtDNA copy number compared to control. We hypothesized that this could be a compensatory mechanism as a reaction to the loss of mitochondrial content.

Using quadruple immunofluorescence, we observed complex IV-deficient cells, via MTCO1 staining, that also lacked expression of complex I, via NDUFB8 staining. These cells displayed reduced mitochondrial content, indicated by VDAC1 staining, and were positive for VIM, demonstrating that they were indeed the previously identified STCs involved in kidney injury and regeneration. Taken together, we believe that the complex IV-deficient cells detected by the COX/SDH assay are STCs. Despite the reduced mitochondrial density in the cells, they might have residual expression which could explain the functional activity of complex II, detected by the COX/SDH assay. Complex IV-deficient cells have been observed in rat kidney via the COX/SDH assay and these cells harbored mtDNA mutations (107). However, these complex IV-deficient cells displayed cell enlargement and nuclear displacement, suggesting that they are different from the human STCs as well as the complex IV-deficient cells described in other tissues (104-106).

Deficiency of complex I increases upon aging in human kidney

Quadruple immunofluorescence also revealed another cell population in the PTs that was deficient of complex I but expressed complex IV. These cells had similar level of VDAC1 staining as that seen for the neighboring PT cells with intact complex I, indicating normal mitochondrial density. Adapting the quadruple immunofluorescence and including the PT marker Lotus Tetragonolobus Lectin (LTL) allowed quantification of the NDUFB8-negative PT cells as well as VIM-

positive PT cells. Given that the frequency of complex I deficiency increased with age in other organs (99, 101-103), we subjected kidney tissue from patients of various ages (1-88 years) to the adapted quadruple immunofluorescent assay and found that a similar situation could be observed in kidney. By linear regression analysis, we demonstrated that the VIM-positive cell population increased upon aging as well and correlation analysis indicated positive, although weak, correlation between the two cell populations. By binning the data into age groups, the increase in complex I deficiency was even more apparent, with complete absence of complex I deficiency in individuals between ages 1-15. However, the difference between VIM-positive PT cells in age groups was non-significant. From this, we interpret complex I deficiency to be involved in the aging process of kidney. Mitochondrial dysfunction is regarded as a hallmark of aging (108, 109) and complex I deficiency has been suggested to occur initially which over time advances to loss of the other complexes (110). The loss of mitochondrial markers in VIM-positive STCs are not necessarily associated with complex I deficiency since the correlation was weak and the increase with age was not as evident in comparison.

Paper IV: Identification of ARMH4 and WIPF3 as human podocyte proteins with potential roles in immunomodulation and cytoskeletal dynamics

The glomeruli contain podocytes which are highly specialized epithelial cells essential for the filtration barrier. During kidney disease, podocytes are often targeted, resulting in impairment of their structure and renal dysfunction (111). Hence, podocyte proteins are important to identify since they may be useful for therapeutic purposes. In Paper IV (112), we aimed to detect novel podocyte markers involved in kidney injury and disease.

ARMH4 and WIPF3 are novel podocyte markers

Using publicly available resources and a multi-step computational approach summarized in **Figure 11**, we aimed to identify podocyte proteins. As a first step, kidney cortex RNA-seq data from the GTEx portal, in the form of transcripts per million, for the previously known podocyte markers *NPHS1*, *NPHS2*, *PTPRO*, and *PLA2R1* were correlated with all other transcripts. By sorting the resulting lists based on the Pearson R-values and cross-referencing the top hits, numerous known podocyte markers were represented in the final overlay. Since the aim was to find novel podocyte markers, we focused on genes lacking any previous literature in a PubMed search of "gene symbol" AND "podocyte". Furthermore, the gene products were screened using HPA where a podocyte staining pattern was desired. Since the glomeruli are located in the kidney cortex, the genes were further filtered by cortical enrichment using cortex and medulla transcripts from the GTEx portal. In a last step, the remaining genes were cross-referenced with genome-wide association study (GWAS) hits for lupus nephritis (113) and albuminuria (114). ARMH4 was a hit for lupus nephritis, while WIPF3 was a hit for albuminuria, suggesting pathological relevance for both proteins which led us to focus on these in our independent confirmation of podocyte localization.

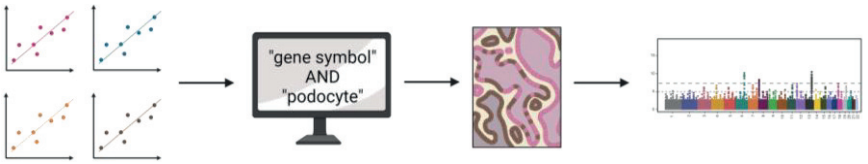

Figure 11. Computational approach for identification of podocyte markers. RNA-seq data from GTEx portal was correlated for the known podocyte proteins *NPHS1*, *NPHS2*, *PTPRO*, and *PLA2R1* and combined, resulting in a list of potential novel podocyte markers. The list was screened using PubMed, where no hits for the search "gene symbol" AND "podocyte" was desired. Screening by glomerular enrichment was performed using the Human Protein Atlas (HPA) for histological expression and cortical versus medullar enrichment using data from the GTEx portal. Pathological relevance was checked by cross-referencing the potential markers against GWAS for lupus nephritis and albuminuria. *Created with BioRender.com.*

To histologically confirm glomerular localization of ARMH4 and WIPF3, we used both immunohistochemistry and immunofluorescence. In the latter case, we also included staining for NPHS2 and WT1, two well-established podocyte markers, which showed positivity for the same cells as the two novel markers. ARMH4 displayed a perinuclear staining, whereas WIPF3 stained the cytoplasm of the podocytes. Results from immunohistochemistry mirrored the ones from immunofluorescence. Moreover, glomerular isolation followed by protein extraction and Western blotting showed enrichment of ARMH4 in glomeruli compared to kidney cortex. ARMH4 staining was also intensified in an immortalized podocyte cell line upon differentiation (59). However, WIPF3 could not be detected, and we proposed that the podocyte cell line might not reach fully mature differentiation. To further confirm their presence in podocytes, we overexpressed the podocyte transcription factor LMX1B in PTECs. Using this approach, we obtained upregulation of both ARMH4 and WIPF3.

ARMH4 shows anti-inflammatory properties

There are previous reports regarding ARMH4 as a negative regulator of AKT (115) and STAT3 signaling (116) via mTORC2 interaction, indicative of a suppressive role in cytokine signaling. In the light of these findings, we overexpressed ARMH4 in PTECs and stimulated

the cells with LPS. Using this approach, we observed reduction of the proinflammatory cytokines IL-1β and IL-8 at the RNA level. In addition, we found that IL-8 protein levels were decreased after ARMH4 overexpression and LPS treatment using Western blotting and ELISA. Conversely, IL-1β transcripts were increased following ARMH4 knockdown via siRNA transfection. Moreover, ARMH4 expression was induced upon differentiation in the podocyte cell line, along with reduction of IL-1β and IL-8 expression. Collectively, these observations suggest an anti-inflammatory role of ARMH4. This is in line with previous reports regarding a suppressive role in cytokine signaling (116) as well as GWAS linking ARMH4 with lupus nephritis (113). Furthermore, we found that ARMH4 was reduced in glomerular diseases via glomerular gene profiles from NephroSeq and immunostaining of renal biopsies from kidney disease patients. Taken together, we hypothesize that loss of ARMH4 contributes to inflammation in glomerular diseases.

WIPF3 regulates cytoskeletal dynamics

Previous studies have presented a complex consisting of WIPF3 and N-WASP involved in actin nucleation (117, 118). Importantly, N-WASP is essential for maintenance of podocyte foot processes and their actin network (119). Moreover, WIPF3-knockout mice show reduction of N-WASP protein in testis (120). On this basis, we overexpressed WIPF3 in both PTECs and podocytes and found an increase in N-WASP protein level. Furthermore, we observed that the intensity of N-WASP increased with WIPF3 overexpression, indicating stabilization of N-WASP by WIPF3. WIPF3 overexpression followed by a wound healing assay resulted in increased cell migration, suggesting a role for WIPF3 in cytoskeletal dynamics. Together with GWAS reporting association between WIPF3 and albuminuria (114), we speculate that WIPF3 stabilizes the cytoskeletal architecture of podocytes which is important for sustaining the glomerular filtration barrier.

Conclusions

The main objective of this thesis was to improve the understanding of the mechanisms of human kidney injury and regeneration by focusing on investigation of the scattered tubular cell phenotype. From Paper I and II, we found that the STC phenotype is indeed inducible in PT cells. Differentiated PT cells were capable of adopting an STC phenotype during cell culture, possibly as an injury response induced by the stress from the cell culture milieu. We also established SOX9 as an STC marker and suggested that the STCs represent a heterogenous population in Paper I. Together, these results indicate that the STCs are de-differentiated PT cells, rather than a pre-existing progenitor population residing in the human kidney.

In Paper I, SOX9-positive cells demonstrated a role in human kidney injury and regeneration, where they showed resistance to injury and proliferative capacity in an explant model of AKI based on human kidney biopsies as well as increased migration rate in a wound healing assay. Moreover, factors acting both upstream and downstream of SOX9 in PT cells were identified. Cytokine treatment resulted in altered SOX9 expression, where TGF-β induced and IFN-γ suppressed SOX9 expression, respectively. *LBR*, *HMGA2*, and *HIPK3* were proposed as downstream targets of SOX9 and potential actors during kidney injury and regeneration.

In Paper II, re-expression of HNF4A in cultured PTECs resulted in partial reversal to the PT phenotype, including expression of genes related to metabolism, transport, and brush border formation. We concluded that HNF4A depletion could induce the expression of some

STC markers, but that other factors are required for development of the full STC phenotype.

In Paper III, we demonstrated that the STCs have a general reduction of mitochondrial markers. Moreover, we identified another cell population in the PT with selective deficiency of mitochondrial respiratory chain complex I. Complex I deficiency was absent in children but increased in frequency upon aging. Interestingly, we observed positive correlation between the STCs and the complex I-deficient cell population, albeit weak.

In Paper IV, we presented ARMH4 and WIPF3 as novel podocyte proteins with roles in kidney injury and disease. ARMH4 displayed anti-inflammatory properties, in line with GWAS linking ARMH4 with lupus nephritis. In turn, WIPF3 overexpression demonstrated a role in stabilization of N-WASP, a protein essential for the actin cytoskeleton of podocytes, indicating a role for WIPF3 in maintenance of the glomerular filtration barrier via stabilization of podocyte foot processes. This notion was further strengthened by GWAS linking WIPF3 with albuminuria.

Future Perspectives

The findings from this thesis support the hypothesis that the scattered tubular cells represent dedifferentiated PT cells. Indeed, the STC phenotype can be induced in PT cells, but the question regarding the origin of the STCs found in healthy human kidney remains unanswered. STCs are found less frequently in rodents, but in the studies where they could be detected, cells that expressed STC markers *de novo* were shown to contribute more actively to kidney repair. This raises the possibility that the STCs represent a resident progenitor or stem cell population but that other surviving PT cells adopt the STC phenotype in response to injury and that these describe two different repair mechanisms of the human kidney. Another possibility is that STCs appear due to microinjuries in otherwise healthy kidneys. Lineage tracing and cell fate tracking studies in rodents imply that dedifferentiation of PT cells is the main repair mechanism of the kidney. Single-cell RNA-seq of human kidney have further strengthened this idea by demonstration of different cellular states or subclusters within the injured or repairing cell populations, indicative of a transient phenotype for the STCs. However, further research is required to fully validate this.

In this thesis, we present two transcription factors of importance for kidney injury and regeneration. SOX9 is activated in response to injury, whereas HNF4A is suppressed. We believe that the transition between PT and STC phenotype is essential to understand and must be controlled for successful repair. SOX9-positive cells were more resistant to injury and capable of proliferation to repair the renal tubules. However, persistent SOX9 expression was reported to result in maladaptive repair and fibrosis. Hence, we propose that HNF4A expression should be introduced after the early injury response to partially suppress the

STC phenotype and allow redifferentiation to the PT phenotype to successfully regenerate the renal tubules. Future studies are needed to test this hypothesis and possibly find a suitable timepoint for HNF4A re-expression.

Primary tubular epithelial cells are commonly used as a model system, although lacking expression of typical PT markers. We demonstrated that HNF4A re-introduction via adenoviral transduction restored aspects of the differentiated PT phenotype in two-dimensional cultured PTECs. However, we did not observe morphological effects of HNF4A transduction, e.g. brush border development. For future improvements, we suggest the combination of HNF4A transduction and culture on Transwell inserts or organoid culture to further improve the resemblance of the PT phenotype *in vivo*.

The observations regarding loss of mitochondrial respiratory chain complexes in the PT of human kidney should be further investigated, importantly in the aspect of whether they are caused by mtDNA mutations. The complex I-deficient cell population show a possible role in renal aging but could also have implications for the development of the STC phenotype with regard to the reduced mitochondrial content observed in the STCs.

ARMH4 and WIPF3 were presented as novel podocyte proteins with roles in inflammation and cytoskeletal dynamics, respectively. The findings were mainly based on *in vitro* experiments, although some functional assays were included. Thus, these results should be complemented with *in vivo* studies of ARMH4 and WIPF3.

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