UKD Universitätsklinikum Düsseldorf

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Modelling nephrogenesis and kidney-associated diseases using urine-derived kidney stem cells and iPS cells

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Düsseldorf, März 2023

aus dem Institut für Stammzellforschung und Regenerative Medizin
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Berichterstatter:

1. Prof. Dr. James Adjaye

2. Prof. Dr. Matias Zurbriggen

Tag der mündlichen Prüfung: 23.08.2023

Die vorliegende Arbeit ist eine kumulative Dissertation
gemäß § 6 (3) der Promotionsordnung der
Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf vom 15.08.2018
zur Verleihung des Grades „Doctor rerum naturalium“

Acknowledgements

First, I deeply thank my supervisor Prof. Dr. James Adjaye for giving me the opportunity to be part of his research group at the Institute for Stem Cell Research and Regenerative Medicine. When I was still an undergraduate student, he welcomed me in his institute and introduced me to the exciting topic of stem cell biology. Thanks to him, I discovered that I wanted to be a stem cell biologist. He supported me during my bachelor and master studies and believed in me, when I was myself not sure, if I was capable of doing my doctoral degree. During my whole scientific life, he showed me different views on the use of stem cells and infected me with enthusiasm and fascination for the kidney organs. Moreover, I am very grateful to Prof. Dr. Matias Zurbriggen as well for accepting my request for co-supervision a second time after already co-supervising my master thesis.

I thank my family, my mother, Thi Mặc Bích Lý, my father, Hoàng Dũng Nguyễn**,** and my sister, Thiên Đức Sylvia Nguyễn**,** for supporting me the whole time. Even without any scientific background, they shared my excitement after each interesting observation and comforted me when things were not going as expected or planned. I could be patient and endure the stress with them strengthening my back. Now I am finally crossing the finishing line and I understand that it is ok when things you have planned out will turn out differently.

I would also like to thank my friends, my second family. They were my personal cheerleaders and them being proud of me, made me trust my own capabilities. When I needed to let out my frustration, they always had an open ear for me and eased my mind when I was struggling.

I thank the ISRM lab member for supporting me during my PhD years. I would like to send a special thank you to the Postdocs Dr. Nina Graffmann and Dr. Lars Erichsen, as they shared their expertise with me, and I could always ask for their opinion and advice. Thank you for creating a supportive and warm environment, Abida Islam Pranty, Christiane Lörch, Chantelle Thimm, Leon-Philip Szepanoswski, Vanessa Cristina Meira de Amorim, Silke Wehrmeyer, Martina Bohndorf and Dr. Wasco Wruck. On the long way of my studies, some of my colleagues became my friends. More than once, I experienced mental breakdowns from stress. Talking to my colleagues and friends about my struggles was comforting and always gave me a new energy boost to keep going. Working side by side but also having random discussions in the office or during lunch break was always delightful for me.

Lastly, I thank all the collaborators from the Heinrich-Heine University, who worked together with me and let me gain insights into their way of research.

My doctoral studies will end with this thesis, but I will be forever grateful for the opportunity to grow as a person and broaden my own horizon. This is just the beginning and I want to keep improving myself and my research to contribute to the scientific future.

Table of Contents

List of Figures

List of Tables

Abbreviations

Abbreviations	Definition
2D	two-dimensional
3D	three-dimensional
3D-NPCs	three-dimensional nephrogenic progenitor cells
ADPKD/PKD	autosomal dominant polycystic kidney disease
AGTR1/2	angiotensin receptor 1/2
AKI	acute kidney injury
ANGII	angiotensin II
ANPEP	Aminopeptidase N
AQP1/2	aquaporin 1/2
ASC	adult stem cell
ATP	adenosine triphospate
B2M	Beta-2-Microglobulin
BMP	bone morphogenetic protein
BUN	blood urea nitrogen
Cas9	CRISPR-associated 9
CD	Cluster of differentiation
CITED1	Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 1
CKD	chronic kidney disease
CLDN10	Claudin 10
CLU	Clusterin
CM	cap mesenchyme
c-MYC/c-Myc	MYC Proto-Oncogene
CRISPR	Clustered Interspaced Short Palindromic Repeats
CSB	Comma-shaped body
CysC	Cystatin C
DNA	Desoxyribonucleic acid
DPP4	Dipeptidyl Peptidase 4
DT	distal tubule
ECM	extracellular matrix
ESRD	endstage renal disease
FABP	fatty acid binding protein
FGF	fibroblast growth factor
FOXD1	Forkhead Box D1
Gb3	glycolipid globotriaosylceramide
GBM	glomerular basement membrane
GDF	growth differentiation factor
GDNF	Glial Cell Derived Neurotrophic Factor
GFP	green fluorescent protein
GFR	glomerular filtration rate
GFRα1	GDNF Familiy Receptor Alpha 1
GO	Gene ontology

GSH	glutathione
GvHD	Graft versus Host Disease
H/R	hypoxia/reoxygenation
HEK	human embryonic kidney
hESC	human embryonic stem cell
HK2	human kidney-2 cells
HLA	human leukocyte antigen
HLA-DR	human leukocyte antigen-DR
hUSC	human urine stem cells
I/RI	Ischemia/reoxgenation injury
iECS	iPSC-derived endothelial cell
IM	intermediate mesoderm
iMSC	iPSC-derived mesenchymal stem cell
iPSC	induced pluripotent stem cell
JNK	c-jun terminal kinase
KDR	kinase insert domain receptor
KIM-1	kidney injury molecule 1
KLF4/Klf4	KLF transcription factor 4
LoH	Loop of Henle
MAPK	mitogen-activated protein kinase
MET	mesenchymal to epithelial transition
MIS	müllerian inhibiting substance
MM	metanephric mesenchyme
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
ND	nephric duct
NGAL	Lipocalin 2
NPC	nephron progenitor cell
NPHS1	Nephrin
NPHS2	Podocin
NR3C2	Nuclear Receptor Subfamily 3 Group C Member 2
OCT4/Oct4	Octamer-Binding Protein 4
OPN	Osteopontin
PAN	Puromycin Aminonucleoside
PAX8	Paired Box 8
PC-1	Polycystin-1/2
PDGFR-β	Platelet Derived Growth Factor Receptor Beta
PHA	phytohemagglutinin
PI3K	phosphoinositide-3-kinase
PLCγ	phospholipase C gamma
PS	primitive streak
PSC	pluripotent stem cell
PSTC	Predictive Safety Testing Consortium
PT	proximal tubule
PTA	pre-tubular aggregate

PTEC	proximal tubule epithelial cell
RAAS	renin-angiotensin-aldosterone system
RC	renal corpuscle
RET	Ret Proto-Oncogene
ROS	reactive oxygen species
RV	renal vesicle
SALL1	Spalt Like Transcription Factor 1
sCr	serum creatinine
SIX2	SIX Homeobox 2
SLC12A1	Solute Carrier Family 12 Member 1
SMAD	Mothers Against Decapentaplegic Homolog
SOX2/Sox2	SRY-Box Transcription Factor 2
SSB	S-shaped body
SSEA-3/4	Stage-specific embryonic antigen 3/4
TFF-3	Trefoil Factor 3
TGFβ	Transforming growth factor beta
TRA-1-60	T-cell receptor alpha locus 1-60 epitope
TRA-1-81	T-cell receptor alpha locus 1-81 epitope
uALB	urinary Albumin
UB	ureteric bud
UdRPC	Urine-derived renal progenitor cell
uTP	urinary total protein
WHO	World Health Organization
WNT	Wingless Type
β-TCP	beta tricalcium phosphate

Abstract

Considering kidney diseases are one of the top leading causes of mortality worldwide, novel therapeutic means instead of the conventional renal replacement therapies are urgently needed. Modelling diseases in an *in vitro* setting is hereby essential to identify disease targets and to push forward the development of potential drugs and treatments. Notably for discovering new medications, urine represents an alternative cell source for renal epithelial and renal stem cells, which can be isolated in a non-invasive and cost-effective way. The multipotent urine stem cells have a broad differentiation potential to renal cell types such as glomerular podocytes and tubular cells but can also be used for iPSC reprogramming. In regard to kidney diseases, glomeruli and proximal tubules within the nephrons are most likely to be damaged, which makes them intriguing for *in vitro* studies on kidney diseases.

In the first study, an isogenic three-dimensional nephrogenic progenitor cell (NPC) model was established using three distinct cell types including UdRPCs, iPSC-derived mesenchymal stem cells and iPSC-derived endothelial cells. The cells differentiated from iPSCs were characterized and hallmarks of mesenchymal stem cells and endothelial cells were identified. Combining the three mentioned cell types at a set ratio resulted in self-condensed three-dimensional structures, referred to as 3D-NPCs. The 3D models were further studied for expression of renal progenitor cell (SIX2, PAX8), podocyte (Nephrin, Podocin) as well as mesenchymal stem cell (Vimentin, PDGFR-β) and endothelial cell (CD31) markers. Since the 3D-NPCs share kidney progenitor characteristics, they have the potential to differentiate into cells of the nephron structure including glomerular podocytes and tubular cells. UdRPCs can be easily isolated from shed urine, therefore, these cells have a promising potential as a tool for nephrotoxicity tests, drugs screenings and disease modelling.

The work described in the second publication aims to model kidney injury on implemented iPSC-based kidney organoids. Often affected by changing cellular conditions are the nephron structures such as the glomeruli and tubules, which are relevant for blood filtration and water and salt re-absorption. By employing Puromycin Aminonucleoside (PAN), kidney injury was induced in the kidney organoids and the effects were evaluated. Findings of this work included the structural disruption of renal glomeruli and tubules within the kidney organoids, DNA damage as well as upregulated gene expression of immune responses such as inflammation and cell death-related processes. In summary, kidney injury mechanisms including anti- and

pro-inflammatory processes, re-arrangement of glomerular cytoskeleton, DNA damage and cell death can be modelled via PAN-induced iPSC-based kidney organoids. This work demonstrated the possibilities to mimic pathological kidney conditions for the cause of disease modelling and the potential use in drug screening and establishment of novel therapies.

The third study portrays the gained insights into signalling pathways involved in the maintenance of self-renewal in multipotent urine-derived renal progenitor cells (UdRPCs). Previous studies have unveiled the role of JNK signalling in the preservation of self-renewal in nephron progenitor cells (NPCs). Due to the similarities between NPCs and UdRPCs, JNK was inhibited in UdRPCs with the small molecule AEG3482 and resulting effects were analysed. Observations included reduced proliferation, shown in transcriptional downregulation of cell cycle-related genes and downregulation of kidney progenitor genes including SIX2, CITED1 and SALL1. JNK inhibition also reduced the level of activated SMAD2/3, which is also involved in the maintenance of self-renewal. Moreover, interference with the JNK pathway resulted in less efficient oxidative phosphorylation and increased lipid peroxidation via the non-apoptotic cell death ferroptosis. Ferroptosis is linked to various forms of kidney disease and with this study, the relevance of JNK signalling for the protection against ferroptosis as well as an involvement in self-renewal was demonstrated. UdRPCs treated with JNK inhibitor are potentially useful for modelling ferroptosis-induced kidney diseases.

This thesis describes the benefits of urine-derived stem cells and three-dimensional kidney organoids for research on kidney diseases with the aim of aiding the future development of new medications and therapy forms.

Zusammenfassung

Weltweit gehören Nierenerkrankungen zu den führenden Todesursachen, weshalb die Entwicklung von neuartigen Therapiemöglichkeiten zu den konventionellen Nierenersatzverfahren eine große Dringlichkeit hat. Um Angriffspunkte von Krankheiten zu identifizieren, ist es essentiell diese *in vitro* zu modellieren, um dadurch die Entwicklung von potentiellen Medikamenten und Behandlungen voranzutreiben. Eine vielversprechende Quelle um renale Epithel-und renale Stammzellen auf nicht-invasive und kostensparende Weise zu gewinnen, ist der Urin. Multipotente Urinstammzellen haben ein breites Differenzierungspotential; sie können zu Nierenzelltypen wie glomerulären Podozyten und tubulären Zellen differenziert und ebenfalls für iPSC Reprogrammierung genutzt werden. Nierenerkrankungen beschädigen besonders die Glomeruli und proximalen Tubuli der Nephrone, weshalb diese Strukturen interessant für in *vitro* Studien sind.

In der ersten Studie wurde ein dreidimensionales, isogenes Nephron Progenitorzellmodell bestehend aus drei distinkten Zelltypen wie renalen Progenitorzellen aus dem Urin, aus induzierten pluripotenten Stammzellen (iPSC)-differenzierten mesenchymalen Stammzellen und aus iPS Zellen-differenzierte Endothelzellen, generiert. Die aus iPS-Zellen differenzierten Zellen wurden charakterisiert und Merkmale von mesenchymalen Stammzellen und Endothelzellen wurden beobachtet. Die Kombination der drei genannten Zelltypen zu einem festgelegten Verhältnis resultierte in selbst-kondensierten, dreidimensionalen Strukturen, die als 3D-NPCs bezeichnet wurden. Die 3D-Modelle wurden tiefergehend auf die Expression von Markern untersucht, die mit renalen Progenitorzellen (SIX2, PAX8), Podozyten (Nephrin, Podocin) sowie mesenchymalen Stamm- (Vimentin, PDGFR-β) und Endothelzellen (CD31) assoziiert sind. Da die 3D-NPCs Gemeinsamkeiten mit Nierenprogenitorzellen teilen, haben sie das Potential zu Nephronzellen wie glomeruläre Podozyten und tubuläre Zellen zu differenzieren. UdRPCs können problemlos aus ausgeschiedenem Urin isoliert werden und haben daher ein vielversprechendes Potential als Werkzeug für Nephrotoxizitätstests, Medikamenten-Screenings und der Modellierung von Krankheiten.

Die beschriebene Arbeit der zweiten Publikation zielt darauf ab Nierenverletzung in iPSC-basierten Nierenorganoiden zu modellieren. Besonders von sich verändernden zellulären Bedingungen betroffen, sind die Strukturen, welche für Blutfiltrierung und Re-Absorption von Wasser und Salzen zuständig sind, darunter die Nephronstrukturen der Glomeruli und Tubuli. Durch die Verwendung von Puromycin Aminonucleoside (PAN) wurde Nierenverletzung in den

Nierenorganoiden induziert und nachfolgende Effekte analysiert. Ergebnisse dieser Arbeit beinhalten strukturelle Zerstörung von renalen Glomeruli und Tubuli innerhalb der Nierenorganoide, DNA Schäden sowie die hochregulierte Genexpression von mit einer Immunantwort-verbundenen Prozesse wie Entzündung und Zelltod. Zusammenfassend kann man sagen, dass zu Nierenverletzung führende Mechanismen wie entzündungshemmende und entzündungsfördernde Prozesse, die Umgestaltung des glomulären Cytoskeletts, DNA-Schäden und Zelltod in aus iPSC-differenzierten Nierenorganoiden durch PAN Induktion modelliert werden können. Diese Arbeit zeigt die Möglichkeiten auf, durch das Imitieren von pathologischen Nierenkonditionen, Krankheiten zu modellieren und die potenzielle Verwendung in Medikamenten-Screenings und die Etablierung von neuen Therapien.

Die dritte Studie gibt tiefere Einblicke in die Signalwege, welche an der Erhaltung der Selbsterneuerung von multipotenten aus Urin gewonnenen renalen Progenitorzellen (UdRPCs) beteiligt sind. Frühere Studien deckten die Rolle des JNK-Signalwegs im Aufrechterhalten der Selbsterneuerung in Nephron Progenitorzellen (NPCs) auf. Daher wurde das JNK Protein in UdRPCs mit dem *„small molecule*“ AEG3482 inhibiert und die daraus resultierenden Effekte wurden analysiert. Gezeigt wurden eine reduzierte Proliferation, ebenfalls sichtbar in der herunterregulierten Expression von Zellzyklus-relevanten Genen und der Herunterregulierung von Nierenprogenitor-assozierten Genen wie SIX2, CITED1 und SALL1. Das Proteinlevel von aktivierten SMAD2/3 wurde durch JNK Inhibierung reduziert, welches auch an der Erhaltung der Selbsterneuerung involviert ist. Darüber hinaus, führte das Inhibieren von JNK zu einer verminderten Effizienz der oxidativen Phosphorylierung und einer erhöhten Lipidperoxidation via des nicht-apoptotischen Zelltods Ferroptose. Ferroptose ist mit verschiedenen Formen von Nierenkrankheiten assoziiert und in dieser Studie wurde veranschaulicht, dass JNK Signale gegen Ferroptose schützen und dabei in der Selbsterneuerung involviert ist. UdRPCs, welche mit dem JNK Inhibitor behandelt wurden, stellen daher einen Weg zur Modellierung von Nierenerkrankungen, welche durch Ferroptose ausgelöst werden, dar.

In dieser Thesis werden die Forschungsergebnisse über zelluläre Prozesse in Urinstammzellen und die Möglichkeiten der *in vitro* Modellierung von Krankheiten in dreidimensionalen Nierenorganoiden beschrieben, welche für die Entdeckung von neuen Medikamenten und Therapien benutzt werden können.

1. Introduction

1.1 The Kidney

1.1.1 Kidney anatomy

The kidneys are a pair of excretory organs essential for maintaining the balance of various metabolic processes. Kidney functions include excretion of toxins and waste products as well as regulation of water and salt homoeostasis (Gopalan & Kirk, 2022). Moreover, the kidneys have endocrine functions, as they secrete hormones and enzymes, are involved in the adjustment of blood pressure via renin-angiotensin-aldosterone system, in the production of erythrocytes as well as the regulation of calcium and phosphate levels (McMahon, 2016).

The bean-shaped organ pair is located in the abdominal region on each side of the spine with the adrenal glands on top (Allan, 2011; Gopalan & Kirk, 2022). Human kidneys consist of several lobes, and they can be divided into two layers, the fibrous renal capsule, which covers the renal parenchyma. The parenchyma is sub-divided into the cortex and the medulla (Fig. 1A, B). Embedded within the medulla are conical regions, called renal pyramids, which are separated by renal columns that run across the cortex and the medulla. The pyramids are connected to several pools, the renal calyces (single: calyx), and end in the renal pelvis emptying into the ureter. The ureter connects to the bladder, where urine is collected until excreted.

The smallest functional units of the kidney are the nephrons, which are generated during a developmental process called nephrogenesis (*see* chapter 1.1.3). Each nephron can be divided into two major compartments: the renal corpuscle and the renal tubule system (Fig. 1C). An adult has approximately one million nephrons, whereas the development of the nephron is completed before birth with post-natal maturation. The renal cortex contains the glomeruli and parts of the proximal tubules, while the medulla houses the remaining part of the proximal as well as the distal and collecting tubules, which connect to the renal calyx for the transport of urine (Krause et al., 2015; McMahon, 2016) (Fig. 1B).

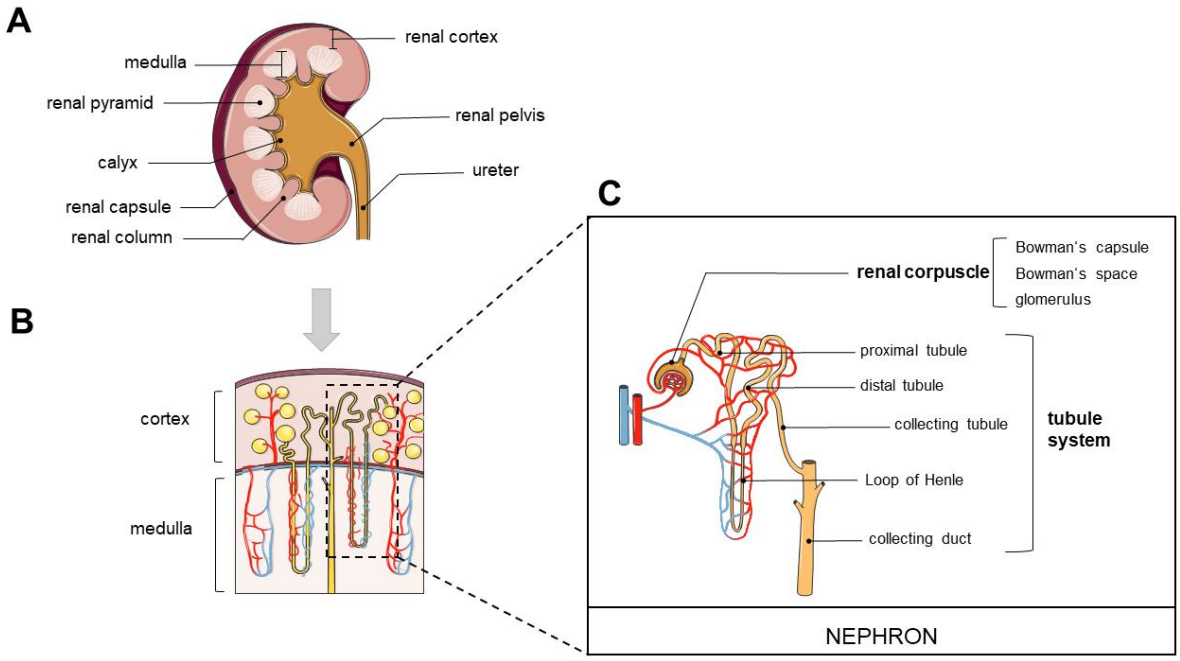

Figure 1. *Kidney anatomy and specific depiction of nephron compartments. A) Sagittal section of a kidney showing the description of the kidney regions. B) Overview of the parenchymal layers, cortex and medulla, depicting the embedding of the nephrons and the vasculature within the parenchyma. C) Segmentation of the nephron structures divided into the renal corpuscle and the tubule system with the description of the specific structures. Original images from Servier Medical Art, as licensed under a Creative Commons Attribution 3.0 Unported License. Text was added to the original images.*

1.1.2 Embryonic development of the kidney

During the embryonal development, the kidneys derive from the intermediate mesoderm (IM) along the rostral-caudal axis of the embryo (M. H. Little et al., 2019). Dividing the IM into anterior and posterior, certain parts of the kidney are originated in the posterior IM, while other parts derive from the anterior IM. In mammals, the organs undergo two transient stages, referred to as pro- and mesonephroi, before developing into the permanent metanephroi (single: pronephros, mesonephros, metanephros). All three kidney organs develop successively from the IM-derived nephrogenic cord (Cullen-McEwen et al., 2015). The kidney development of higher vertebrates is evolutionarily conserved, which can be observed via the successive appearance of the transient renal structures (Aboul Mahasen, 2016).

The pronephroi develop epithelial structures, the nephrotomes, which are simple excretory tubules in some amphibians and fish, while they are non-functional in mammals (Smyth et al., 2017; Winyard, 2019). The caudal part of the pronephroi elongate and eventually connect with the cloaca (Smyth et al., 2017). This structure is termed the Wolffian duct or nephric duct, and it persists further, while all rostral parts regress. The Wolffian duct then further elongates in caudal direction and is connected with the later developed mesonephric kidneys (Cullen-McEwen et al., 2015). During mammalian embryogenesis renal functions are rudimentarily

fulfilled by the mesonephros (M. Little et al., 2010). Most of the mesonephroi degenerate at later developmental stages. However, a few segments of the mesonephroi are involved in the development of other parts of the urogenital system such as the male gonads, the adrenal glands and also take part in the development of erythrocytes (Krause et al., 2015).

The next developmental stage is the metanephros, which develops into the permanent kidney organ by a process termed nephrogenesis. The main actors of nephrogenesis are the metanephric mesenchyme and the nephric duct-derived ureteric bud. As mentioned before, the metanephric mesenchyme originates from the posterior intermediate mesoderm, while the ureteric duct derives from the anterior intermediate mesoderm.

1.1.3 Nephrogenesis

The nephron development begins around gestation week 4-5 in humans when the epithelial ureteric bud (UB) forms from the nephric duct (Cullen-McEwen et al., 2015). The UB and the surrounding metanephric mesenchyme (MM) secrete growth factors, which induce reciprocal events. Two events take place in parallel:

First, renal branching morphogenesis starts when the outgrowth of the UB into the MM is induced by glial cell-derived neurotropic factor (GDNF), a growth factor secreted by the MM (Fig. 2A). GDNF binds to the glycosylphosphatidylinsotol-linked cell surface receptor GFRα1 to interact with the receptor tyrosine kinase ret proto-oncogene (RET). RET is then autophosphorylated and activates downstream target pathways and genes. RET is present on the UB epithelium and GFRα1 is expressed in both the MM and the UB (Krause et al., 2015). The stimulated UB initially branches in a dichotomous and symmetric way, followed by continuous branching until the 34th gestation week in humans (Bush et al., 2012). However, the branching is never completely identical and symmetrical (Alpern et al., 2013; Costantini, 2015). Maintenance of the ongoing branching of the UB is based on the interplay between different signalling pathways including GDNF-RET, Fibroblast Growth Factor (FGF) and Wingless-type 1 (WNT) signalling (Smyth et al., 2017). Additionally, phosphoinositide 3-kinase (PI3K) and Phospholipase C γ (PLCγ) pathways are likely to be involved in the branching rate (Smyth et al., 2017). Continuous branching ultimately derives the tree-like collecting system of the adult kidney, consisting of the collecting ducts, the ureters, the calyces and the renal pelvis (Alpern et al., 2013; Costantini, 2015; Winyard, 2019).

Simultaneously to the branching, WNT9B signals from the UB induce differentiation starting with a mesenchymal to epithelial transition (MET) in the MM. This leads to the condensation of the MM surrounding the tips of the UB, which is then referred to as cap mesenchyme (CM) (M. Little et al., 2010). The area around the UB tips represent nephrogenic niches containing the RET^+ UB tip, $SIX2^+$ nephron progenitors and $FOXD1^+$ cortical stromal progenitors (M. Little et al., 2010; Wilson & Little, 2021). Signals from the UB tip maintain the self-renewal and proliferation of the progenitors, but also induce nephrogenesis (Wilson & Little, 2021). Besides the nephrons and the stroma, interstitial cells originate in the MM (Al-Awqati & Oliver, 2002). After the CM derives, an epithelial pre-tubular aggregate (PTA) is formed beneath the UB (Koning et al., 2020). Non-canonical WNT signalling via WNT4 induces MET, which ultimately results in the formation of an epithelial structure called renal vesicle (RV) (Koning et al., 2020; M. H. Little et al., 2019). The RV is polarized, it has a proximal and distal part, which contain precursors later developing into the glomerulus and the tubule compartments (Kaku et al., 2017). The RV is elongated, and two clefts are formed one by one, which firstly leads to transitioning to the comma-shaped (CSB) and then the S-shaped body (SSB) (Figure 2A). During that time, the SSB is patterned along the proximo-distal axis, resulting in the differentiation of tubule regions and the formation of the renal corpuscle. The patterning from the distal to the proximal end of the SSB derives the distal tubule, then the loop of Henle (LoH) and the proximal tubule, based on a gradient of WNT signalling from distal to proximal region (M. H. Little & Combes, 2019) (Figure 2B). The distal part of the SSB is then connected to the UB-derived collecting duct system (Bush et al., 2012). The proximal end of the SSB gives rises to the renal corpuscle consisting of the Bowman's capsule, the Bowman's space, the glomerulus, the glomerular filtration barrier, and the capillary tuft.

The origin of the renal vasculature is still not completely clarified. One theory indicates that endothelial and mesangial precursor cells derive from the IM and migrate to the kidney (Pietilä & Vainio, 2014). The other theory postulates that the endothelium may originate from the renal anlage itself (Pietilä & Vainio, 2014). Moreover, it is unclear, whether the renal vasculature emerges from vasculogenesis or angiogenesis (Smyth et al., 2017). Vasculogenesis is the process, where blood vessels are formed *de novo*, while angiogenesis describes the sprouting of new vasculature from pre-existing vessels (Smyth et al., 2017; Winyard, 2019). Many researchers follow the hypothesis that the renal vasculature derives from a mix of both processes (Khoshdel-Rad et al., 2022).

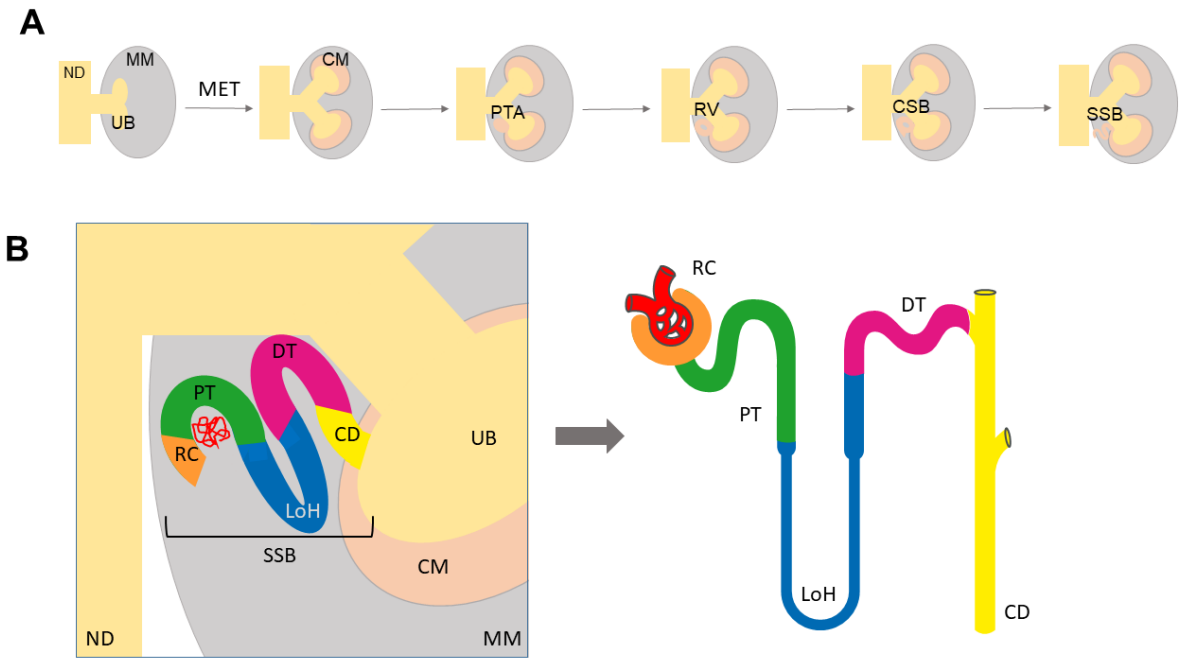

Figure 2. *Overview of the nephrogenesis. A) Chronological order of cellular events during nephrogenesis. B) Breakdown of the different sections of the nephron and their origin in the SSB. ND= nephric duct, MM= metanephric mesenchyme, UB= ureteric bud, CM= cap mesenchyme, PTA= pre-tubular aggregate, RV= renal vesicle, CSB= C-shaped body, SSB= S-shaped body, RC= renal corpuscle, PT= proximal tubule, LoH= Loop of Henle, DT= distal tubule, CD= collecting duct. Original images from Servier Medical Art, as licensed under a Creative Commons Attribution 3.0 Unported License. Text was added to the original images.*

1.1.4 Filtration of the blood

More than 20 different cell types assemble the nephron. The main unit of the filtration apparatus, the renal corpuscle, is sub-divided into the Bowman's capsule, the Bowman's space, and the glomerulus (Figure 1C). The glomerulus is an encapsulated structure surrounding a tight capillary tuft. Essential for the filtration is the glomerular filtration barrier, which consists of three layers: i) the glomerular endothelium; ii) the glomerular basement membrane and iii) a layer of tightly interdigitating podocytes (BENZING, 2020; Rayner et al., 2016). Water and other smaller-sized molecules are able to move freely through the filtration barrier (Kriz, 2010). Two characteristics of the filtration barrier contribute to the filtration function. Firstly, the filtration barrier is highly negatively charged (Rayner et al., 2016). The cell surface of glomerular endothelial cells is layered with the negatively charged glycocalyx made of proteoglycans, glycoproteins and glycolipids (The glomerular filtration barrier: a structural target for novel kidney therapies, 2021). The composition of the glomerular basement membrane (GBM) adds to the negative charge (Kriz, 2010). The charge contributes to the charge selectivity of the filtration barrier, which prevents highly negatively charged molecules

such as albumin from passing through (Rayner et al., 2016; Scott & Quaggin, 2015). In addition to the charge selectivity, the filtration barrier is also size-selective, which is ensured by the visceral epithelium. Podocytes cover the capillaries and spread out interdigitating cellular outgrowths called foot processes, which are interconnected via slit diaphragms, which restrict bigger-sized molecules such as Albumin and cells like erythrocytes from passing through the filtration barrier (BENZING, 2020; Kriz, 2010).

When the blood flows into the glomerulus, water and smaller-sized molecules, such as salts and urea can pass the glomerular filtration barrier. This filtrate is the primary urine, which is further concentrated in the next steps. Connected to the renal corpuscle is the tubule system, sub-divided into the proximal tubule, loop of Henle, distal tubule, collecting tubule and collecting duct (Figure 1C). The proximal tubules are important for the re-absorption of water, proteins and other molecules. Due to an abundant number of water permeable pores (aquaporins), water can be easily recovered (Kriz, 2010). Additionally, active transporters ensure the re-absorption of salts, sugars, amino acids and bicarbonate into the blood (Cole & Kramer, 2016). The main task of the loop of Henle is the re-absorption of salts. High numbers of aquaporins make the first segment of the LoH, the thin descending limb, highly water permeable (Kriz, 2010). Water diffuses into the blood, which enriches the secondary urine with salts and creates an inward-oriented concentration gradient (Akilesh, 2014). Due to this gradient, water cannot penetrate the thin ascending limb and thick ascending limb (Akilesh, 2014; Kriz, 2010). Further concentration of the fluid is acquired by active salt co-transporters in the thick ascending limb (Akilesh, 2014; Kriz, 2010). The distal tubule also actively re-absorbs electrolytes from the filtrate, including calcium, phosphate and potassium ions (Cole & Kramer, 2016). Before the secondary urine leaves through the ureter further to the bladder and is excreted through the urethra, water and salts are re-absorbed by the collecting duct in a final step (Kriz, 2010). Kidney diseases, infections and medications affect the nephron integrity and often come with an irreversible loss of nephrons (Connelly et al., 2020).

1.1.5 Kidney diseases and their causes

According to the statistics of the World Health Organization (WHO), in 2019, kidney diseases were one of the top ten leading causes of death (WHO, 2020). Kidney diseases such as acute kidney injury (AKI) and chronic kidney disease (CKD) are a worldwide health burden. While AKI describes a condition of reduced kidney function in less than three months, CKD is defined as

a gradual loss of kidney function for a minimum duration time of three months (Webster et al., 2017). Leading etiologies for AKI include renal ischemia/ reperfusion (I/R) injury, drugs and medications, but also surgical procedures can cause AKI (Hosohata et al., 2022; *Appendix I.1.ii, p. 20*) (Fig. 3). During I/R injury, blood flow to the kidney and consequently oxygen supply is interrupted and this condition can appear after infarction, renal artery embolism or organ transplantation (Malek & Nematbakhsh, 2015; Spiliopoulos et al., 2020). Under ischemic conditions, production of reactive oxygen species (ROS) and inflammatory processes are induced. The resulting cell damage can cause ferroptosis, an iron-dependent cell death, which is characterized by the accumulation of iron and lipid peroxidation (Hosohata et al., 2022). Under normal conditions, anti-oxidative enzymes remove the lipid peroxides and prevent their accumulation. However, pathological conditions can cause increased ROS production and often go together with decreased levels of antioxidants. Accumulated ROS activate inflammatory- and cell death-associated processes and can trigger AKI leading to damage of the functional cells, like tubular cells (Hosohata et al., 2022). In recent years, dysregulated iron metabolism caused by ferroptosis was also linked to the emergence of AKI (Borawski & Malyszko, 2020). Therefore, ferroptosis inhibitors preventing the cellular accumulation of iron or lipid peroxides represent a potential therapy for AKI patients (Borawski & Malyszko, 2020; Hosohata et al., 2022).

CKD is diagnosed under the conditions of increased urinary protein secretion and reduced glomerular filtration rate during a time span of three months (Kovesdy, 2022). Causes for CKD are multifaceted. Especially diabetes mellitus and high blood pressure are high risk factors for the emergence of CKD (Kovesdy, 2022; Noble & Taal, 2019) (Fig. 3). Hypertension has drastic effects on the glomeruli, as the delicate capillary systems are easily damaged by high blood pressure (D. Sun et al., 2020). Since glomerular cells are not capable to self-renew, permanent loss of glomerular cells and function is inevitable and ultimately results in disease progression (BENZING, 2020). Besides pathological kidney conditions such as AKI or glomerulonephritis, cardiovascular diseases and drugs can equally cause CKD (Noble & Taal, 2019). AKI and CKD are interlinked pathophysiological conditions. AKI can progress to CKD due to various circumstances such as inflammation, developing fibrosis and hypoxia among others(Fiorentino et al., 2018). On the other hand, patients suffering from CKD are more susceptible for further kidney damages due to CKD-induced AKI (Ferenbach et al., 2017; Fiorentino et al., 2018)(Fig. 3). The progression of CKD to the final stage is described as end-

stage renal disease (ESRD), which is defined as an irreversible loss of kidney function (Abbasi et al., 2010) (Fig. 3).

Early diagnosis and treatment can reduce the potential progression to ESRD, as this condition is fatal for patients if not treated with kidney replacement therapy including dialysis or organ transplantation (Abbasi et al., 2010). Other than the high costs, dialysis is time-consuming and restricts the daily life of the patients drastically (Himmelfarb et al., 2020). On the other hand, donor organs are rarely available due to organ shortage and lacking immune compatibility (Beyar, 2011). Taking into account that there are desensitization approaches, which allow allograft-transplantation and counter immune incompatibility, the procedure goes together with high costs and long-life use of immunosuppressive medications to prevent antibody-mediated rejection (Beyar, 2011; Gloor & Stegall, 2010).

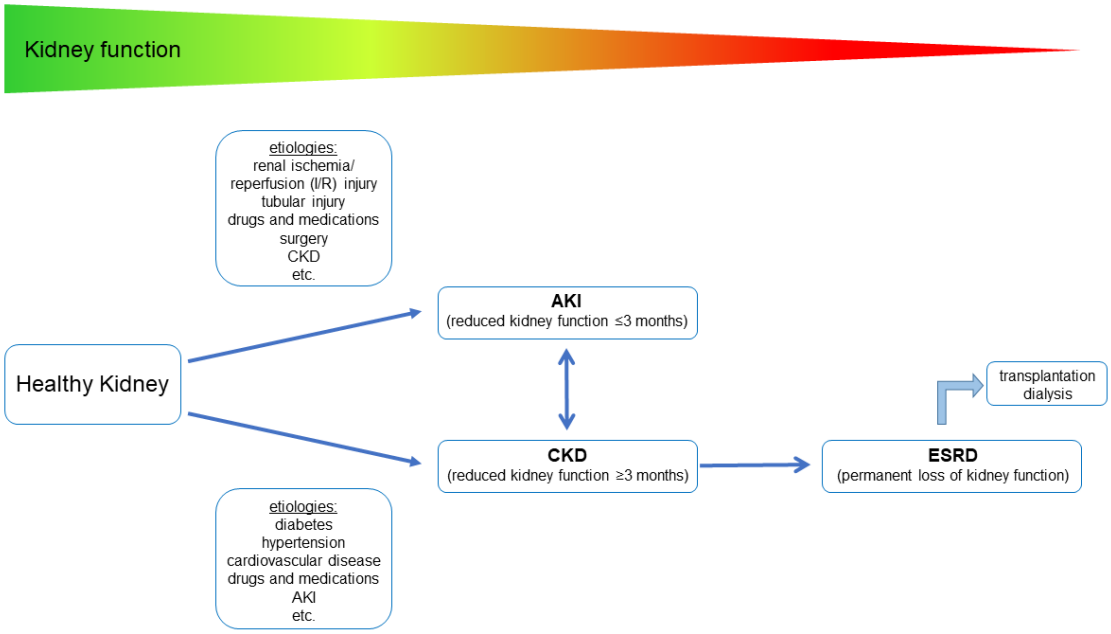

Figure 3.Correlation between AKI, CKD and ESRD. Described are the etiologies leading to the emergence of AKI and CKD as well as the connection between the pathologies. Decline of kidney functions is depicted in a coloured arrow.

1.1.6 Nephrotoxicity

One contributor to kidney diseases can be nephrotoxicity. Nephrotoxicity is described as alterations of kidney functions and structures due to acute or chronic exposure to toxicants such as drugs and medications (S. Y. Kim & Moon, 2012; Y. Y. Zhao & Lin, 2014). Affected by drug-induced renal toxicity may be tubules, interstitial tissues, glomeruli, or the renal microvasculature (Faria et al., 2019). The emergence of pathological consequences is often

reversible upon discontinuation of drug use (Faria et al., 2019). Several circumstances contribute to the kidney's susceptibility to drug-induced injury: i) kidneys receive a quarter of the resting cardiac output and are prone to circulating drugs ii) high exposure to xenobiotics due to tubular function of toxin removal iii) tubular accumulation of drugs, and iv) high tubular energy demand, which makes tubules vulnerable to renal toxicity (Griffin et al., 2019). Owning to the functions of solute re-absorption and the metabolism of xenobiotics, tubules are the main target of nephrotoxic substances. Numerous drugs are taken up by endocytosis and active transporters from the blood, which makes a tubular accumulation very likely (Hall et al., 2022; K. D. Liu & Palevsky, 2022). Tubular injury or loss can be induced by medications such as antibiotics, anti-viral drugs like tenofovir and chemotherapeutics like cisplatin (Hall et al., 2022; S. Y. Kim & Moon, 2012; Perazella & Rosner, 2022). Within the tubules, xenobiotics often induce disruption of the tubular cell polarity and mitochondrial damage accompanied by increased oxidative stress, which impair the correct tubular functions (Faria et al., 2019; K. D. Liu & Palevsky, 2022).

In the recent years, researchers developed different *in vitro* approaches to study nephrotoxicity with a focus on proximal tubular cells. Close to the *in vivo* state are primary cells, which can only be maintained for a short period since they are limited in their growth and rapidly lose their phenotype *in vitro* (Barnett & Cummings, 2018). On the other hand, immortalized cells can be cultured indefinitely, but may carry inadvertent alterations in their characteristics (Faria et al., 2019). Alternatively, three-dimensional kidney models were developed including pluripotent stem cell (PSC)-derived kidney organoids, bioengineered kidneys and organ-on-a-chip systems (*see* chapter 1.2.61.2.6) (Cintron Pregosin et al., 2021; Faria et al., 2019; Rizki-Safitri et al., 2021). Various kidney models were generated by applying immortalized kidney cells and PSC-derived kidney organoids on biological and non-biological scaffolds such as Matrigel, hollow fibres and de-cellularized kidneys (Cintron Pregosin et al., 2021; Faria et al., 2019; Rizki-Safitri et al., 2021).

Drug-induced nephrotoxicity can be evaluated via blood tests, indicated by high blood urea nitrogen (BUN) levels, increased serum creatinine (sCr) levels and reduced glomerular filtration rate (GFR) (S. Y. Kim & Moon, 2012). However, the serum biomarkers BUN and serum creatinine are not highly specific toxicity markers, as serum levels can be altered by renal and non-renal factors other than drug-induced kidney injury (Griffin et al., 2019). Besides blood, urine represents a non-invasive source for the analysis of nephrotoxic biomarkers.

Nephrotoxicity biomarkers in the urine, which were approved by the Predictive Safety Testing Consortium (PSTC) included kidney injury molecule-1 (KIM-1), trefoil factor 3 (TFF-3), beta-2 microglobulin (B2M), cystatin C (CysC), urinary albumin (uALB), urinary total protein (uTP) and Clusterin (CLU) and later neutrophil gelatinase-associated lipocalin (NGAL) and osteopontin (OPN) (Faria et al., 2019; Griffin et al., 2019). Drug-induced dysfunction of tubules can lead to urinary protein losses and in the worst case result in AKI (Hall et al., 2022). Under chronic circumstances, tubular injury activates renal inflammation and fibrosis via numerous pathways, which drives disease progression (B. C. Liu et al., 2018).

1.2 Stem Cells

1.2.1 Classification of stem cells

Cells of various tissues in the body must be regularly renewed due to age, damage, or injury, which is important for tissue regeneration. This task is fulfilled by stem cells. Other than somatic cells, stem cells are undifferentiated and can self-renew. This implies they are able to divide into two daughter cells, where one differentiates to the target cell and the other remains as an undifferentiated stem cell. Stem cells can be categorized by their potency to differentiate. The zygote represents the totipotent state, as it is able to differentiate into a complete embryo as well as extra-embryonic tissues (Zakrzewski et al., 2019). Embryonic stem cells (ESCs), from the inner cell mass of the blastocyst, are pluripotent and can differentiate in every cell type of the germ layers ectoderm, mesoderm, and endoderm. These cells are often extracted from supernumerary embryos, which were generated by *in vitro* fertilization (Thomson, 1998). Classified as adult stem cells (ASCs) are multipotent and unipotent stem cells. Multipotent stem cells are restricted to differentiate into cell types of one lineage, while unipotent stem cells can only differentiate into one specific cell type. Tissue damage caused by injury or pathological conditions may activate adult stem cells for regeneration (Urinary stem cells as tools to study genetic disease: Overview of the literature, 2019).

1.2.2 Induced pluripotent stem cells

While embryonic stem cells represent a promising tool for regenerative medicine for their great potential, ethical concerns were risen, since the cells are isolated from embryos. In 2006, the Yamanaka lab firstly generated pluripotent mice stem cells from somatic mice cells *in vitro*

via a technique called reprogramming (Takahashi & Yamanaka, 2006). One year later, the same lab reproduced this experiment, and successfully produced human induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007). In short, that process included the retroviral introduction of four transcription factors into adult somatic cells, later called the Yamanaka cocktail: Oct4, Sox2, c-Myc and Klf4 (Takahashi et al., 2007; Takahashi & Yamanaka, 2006). The generated cells demonstrated characteristics typical for pluripotent embryonic stem cells, including unlimited self-renewal and the ability to spontaneously generate cell types of all three germ layers via embryoid body formation (Takahashi et al., 2007). Additional features included telomerase activity, expression of hESC surface markers including SSEA3/4, TRA-1-60, TRA-1-81 and alkaline phosphatase (Takahashi et al., 2007; Thomson, 1998). Another hallmark of ESCs include teratoma formation when transplanted, which could also be demonstrated with human iPSCs (Takahashi et al., 2007).

The prospect for the medical sector is quite high, as patients could have autologous iPSC-derived tissues for regenerative purposes without the risk of immune rejection (Zakrzewski et al., 2019). In the last decade, efforts in using iPSCs for the generation of different cell models for regenerative medicine led to an immense number of studies actively working towards this goal. Similar to ESCs, iPSCs have drawbacks, which makes it complicated to use them in the clinical setting. Their high differentiation and proliferation potential increases the risk of tumorigenicity. When iPSCs are differentiated for tissue regeneration or similar purposes, the possibility of remaining undifferentiated iPSCs is quite high (Doss & Sachinidis, 2019; Zhong et al., 2022). These undifferentiated iPSCs have a high risk to develop into tumours. In case of autologous iPSCs, a potential tumour formation cannot be recognized by the immune system (Kanemura et al., 2014). Moreover, high passage number and other circumstances can lead to karyotypic aberrations, which also increases the risk for tumour formation (Qiao et al., 2020). Additionally, many tissues derived from iPSCs resemble the fetal counterpart and therefore do not fully recapitulate the adult functions.

1.2.3 Stem cells isolated from the urine

Urine-derived stem cells (USCs) represent an alternative to the conventional iPSCs. These cells are stem cells isolated from urine. Besides urine stem cells, also renal epithelial cells, such as podocytes and proximal tubule epithelial cells (PTECs) have been found within the shed urine

(Manaph et al., 2018). Due to the easy access of voided urine, urine stem cells are a good candidate for the reprogramming of iPSCs for personalized medicine (Bento et al., 2020). With urine-derived iPSCs the problem with allogenic donor cells, which come with immunosuppressive measurements, can be easily avoided.

Typically, urine stem cells have a rice grain-like and spindle-shaped morphology and are capable to grow on plastic surfaces. Some USC clones express stemness-related genes such as OCT3/4, c-MYC, SSEA-3/4 and KLF4 (Huang et al., 2022). Besides that, USCs have long telomeres, and telomerase activity was measured in some clones, while no tumorigenicity in *in vivo* studies could be demonstrated (Chun et al., 2012; D. Zhang et al., 2014). Moreover, renal markers such as the progenitor marker SIX2, the podocyte marker NPHS1, and the tubular markers AQP1, AQP2, and SLC12A1 were expressed in USCs (A. J. Chen et al., 2020). The presence of SIX2 together with CD24 and CD133 indicates that some urine cells are renal progenitor cells (Lazzeri et al., 2015; Pavathuparambil Abdul Manaph et al., 2018; Rahman et al., 2020[see *Appendix I.i*]). In this regard, the name urine-derived renal progenitor cells (UdRPCs) was utilized to reflect this in greater detail[1] (Rahman et al., 2018, 2019). The origin of UdRPCs is not completely clarified. One possible origin is the upper urinary tract, indicated by the expression of typical markers and the presence of Y-chromosomes in urine cells from a female transplantation patient who received a male donor organ (Bharadwaj et al., 2013). UdRPCs have a multipotent differentiation potential. Differentiation of urine stem cells to urothelial cells, endothelial cells, smooth muscle-like cells, neurons, and even beta cells and hepatocytes was documented before (Huang et al., 2022; Hwang et al., 2019; G. Liu et al., 2018; Ouyang et al., 2014; S. Wu et al., 2011; Xu et al., 2019; M. Zhou et al., 2020).

On the cellular level, these cells resemble mesenchymal stem cells (MSCs) as they exhibit the MSC characteristics introduced by the International Society for Cellular Therapy: i) plastic adherence, ii) expression of the mesenchymal surface marker CD73, CD90, CD105 and CD133, iii) absence of hematopoietic surface markers CD14, CD34 and CD40, iv) tri-lineage differentiation potential to adipocytes, chondrocytes and osteoblasts (Dominici et al., 2006). Moreover, immune-modulatory properties are one of their characteristics, as they express class II HLA glycoproteins, common for immune cells such as macrophages and B-lymphocytes but not the immunogenic HLA-DR glycoproteins (Bento et al., 2020). Besides that, the urine

[1] The term UdRPCs will be used when the cells are characterized as in (Rahman et al., 2018), while the term USCs will refer to the cells of variable cell characteristics based on the quoted studies.

stem cells were able to inhibit phytohemagglutinin (PHA)-activated lymphocyte cluster formation (Schosserer et al., 2015). Another similarity to MSCs is the capability to secrete paracrine factors through exosomes and extracellular vesicles, which can for instance alleviate inflammatory processes (Bento et al., 2020; Huang et al., 2022).

Due to their non-invasive isolation from urine, UdRPCs represent an interesting cell source for paediatric therapy (Bento et al., 2020). In contrast to conventional skin biopsies, urine cells represent a less problematic cell source for patients suffering from skin diseases including inherited epidermolysis bullosa as it reduces the burden on the patients (Schosserer et al., 2015). Besides being easily reprogrammed to iPSCs, native UdRPCs are valuable for disease modelling and the diagnosis of genetic disorders (Bento et al., 2020). Urine stem cells furthermore represent a promising tool for cell therapy approaches.

1.2.4 Application of urine-derived renal progenitor cells

The use of cells isolated from urine for clinical applications and therapeutic means has been investigated in the recent years, which will be reviewed in the following based on a few studies (*Table 1*). Urine stem cells are a cost-effective, non-invasive cell source with minimal ethical concerns, and therefore, are especially attractive for the field of regenerative medicine. Their differentiation potential to cell types of the urogenital tract make them possibly an interesting cell source for reconstruction or regeneration of urogenital tissues (Qin et al., 2014).

For tissue regeneration, UdRPCs can be directly incorporated into the injured tissue (Li et al., 2020). Tian *et al.* reported the injection of urine stem cells to treat the effects of AKI in a rodent model (Tian et al., 2017). They were able to show that the incorporation of USCs ameliorated AKI, detected by reduced sCr and BUN levels, decreased tubular injury score, less numbers of apoptotic cells and high levels of anti-inflammatory cytokines. Moreover, GFP labelling demonstrated the proliferation and differentiation of incorporated USCs to tubular epithelial cells supporting tissue regeneration after tubular injury (Tian et al., 2017). The broad potential for the use of UdRPCs was also shown by regeneration studies of tissues other than tissues of the urogenital system. For instance, researchers administered composites of USCs and small intestine submucosa hydrogels to heat-injured vocal folds in rabbit models and observed the promotion of vocal fold recovery seen by enhanced anti-inflammatory responses, neovascularization and suppression of fibrosis (Hu et al., 2022). The combination of USCs with

the bone tissue engineering scaffold, β-TCP, was moreover used to reconstruct bone tissue, as USCs have an osteogenic differentiation potential (Guan et al., 2015). Patient-derived USCs were also demonstrated to help in the recovery of liver function and the alleviation of tissue damages induced by liver injury (N. Zhang et al., 2021). But also for multi-organ diseases such as diabetes, UdRPCs may be beneficial, as demonstrated by a study, where application of USCs led to improvement of blood glucose levels, regeneration of islet vasculature and increased β-cell survival as well as an overall increased median survival rate of diabetic mice (T. Zhao et al., 2018). One subsequent pathology of diabetes affecting the kidneys can be diabetic nephropathy and the effects of applying hUSCs was also studied before (Xiong et al., 2020). Mouse models of diabetic nephropathy were treated with hUSCs, and the cells were demonstrated to preserve kidney function, have anti-inflammatory effects and reduce renal fibrosis as well as oxidative stress (Xiong et al., 2020).

However, the exact mechanisms of how urine stem cell function in regeneration processes are not fully understood. Similar to MSCs, USCs have high immunomodulatory properties, which suggests, that secreted paracrine factors from USCs may play an essential role in tissue repair and regeneration. The paracrine factors have anti-inflammatory and anti-oxidative effects, and may be beneficial for transplantation patients as they reduce immune rejection and graft versus host disease (GvHD) (Q. Zhou et al., 2022). Therefore, alternative approaches besides direct cell incorporation was tackled by utilizing the secreted paracrine factors (Li et al., 2020; Q. Zhou et al., 2022). Li *et al.* demonstrated in their study that injected USCs into an I/RI rat model ameliorated renal injury shown by decreased levels of sCr and BUN, a reduced pathological score for renal injury, reduced apoptosis, a lower amount of infiltrating neutrophils and reduced levels of ROS (Li et al., 2020). Additionally, exosomes isolated from USC conditioned medium were used on hypoxia/reoxygenation (H/R) injury-induced HK2 cells (Li et al., 2020). Similar to the previous results, the USC-exosomes had an anti-apoptotic, anti-inflammatory, anti-oxidative and anti-fibrotic effect on H/R-HK2 cells (Li et al., 2020). Extracellular vesicles secreted by USCs were also shown to support angiogenesis and muscle regeneration after ischaemia/hypoxia insult in a mouse model (Zhu et al., 2018).

Besides the therapeutic potential of USCs and the secreted paracrine factors, cells isolated from patient urine can also be used for predicting the course of kidney diseases (Gerges et al., 2022). Gerges *et al.* demonstrated the presence of proliferative tubular progenitor cells in urine from recovering AKI stage 3 patients, which resemble the findings of recovering patients

suffering from transplantation-induced AKI (Gerges et al., 2022). This shows the potential of urine as a future prognostic tool associated with renal recovery from AKI, which has to be further investigated. Cells isolated from patients' urine, especially podocytes and tubular epithelial cells, are moreover interesting for disease modelling of genetic diseases and a potential hub for the development of novel therapeutics (Bondue et al., 2021). Other options for the use of USCs can include the reprogramming to iPSCs and further differentiation to various cell types for disease modelling as well as for the generation of 3D cell models (Bohndorf et al., 2017).

Table 1. Applications of urine stem cells for medical research regarding possible treatment options.

Cell source	pathology	methods	Major findings	reference
Human urine stem cells	I/R-induced AKI	Injection of USCs in left kidney of I/R injury rat model	Promoted kidney repair, led to reduction of sCr and BUN, decreased tubular injury score, reduced apoptosis, and increased proliferation, increased levels of anti-inflammatory cytokines	(Tian et al., 2017)
Human urine stem cells	Voice disorders, voice fold scarring	USCs+ small intestine submucosa hydrogels in heat-injured vocal folds of rabbit model	Support of anti-inflammatory polarization of M2 macrophages, neovascularization, re-epithelialization and suppressed fibrosis	(Hu et al., 2022)
Human urine stem cells	Bone fractures	USCs+bone tissue engineering scaffold b-TCP	Reconstruction of bone tissue, osteogenic differentiation of USCs and promotion of new bone formation	(Guan et al., 2015)
Human urine stem cells	Liver injury	Transplantation of patient-derived USCs into CCL4-induced liver fibrosis in mouse model	Recovery of liver function, improvement of alanine aminotransferase and aspartate levels, and decrease of liver tissue injury	(N. Zhang et al., 2021)
Human urine stem cells	diabetes	Transplantation of USCs in streptozotocin-induced diabetes mouse model	Improvement of median survival rate of mice, recovery of glucose homoeostasis, islet re-vascularization and pancreatic β-cell survival	(T. Zhao et al., 2018)
Human urine stem cells	Diabetic nephropathy (D/N)	Injection of USCs in streptozotocin-induced D/N mouse model	Alleviation of inflammation and oxidative stress, reduction of renal fibrosis, protection of renal functions	(Xiong et al., 2020)
Human urine stem cells	I/R injury-induced AKI	Injection of USCs into I/R injury rat model	USCs reduced sCr and BUN, apoptosis, inflammation, oxidative stress and fibrosis.	(Li et al., 2020)

		Exosomes from USC-conditioned medium used on H/R injury HK2 cells	Exosomes promoted reduction of inflammation, apoptosis, oxidative stress and fibrosis	
Human urine stem cells	Ischemia/hypoxia	Transplantation of extracellular vesicles of USCs into ischemic mouse hind-limb	Supported angiogenesis and muscle regeneration	(Zhu et al., 2018)
Human tubular progenitor cells	Non-transplantation AKI		Presence of proliferative tubular progenitor cells in urine of non-transplantation AKI patients, indication for AKI recovery	(Gerges et al., 2022)

(continued Table 1)

1.2.5 Organoids

In order to discover new therapies and medications disease models are widely used or currently in development. 2D cell culture models include immortalized cell lines (tumour cell lines like HEK, HEPG2, etc), primary cells and *in vitro* differentiated cells originating from pluripotent stem cells. These cell cultures can be used for studying cellular processes and disease mechanisms in adult cells. However, immortalized cells are not suitable to study diseases related to the cell cycle, since this cell cycle-related processes are abnormal in these cells. On the other hand, primary cells often lose their functionality or their in *vivo* phenotype under *in vitro* cultivation conditions (Tang et al., 2022). Additionally, many diseases are multi-faceted and also result from disorders in various organs, which cannot be mimicked in single cell layer cultures generated from PSCs (B S Freedman, 2015). Despite the easy cultivation, cell cultures also cannot fully recapitulate the cellular complexity in regards of the structure and cell diversity of the target tissue or organ (Tang et al., 2022). The required tissue and organ complexity is ensured by using model organisms. Depending on the animal model, robustness, a high generation turnover rate and low maintenance costs are some of the advantages (J. Kim et al., 2020). Studying diseases on animal models requires basic knowledge about the underlying disease mechanisms, but new findings cannot be fully extrapolated to humans due to interspecies differences (J. Kim et al., 2020). For instance, genetic mutations causing diseases in humans can have another effect in an animal model (B S Freedman, 2015). Moreover, human-specific pathologies caused by an imbalance of the human microbiome or the general interactions of human pathogens cannot be studied in mammalian animal models (J. Kim et al., 2020).

In that regard, alternatives were in urgent need and in the last decade several 3D *in vitro* models were developed to better recapitulate the composition and functions of the target tissue or organ. Sato *et al.* were one of the first to generate intestinal organoids, self-organizing cell aggregates reflecting the structure of intestinal crypts (Sato et al., 2009). These 3D cell models are generated by mimicking the tissue or organ development *in vitro*. Organoids have three major characteristics: i) they are composed of organ-specific cell types, ii) the spatial organization resembles that of the target organ, iii) they recapitulate organ functions.

Various protocols use pluripotent stem cells for the generation of organoids by cultivating these cells with a cocktail of growth factors and developmental cues, which differentiate the

PSCs towards the target fate, such as the optic cup, cerebral cortex, kidney and liver (Eiraku et al., 2011; Lancaster et al., 2013; Takasato et al., 2015; Takebe et al., 2015). The close resemblance to human physiology make organoids a promising tool for research. In most cases, organoids generated from pluripotent stem cells resemble the fetal organ more than the adult organ (H. Wu et al., 2018). Therefore, these PSC-derived organoids are more applicable for the study of embryonal development and thus can be used to broaden the basic knowledge of developmental processes (J. Kim et al., 2020; Tang et al., 2022). Other protocols also describe organoids derived from primary cells and adult stem cells, which are extracted from the target organs by biopsy (Hofer & Lutolf, 2021). Diseases, which are not extensively studied or occur quite rarely can be studied via the generation of organoids from patient-derived cells (J. Kim et al., 2020). Knowledge of the stem cell niche is essential to maintain ASCs and give the appropriate signals for the differentiation to the target cell types (J. Kim et al., 2020). These organoids are less complex due to the cells' restricted potency and consist of a single epithelial cell layer (Tang et al., 2022). They are closer to the adult tissue or organ than the PSC-derived organoids and are therefore more suitable for the study of tissue repair and viral infection disease (Tang et al., 2022). Since ASCs are more difficult to obtain and due to their restricted potential to differentiate to a certain tissue type, PSCs with their potential to generate all tissues of the body represent a more promising option for organoid formation (J. Kim et al., 2020). Alternatively, organoids were formed by mixing various cell types including iPSC-derived organ-specific cells, endothelial cells, mesenchymal stem cells and/or smooth muscle cells (Takebe et al., 2013; Wörsdörfer et al., 2019). In contrast to the *in vivo* organ, organoids mostly do not have a mature, diffusible vasculature. The vascular network is indispensable for the supply with oxygen and nutrients and is also relevant for structural support (M. H. Little & Combes, 2019; S. Zhang et al., 2021). However, in organoids nutrients can only be provided by diffusion to a certain degree, which often leads to a necrotic core (Lancaster et al., 2013). Previous studies have demonstrated that included endothelial cells formed *in vitro* vessels, which connected to the host vasculature upon transplantation and led to an improvement of the overall maturity of the organoids (Takebe et al., 2013; Wörsdörfer et al., 2019). Besides the co-culture with endothelial cells, other attempts to improve the vascularization of organoids include the use of microfluidic chambers, and 3D bioprinting for better recapitulation of the *in vivo* organ organization (Hofer & Lutolf, 2021; Khoshdel-Rad et al., 2022). The organoid technology is a huge step towards the rapid developing future of

personalized medicine, as the patient-derived organoids can be used to evaluate disease profiles and drug screenings adapted to the individual patient (J. Kim et al., 2020).

Limitations of the organoid technology include the low maturity and off-target cell populations (M. H. Little & Combes, 2019). Regardless of the high complexity compared to 2D cell cultures, the organoid technology cannot compensate the lack of mesenchyme and the microbiome, which also contribute to the organ complexity (Hofer & Lutolf, 2021). Additionally, there is a lack of immune system and a restriction in the study of multiorgan diseases since interorgan communication is mostly not given (J. Kim et al., 2020; M. H. Little & Combes, 2019; Phipson et al., 2019; Stein et al., 2021).

Despite the limitations, the organoid technology poses a promising future for regenerative and personalized medicine. Plenty studies describe the use of organoids for developmental research, for disease modelling, for drug discovery and toxicity tests and they have sheer endless possibilities for personalized therapies.

1.2.6 Kidney organoids

In the last years, researchers developed various differentiation protocols for the generation of kidney organoids. In most cases, kidney organoids are generated from PSCs (Low et al., 2019; Morizane & Bonventre, 2017; Takasato et al., 2015). These differentiation protocols were developed based on the knowledge about kidney development gained so far. Shortly, PSCs are induced to form the primitive streak. After that, intermediate mesoderm follows, whereas depending on the time frame of WNT signal, development towards the anterior or posterior intermediate mesoderm is induced. The next step is then the induction of metanephric mesoderm or ureteric bud. As mentioned before (*see* 1.1.3), the nephron derives from the metanephric mesenchyme, while the ureteric bud gives rise to the collecting duct system. Most protocols induce the formation of nephrons, resulting in kidney organoids with nephron systems but lacking collecting duct systems. This mainly is attributed to the different origins of the tissues. While the nephrons derive from the posterior intermediate mesoderm, the collecting duct system is a derivate of the anterior intermediate mesoderm. *Taguchi & Nishinakamura* (2017) described a protocol for the separate generation of nephron progenitors and ureteric buds from murine PSCs and a combination of the tissues with isolated stromal progenitors led to the assembly of higher-order organoids, which recapitulated the

renal branching morphogenesis (Taguchi & Nishinakamura, 2017). Similar experiments with human PSCs did not yield the same results, which was contributed to the lack of stromal cells (Taguchi & Nishinakamura, 2017). Besides the nephron and collecting duct system, the kidney tissue also consists of interstitial and stromal cells. The stroma is likely involved in the nephrogenesis (Wilson & Little, 2021). Interestingly, *in vivo* stromal cells are not only derivates from the renal anlagen, but a subpopulation of stromal progenitor cells also migrate from other sites to the fetal kidney, which is the reason why most induction protocols do not derive stromal cells (Khoshdel-Rad et al., 2022). Similarly, endothelial cells are proposed to be partly differentiated from a certain $SIX2^+/KDR^+$ population of NPCs, while a portion likely migrates from another tissue site (Low et al., 2019). Attempts to counter the lack of a mature vasculature, include subcapsular transplantation into the kidney of immune-compromised mice, which led to a host-derived invasion of vasculature and increased the organoid maturity and functionality (van den Berg et al., 2018). Besides, a particular composition of ECM as well as shear-stress was observed to improve the maturity of vessels in kidney organoids (Homan et al., 2019).

Since the organoid technology is constantly improved to better recapitulate the kidney physiology in an *in vitro* setting, the potential of using kidney organoids for the clinical setting increases step by step.

1.2.7 Disease modelling with kidney organoids

The study of kidney diseases and potential treatments were highly improved by the introduction of kidney organoids. Especially for the discovery of novel drugs and medications, kidney organoids represent a valuable tool to the conventional animal models. However, it has to be acknowledged that fundamental knowledge about diseases was gained by animal testing, which is essential for the successful modelling in organoids.

Kidney diseases caused by genetic mutations, such as autosomal-dominant polycystic kidney disease (ADPKD), can be modelled in kidney organoids via CRISPR-Cas9-guided gene editing, since the disease is commonly caused by mutations in two genes, polycystin-1 (PC-1) and polycystin-2 (PC-2) (Cruz et al., 2017; Benjamin S. Freedman et al., 2015). As the name suggests, PKD is characterized by the formation of fluid-filled cysts, which form from tubular epithelia and lead to functional loss. Modelling PKD in kidney organoids has helped in the

understanding of cystogenesis in the kidney, the microenvironmental influence on cyst formation and can be a potential platform for drug screening (Cruz et al., 2017; Benjamin S. Freedman et al., 2015; Shimizu et al., 2020). Other than that, gene editing was also applied to model Fabry disease, a metabolic multisystemic disease affecting not only the kidney but various other organs (J. W. Kim et al., 2021). Caused by a X-linked mutation in the α-galactosidase A enzyme, the metabolism of sphingolipids is interfered, which finally result in life-threatening pathological conditions of the kidney, brain and heart (J. W. Kim et al., 2021). To model this disease, Kim *et al.* induced a knockout of the gene encoding the α-galactosidase A in human iPSCs via CRISPR-Cas9 and generated kidney organoids. The kidney organoids presented the phenotype of the Fabry disease, such as lipid deposits in form of zebra bodies and the accumulation of the glycolipid globotriaosylceramide (Gb3) in podocytes and tubular cells (J. W. Kim et al., 2021). Accompanying were higher oxidative stress and apoptosis. With the *in vitro* model of Fabry disease the role of glutathione as a potential therapy approach, as it alleviated the level of oxidative stress, was discovered (J. W. Kim et al., 2021).

Alternatively, iPSC reprogramming paved the way for disease modelling of genetic aberrations by using patient-derived cells. Few studies reported the generation of patient-derived kidney organoids, which recapitulated various disease phenotypes (De & Nishinakamura, 2022). One disease, which was studied via patient-derived kidney organoids is the Finnish-type congenital nephrotic syndrome (Tanigawa et al., 2018). Characteristic for the initial stage of Finnish-type congenital nephrotic syndrome is a dysfunctional filtration barrier due to abnormal proteins, resulting in massive protein shedding into the urine. NEPHRIN is involved in the formation of the slit diaphragm and a point mutation in the gene hinders the correct folding, which finally results in congenital nephrotic syndrome. Tanigawa *et al.* have generated kidney organoids from patient-derived iPSCs, which recapitulated the disease phenotype and demonstrated that the correction of the gene leads to correct slit diaphragm formation (Tanigawa et al., 2018). Thus, they were able to show the potential of patient-derived kidney organoids for the understanding of underlying disease mechanisms and encouraging possibilities for the use of gene therapy.

Even though a few studies demonstrated gene correction to reverse phenotypic causes of diseases, the use of kidney organoids for regenerative medicine is still in development. Due to their immaturity, the lower numbers of nephrons and the lower cell complexity than the *in vivo* organ, the lack of vasculature and lower functionality, the use of kidney organoids for the

clinical setting such as renal replacement purposes is far from applicable for medical purposes (Khoshdel-Rad et al., 2022; M. H. Little & Combes, 2019).

2. Aims and Objectives

The aim of this thesis was to understand cellular processes within the kidney organ in order to implement this knowledge for the improvement of kidney cell cultivation, the generation of kidney models as well as to identify renal disease mechanisms.

Considering the fact that renal diseases often affect the structures of the renal filtration apparatus, various renal pathologies can be better comprehended by studying the nephrons. Therefore, the goal was to generate *in vitro* kidney models, which recapitulate the nephron structures. Firstly, a three-dimensional multi-cellular kidney model based on urine-derived renal progenitor cells, mesenchymal stem cells and endothelial cells. Using adult stem cells such as UdRPCs with a restricted differentiation potency results in less complex, epithelial organoids, which are not sufficient for studying disease mechanisms involving the nephron structures. Therefore, the goal was to improve the renal organoid system by alternatively using induced pluripotent stem cells, and further model kidney injury via induction with the nephrotoxin Puromycin Aminonucleoside. UdRPCs, isolated from patient urine, represent another way to investigate disease mechanisms and have a promising prospect for regenerative medicine. Thus, another objective was to continue previous studies on UdRPCs and further decipher signalling pathways relevant for self-renewal and maintenance of the progenitor state.

Finally, both approaches in form of the organoid technology and the promising cell source of UdRPCs, improve the understanding of disease mechanisms and aid the future development of novel therapies.

Structure of this Thesis

In this thesis three publications are presented, which address the potential use of urine-derived renal progenitor cells and iPSC-derived three-dimensional model of the kidney organ to study kidney diseases:

1. Nguyen L, Spitzhorn LS, Adjaye J (2019). **Constructing an Isogenic 3D Human Nephrogenic Progenitor Cell Model Composed of Endothelial, Mesenchymal, and SIX2-Positive Renal Progenitor Cells.** *Stem Cells Int*;2019:3298432. (original research)

2. Nguyen L, Wruck W, Erichsen L, Graffmann N, Adjaye J (2022). **The Nephrotoxin Puromycin Aminonucleoside Induces Injury in Kidney Organoids Differentiated from Induced Pluripotent Stem Cells.** *Cells*;11(4):635. (original research)

3. Nguyen L, Westerhoff M, Thewes L, Wruck W, Reichert A, Berndt C, Adjaye J. **JNK signalling regulates self-renewal of proliferative urine-derived renal progenitor cells via inhibition of ferroptosis.** Under revision at *iScience Journal*. (original research)

A general synopsis of the developmental processes in the kidney organ as well as an overview of stem cell-based technologies was presented in the introduction, while the publications give deeper insights into the background and methodological information. Finally, a summary of the publications will be provided and the findings will be discussed in the scientific context of recent literature and their contribution to the future research.

3. Publications

3.1 Constructing An Isogenic 3D Human Nephrogenic Progenitor Cell Model Composed of Endothelial, Mesenchymal and SIX2-Positive Renal Progenitor Cells

Lisa Nguyen, Lucas-Sebastian Spitzhorn, James Adjaye

Stem Cells International, Volume 2019, Article ID 3298432

Abstract:

Urine has become the source of choice for noninvasive renal epithelial cells and renal stem cells which can be used for generating induced pluripotent stem cells. The aim of this study was to generate a 3D nephrogenic progenitor cell model composed of three distinct cell types—urine-derived SIX2-positive renal progenitor cells, iPSC-derived mesenchymal stem cells, and iPSC-derived endothelial cells originating from the same individual. Characterization of the generated mesenchymal stem cells revealed plastic adherent growth and a trilineage differentiation potential to adipocytes, chondrocytes, and osteoblasts. Furthermore, these cells express the typical MSC markers CD73, CD90, and CD105. The induced endothelial cells express the endothelial cell surface marker CD31. Upon combination of urine-derived renal progenitor cells, induced mesenchymal stem cells, and induced endothelial cells at a set ratio, the cells self-condensed into three-dimensional nephrogenic progenitor cells which we refer to as 3D-NPCs. Immunofluorescence-based stainings of sectioned 3D-NPCs revealed cells expressing the renal progenitor cell markers (SIX2 and PAX8), podocyte markers (Nephrin and Podocin), the endothelial marker (CD31), and mesenchymal markers (Vimentin and PDGFR-*β*). These 3D-NPCs share kidney progenitor characteristics and thus the potential to differentiate into podocytes and proximal and distal tubules. As urine-derived renal progenitor cells can be easily obtained from cells shed into urine, the generation of 3D-NPCs directly from renal progenitor cells instead of pluripotent stem cells or kidney biopsies holds a great potential for the use in nephrotoxicity tests, drug screening, modelling nephrogenesis and diseases.

Author contribution: 80%
L.N. designed and executed experiments, analysed data, wrote and edited the manuscript.

Status: Published in *Stem Cells International* (doi.org/10.1155/2019/3298432).

Hindawi
Stem Cells International
Volume 2019, Article ID 3298432, 11 pages
https://doi.org/10.1155/2019/3298432

Research Article

Constructing an Isogenic 3D Human Nephrogenic Progenitor Cell Model Composed of Endothelial, Mesenchymal, and SIX2-Positive Renal Progenitor Cells

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Received 20 December 2018; Accepted 20 March 2019; Published 2 May 2019

Academic Editor: Stefan Liebau

Urine has become the source of choice for noninvasive renal epithelial cells and renal stem cells which can be used for generating induced pluripotent stem cells. The aim of this study was to generate a 3D nephrogenic progenitor cell model composed of three distinct cell types—urine-derived SIX2-positive renal progenitor cells, iPSC-derived mesenchymal stem cells, and iPSC-derived endothelial cells originating from the same individual. Characterization of the generated mesenchymal stem cells revealed plastic adherent growth and a trilineage differentiation potential to adipocytes, chondrocytes, and osteoblasts. Furthermore, these cells express the typical MSC markers CD73, CD90, and CD105. The induced endothelial cells express the endothelial cell surface marker CD31. Upon combination of urine-derived renal progenitor cells, induced mesenchymal stem cells, and induced endothelial cells at a set ratio, the cells self-condensed into three-dimensional nephrogenic progenitor cells which we refer to as 3D-NPCs. Immunofluorescence-based stainings of sectioned 3D-NPCs revealed cells expressing the renal progenitor cell markers (SIX2 and PAX8), podocyte markers (Nephrin and Podocin), the endothelial marker (CD31), and mesenchymal markers (Vimentin and PDGFR-β). These 3D-NPCs share kidney progenitor characteristics and thus the potential to differentiate into podocytes and proximal and distal tubules. As urine-derived renal progenitor cells can be easily obtained from cells shed into urine, the generation of 3D-NPCs directly from renal progenitor cells instead of pluripotent stem cells or kidney biopsies holds a great potential for the use in nephrotoxicity tests, drug screening, modelling nephrogenesis and diseases.

1. Introduction

Many disease conditions, including renal diseases, require replacement of tissues or organs. Organ or tissue transplantation is the only effective and most widely used medical treatment [1]. As stem cells can be used for the generation of autologous, specialized cell types, stem cell-based therapies are an alternative to transplantation [2]. However, both treatments face major problems: worldwide donor shortage, poor immunohistocompatibility between the donor and recipient, and the probability of side effects such as teratoma and tumor formation upon stem cell therapy. An alternative to kidney transplantations is the use of renal progenitor cells, which can be isolated from human urine [1], in order to generate kidney cell types and subsequently transplantable renal tissues. Physiological processes in the kidney result in thousands of viable kidney cells being shed into the urine [1, 3]. The cell type of interest, i.e., urine stem cell or urine-derived renal progenitor cell (UdRPC), is required for the renewal of kidney cells [4]. UdRPCs have a rice grain-like morphology [3] and share stem cell characteristics including clonogenicity, high expansion capacity, multipotent differentiation potency, and self-renewal driven by SIX2 [3, 5, 6]. In addition, these cells have the potential to be differentiated into numerous cell types present within the kidney.

The three-dimensional organoid technology is another alternative. Here, cells of the organ of interest, such as heart, liver, or kidney, are generated from induced pluripotent stem cells (iPSCs) in a 3D manner, named organoids. Because these organoids are composed of organ-specific cells which can self-organize, they are able to recapitulate some of the typical organ structures and functions [7, 8]. Other three-

dimensional models include gastruloids, defined as *in vitro* multicellular models capable of mimicking the gastrulation process [9]. Published reports have shown successful generation of organoids derived from tissues such as the optic cup [10], hypophysis epithelium [11], intestine [12], cerebrum [13], and kidney [14]. Current shortfalls of existing organoid models include the lack of vascularization and the associated supply with nutrients and oxygen through blood flow as well as the organization of complex structures. Moreover, this kind of tissue engineering is based on the use of specific inducing factors and scaffolds, which cannot fully recapitulate the *in vivo* microenvironment needed for cell-cell interactions in the changing fluidity during organogenesis [15]. In light of these shortfalls, the generation of organoids by imitating the multicellular interactions in the *in vivo* organ is the next step needed to enhance organoid technology, especially in the kidney.

Here, we describe the generation and characterization of 3D-NPCs (three-dimensional nephron progenitor cells) composed of three cell types—SIX2-positive urine-derived renal progenitor cells (UdRPCs), UdRPC-iPSC-derived mesenchymal stem cells (UdRPC-iMSCs), and endothelial cells (UdRPC-iECs) to mimic the multicellular organization of the *in vivo* organ. The combination of the aforementioned cell types resulted in self-condensed 3D-NPCs, maintaining the expression of the renal progenitor marker SIX2 when cultured in self-renewal supportive medium. 3D-NPCs can be harnessed for efficient generation of kidney organoids useful as a platform for studying nephrogenesis, kidney disease modelling, and nephrotoxicity testing.

2. Materials and Methods

2.1. iPSCs from Urine-Derived Renal Progenitor Cells (UdRPCs). The iPSC line used, ISRM-UM51, here called UdRPC-iPSCs, was reprogrammed from renal progenitor cells (UdRPCs) isolated from urine samples as described before [16, 17]. ISRM-UM51 is of known HLA and has a CYP2D6 status of an intermediate metabolizer [17].

2.2. Differentiation of UdRPC-iPSCs to Endothelial Cells (UdRPC-iECs). Prior to differentiation, UdRPC-iPS cells were adapted to E8 medium (STEMCELL Technologies) on Matrigel-coated plates (Corning Incorporated, #354277). At 80–90% confluency, cells were dissociated with 0.05% ED TA/PBS and single cells were seeded on Matrigel-coated plates with an addition of ROCK inhibitor Y-27632 (10 μM) (Tocris Bioscience, #1254/1) for the first 24 h to improve cell survival. When the cell density reached 70-80%, mesoderm formation was induced for 44 h by cultivating the cells in E8 medium containing 25 ng/ml activin A (PeproTech, #120-14E), 5 ng/ml BMP4 (PeproTech, #120-05ET), and 1 μM CHIR99021 (Tocris Bioscience, #TB4423-GMP/10) [18]. The medium was changed to E7 medium (STEMCELL Technologies, #05910) supplemented with 50 ng/ml BMP4, 5 μM SB431542 (Tocris Bioscience, #1614), and 50 ng/ml VEGF-A (PeproTech, #100-200) for three to five days. Endothelial cells were maintained in E7 medium supplemented with 50 ng/ml VEGF-A or Medium 200 (Gibco, #M200500) at 37°C and 5% CO_2. Human umbilical cord vein endothelial cells (HUVECs) were used as a control.

2.3. Differentiation of UdRPC-iPSCs to Mesenchymal Stem Cells (UdRPC-iMSCs). The UdRPC-iPSCs were split into single cells at a confluency of 90-100% by incubating with TrypLE (Gibco, #12604021) for 4 min. Single cells were seeded on Matrigel-coated 6-well plates. As described before, differentiation was prepared at 60-70% confluency [19, 20]. Maintenance medium was replaced with mesenchymal stem cell (MSC) differentiation medium composed of Minimum Essential Medium Eagle (α-MEM) (Sigma-Aldrich, #M8042-6x500ml), 10% FBS (Gibco, #10500064), 1% P/S (Invitrogen, #15140122), 1% GlutaMAX (Gibco, #35050061), and 10 μM of the TGFβ-receptor inhibitor SB431542. Cell differentiation was carried out for 14 days, and medium was changed every second day. Afterwards, the cells were passaged with TrypLE and were plated onto uncoated flasks. Passaging was continued until the cells attained an MSC-like morphology. The cells were kept in MSC cultivation medium (α-MEM, 10% FBS, 1% P/S, and 1% GlutaMAX) lacking SB431542. Differentiation of resulting UdRPC-iMSCs was carried out afterwards to evaluate their trilineage differentiation potential. In addition, the expression of typical MSC cell surface markers and the absence of hematopoietic markers were analysed via flow cytometry.

2.4. In Vitro Differentiation Assays

2.4.1. Adipogenesis. Induction of adipogenesis was performed by incubating UdRPC-iMSCs in adipoinductive medium (Gibco, #A1007001) for three weeks with medium changes every second day. Formation of lipid droplets was detected via Oil Red O staining (Sigma-Aldrich, #1320-06-5).

2.4.2. Chrondrogenesis. Chondrogenesis of UdRPC-iMSCs was induced with chondroinductive medium (Gibco, #A10 07101), and cells were cultivated for three weeks with regular medium changes every second day. Cartilage formation was confirmed with Alcian Blue staining (Sigma-Aldrich, #33864-99-2).

2.4.3. Osteogenesis. UdRPC-iMSCs were seeded in two wells of a 24-well plate and were incubated in osteoinductive medium (Gibco, #A1007201) for three weeks with medium changes every second day. To demonstrate the successful differentiation, calcium depots were identified with Alizarin Red staining (Sigma-Aldrich, #130-22-3).

2.5. Immunophenotyping of UdRPC-iMSCs. For the immunophenotyping, two biological replicates per cell type, namely, UdRPC-iMSCs, native UdRPCs and native human fetal MSCs [21], were analysed. Each replicate was divided into two aliquots, each containing 1×10^5 cells. MSC phenotyping cocktail (cocktail of fluorochrome-conjugated monoclonal antibodies: CD14-PerCP, CD20-PerCP, CD34-PerCP, CD45-PerCP, CD73-APC, CD90-FITC, and CD105-PE) or the isotype control cocktail (cocktail of fluorochrome-conjugated monoclonal antibodies: mouse IgG1-FITC, mouse IgG1-PE, mouse IgG1-APC, mouse IgG1-PerCP, and mouse IgG2a-PerCP) was added to the samples. The

Table 1: List of antibodies and dilution for immunofluorescence staining.

Immunofluorescence antibody	Specificity	Dilution	Company	Cat. No.
CD31	Mouse	1 : 300	R&D	BBA7
NPHS1	Rabbit	1 : 200	Invitrogen	PA5-20330
NPHS2	Rabbit	1 : 400	Proteintech	20384-1-AP
PAX8	Rabbit	1 : 200	Cell Signaling	59019
PDGFR-β	Rabbit	1 : 100	Cell Signaling	3169
SIX2	Rabbit	1 : 200	Proteintech	11562-1-AP
Vimentin	Rabbit	1 : 200	Cell Signaling	5741
Alexa 488	Rabbit	1 : 500	Invitrogen	A-11034
Alexa 555	Rabbit	1 : 500	Invitrogen	A-21428
Cy3	Mouse	1 : 500	Invitrogen	A10521
NANOG	Rabbit	1 : 800	Cell Signaling	4903T
SSEA4	Mouse	1 : 1000	Cell Signaling	4755T
TRA-1-81	Mouse	1 : 1000	Cell Signaling	4745T

cells were incubated with the respective antibody cocktail for 10 min at 4°C in the dark with occasional swaying of the tubes. Cells were washed afterwards, and the fixed samples were measured using the CyAn ADP (Beckman Coulter, CA, USA) and analysed using the Summit 4.3 software.

2.6. Immunofluorescence-Based Staining. Paraformaldehyde (Polysciences, #18814-10) fixed samples were washed with 1% Triton X-100/PBS (Merck, #9002-93-1). If staining for cell surface markers was intended, washing was done with PBS instead. After this step, samples were washed twice with PBS. To block unspecific binding sites, the sample was incubated with blocking buffer for 2 h at room temperature.

The primary antibody was incubated overnight at 4°C. The respective antibody was diluted following the instructions in Table 1. The following day, samples were washed three times with 0.05% Tween/PBS (Merck, #9005-64-5). The secondary antibody (solved 1 : 500 in blocking buffer/PBS of a ratio 1 : 2) and Hoechst (Thermo Fisher, #H3570) (1 : 5000) were added and incubated for 1 h in the dark at room temperature. After washing the samples twice with 0.05% Tween/PBS, the plates were kept in 1% PS/PBS at 4°C until evaluation under a fluorescence microscope X-Cite series 120 Lumen Dynamics (Zeiss).

2.7. Generation of 3D-NPCs Based on the Coculture of UdRPCs, UdRPC-iMSCs, and UdRPC-iECs. The medium for 3D-NPC maintenance was prepared by adding 5 ng/ml VEGF-A, 1 μg/ml heparin, and 5 ng/ml EGF (PeproTech, #100-47) to renal progenitor maintenance medium (RPMM) [16, 17]. Confluent wells of UdRPCs, UdRPC-iMSCs, and UdRPC-iECs were incubated with TrypLE at 37°C until cells detached; thereafter, RPMM was added to stop the enzymatic reaction. UdRPCs and UdRPC-iMSCs were centrifuged at 250 ×g for 5 min, and UdRPC-iECs were centrifuged at 150 ×g for 5 min. After aspirating the supernatant and replenishing with fresh medium, cells were counted. The seeding ratio between the three cell types was 10 : 7 : 2 (UdRPCs, UdRPC-iMSCs, and UdRPC-iECs). The required cell number of one combination process was as follows: 1×10^6 UdRPCs, 0.7×10^6 UdRPC-iMSCs, and 0.2×10^6 UdRPC-iECs. The cell types were resuspended in 1 ml RPMM. After mixing the three cell types, the cell suspension was added to a T25 flask with 7 ml RPMM, filling up to a total volume of 10 ml. ROCK inhibitor Y-27632 (10 μM) was added on day one to ensure cell survival. The flask was placed in an upright position in the incubator at 37°C and 5% CO_2. After 14 days of cultivation, condensed 3D-NPCs were transferred into a petri dish and kept at 37°C and 5% CO_2 in a rotating incubator. Approximately 90% of the condensation experiments resulted in three-dimensional, non-adherent 3D-NPCs.

3. Results and Discussion

3.1. Derivation of UdRPC-iMSCs from UdRPC-iPSCs. In this study, UdRPC-iMSCs were successfully generated from the iPSC line UM51 reprogrammed from UdRPCs [17]. The criteria defining mesenchymal stem cells include plastic adherence, trilineage differentiation potential to adipocytes, chondrocytes, and osteoblasts, expression of cell surface markers CD73, CD90, and CD105 (95% and higher), and absence of hematopoietic markers CD14, CD20, CD34, and CD45 [22]. The UdRPC-iMSCs displayed a fibroblast-like and spindle-shaped morphology and were able to adhere to plastic surfaces (Figure 1(a)). Their potential to differentiate to clinical relevant chondrogenic and osteogenic fate was observed by Alcian Blue and Alizarin Red staining (Figure 1(a)). Additionally, the potential to differentiate into adipocytes was shown by Oil Red O staining (Figure 1(a)).

Immunophenotyping of the UdRPC-iMSCs confirmed the expression of the typical MSC cell surface markers CD73, CD90, and CD105 and absence of the hematopoietic markers CD14/CD20/CD34/CD45 (1.59 ± 0.7%, Figure 1(b)). The levels of CD73 and CD105 were 98.30 ± 0.3% and 98.27 ± 0.3%, respectively (Figure 1(b)). UdRPC-iMSCs had a lower level CD90 (25.25 ± 6.1%) compared to bone marrow MSCs (Figure 1(b)). The reference gold standard bone

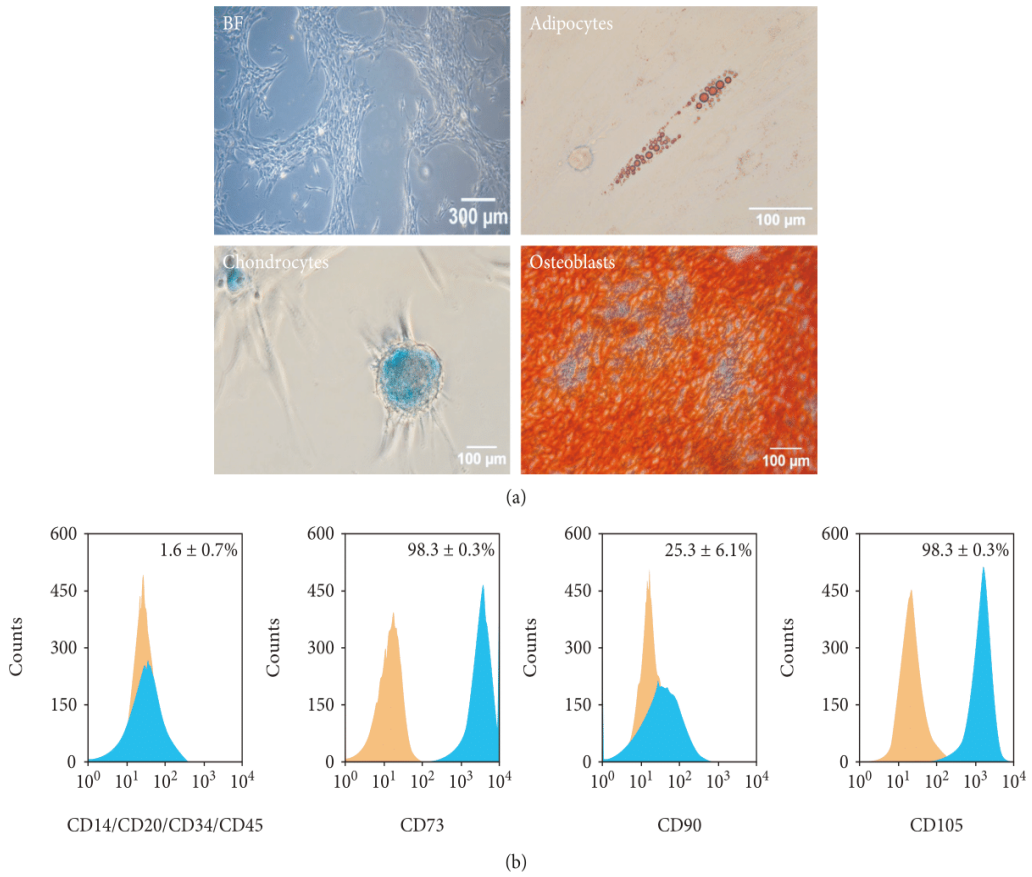


FIGURE 1: UdRPC-iMSCs are MSCs and bear characteristic MSC features. (a) Cell morphology and trilineage differentiation potential of UdRPC-iMSCs. UdRPC-iMSCs are elongated and spindle-shaped and possess trilineage differentiation potential to adipocytes, chondrocytes, and osteoblasts. (b) Immunophenotype of the generated UdRPC-iMSC line. Expression of MSC cell surface markers CD73, CD90, and CD105 and the hematopoietic markers CD14, CD20, CD34 and CD45 was analysed. Histograms of IgG control are displayed in orange, and histograms of MSC markers are displayed in blue ($n = 2$).

marrow-derived MSCs have more than 95% $CD90^+$ cells (Figure S1A). However, MSCs isolated from distinct organs and origins are known to express a diverse set of MSC cell surface markers and even with varying degrees of expression [23]. In contrast, the native UdRPCs, from which the UdRPC-iMSCs originate, have high levels of CD73 ($99.11 \pm 0.3\%$) and CD90 ($79.28 \pm 3.6\%$) and a low level of CD105 ($10.92 \pm 0.6\%$) (Figure S1B). Urine-derived stem cells have been described to express high levels of CD29, CD44, and CD73 (>98%) and a variable expression of CD54, CD90, CD105, and CD166 [24, 25]. These variations between MSCs may be due to inherent functional differences and the fact that the cells are part of a heterogeneous subpopulation within tissues [23]. Since UdRPC-iMSCs bear MSC features other than 95% CD90 expression, i.e., plastic adherence and the trilineage differentiation to adipogenic, chondrogenic, and osteogenic fate, UdRPC-iMSCs are considered MSC-like.

Additionally, immunofluorescence-based staining also revealed the expression of the MSC markers α-SMA, Vimentin, and PDGFR-β (Figure 2). As MSCs are found in almost all tissues of the human body, UdRPC-iMSCs are perfectly suited for the generation of organoids consisting of distinct cell types. MSCs have been described to be important for the process of self-condensation in the generation of organoids where contractions of the actomyosin cytoskeletal axis of MSCs play the key role [2, 26]. Condensation did not occur in the absence of MSCs and organoids could not form [2]. This observation was also made in this study; even though UdRPCs are MSCs, incubation of UdRPCs alone only led to emerging 3D cell aggregates without the typical round organoid structures with borders typical of 3D-NPCs (data not shown). It is known from embryonic invagination that Myosin II is active during this developmental process which leads to inward dislocation of cell-cell junctions [2, 26]. Takebe et al. were able to show that in MSCs, Myosin II was highly expressed just before condensation took place [2]. Furthermore, it has been shown that progressive recruitment of mesenchymal progenitors plays a fundamental role in cell fate acquisition during nephrogenesis in mice and human [27]. Another important role of MSCs was described by Tögel et al., where MSCs were injected into rat models

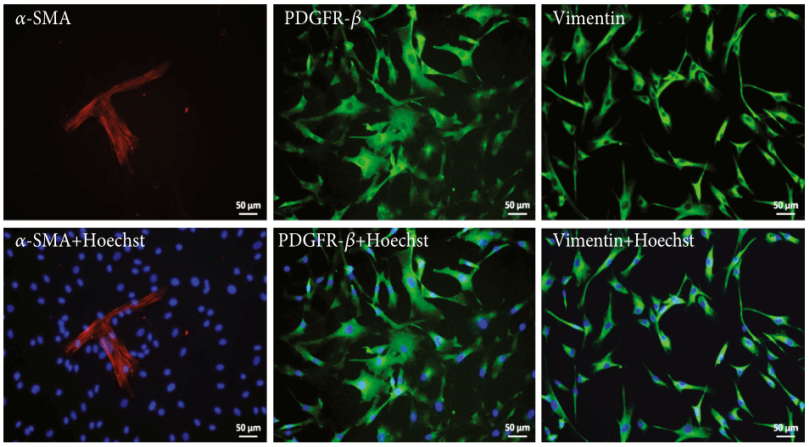


FIGURE 2: Expression of MSC markers in UdRPC-iMSCs. Stainings were carried out for the expression of the mesenchymal markers—α-SMA, Vimentin, and PDGFR-β. Cell nuclei were stained with Hoechst. Pictures were taken under 20x magnification.

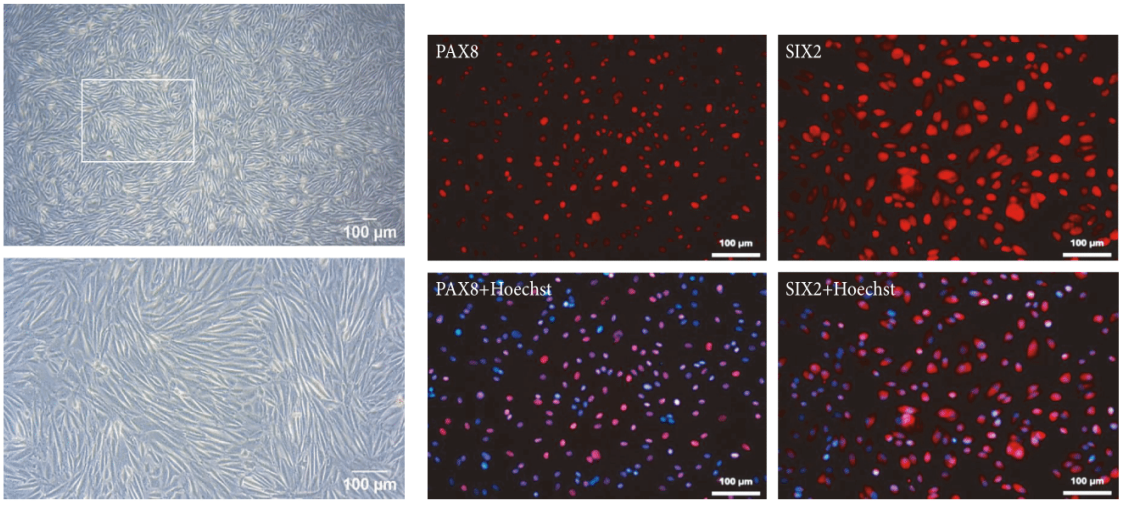


FIGURE 3: Phase contrast image of UdRPCs and expression of kidney-related markers. (a) Cell morphology of UdRPCs. (b) Stainings were carried out for PAX8 and the nephron progenitor marker SIX2. Cell nuclei were stained with Hoechst. Pictures were taken under 10x and 20x magnification.

suffering from reperfusion-induced acute renal failure [28]. The injected MSCs were able to protect renal cells from further damage and partly restored renal functions by secretion of anti-inflammatory factors.

3.2. Urine-Derived Renal Progenitor Cells (UdRPCs). UdRPCs were isolated from voided urine of a male donor of African origin [17]. When kept in proliferation medium, they retained the typical rice grain-like morphology (Figure 3(a)) and expressed PAX8 and SIX2 (Figure 3(b)).

3.3. Generation of Endothelial Cells from UdRPC-iPSCs. UdRPC-iPSCs were differentiated to endothelial cells (UdRPC-iECS) using a modified two-step protocol [18]. The differentiated cells had a cobblestone-like morphology with broad cell bodies and grew as a thin adherent cell layer (Figure 4(a)). Like HUVECs, UdRPC-iECs uniformly expressed the endothelial cell surface marker CD31 (Figure 4(b)). Cell sizes of UdRPC-iECs were smaller than those of HUVECs (Figure 4(b)) which could be explained by a lower passage number and the fact that they were derived from iPSCs which are small in size themselves. Since *in vivo* vasculature for nutrient and oxygen supply is established in the early embryonal development, UdRPC-iECs were used for the formation of kidney preorganoids which should support the sufficient availability with nutrients and oxygen and allow further maturation of kidney structures.

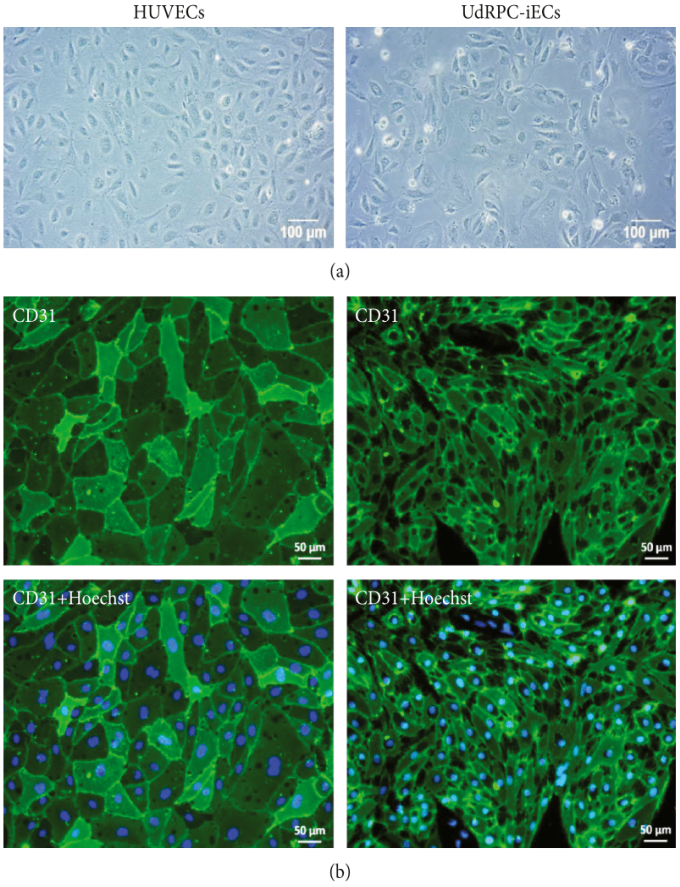


FIGURE 4: Comparison of UdRPC-iECs with HUVECs. (a) UdRPC-iECs had a broad, cobblestone-like morphology similar to HUVECs. (b) Expression of the endothelial cell surface marker CD31 in HUVECs and UdRPC-iECs. Cell nuclei were stained with Hoechst.

3.4. Formation of 3D-Nephron Progenitor Cells. Three-dimensional nephron progenitor cells (3D-NPCs) were generated by combining urine-derived SIX2-positive renal progenitor cells (UdRPCs), UdRPC-iMSCs, and UdRPC-iECs at a ratio of 10 : 7 : 2. The cell mixture self-condensed after 2 to 4 days forming round-shaped, three-dimensional structures with sharp borders (Figure 5). The 3D-NPCs were transferred to petri dishes 14 days after the respective cells were combined.

3.5. Expression of Renal, Endothelial, and Mesenchymal Markers in 3D-NPCs. After three to four weeks of cultivation, 3D-NPCs were fixed, dehydrated, and subsequently embedded in the preparation of cryosectioning. The sections were then stained for the expression of several kidney-specific markers, such as SIX2, PAX8, Nephrin, and Podocin, endothelial marker- CD31, and mesenchymal markers, PDGFR-β and Vimentin (Figure 6).

3D-NPCs express the renal progenitor marker SIX2 which in mice has been shown to be expressed during early kidney development, especially in the cap mesenchyme, a region consisting of progenitor cells committed to the nephron fate [27, 29]. This gene is involved in the maintenance of the progenitor state, and the depletion of SIX2 leads to the differentiation of the progenitor cells towards cell types making up the nephron, the functional unit of the kidney, including podocytes and distal and proximal tubules.

The early renal marker PAX8 is uniformly expressed in 3D-NPCs (Figure 6). PAX8 expression is maintained throughout nephron morphogenesis, emerging at the renal vesicle stage, and regulates kidney organogenesis [30, 31]. Cytoplasmic expression of Nephrin was not as uniform as seen for SIX2 and PAX8, but more localized (Figure 6). Nephrin is a protein of the immunoglobulin superfamily of cell adhesion receptors and is present in epithelial podocytes which wrap around the glomeruli and are part of the glomerular filtration barrier [32]. The podocytic foot processes are interconnected via slit diaphragms which are formed by Nephrin, Podocin, TRPC6, and FAT1 [33, 34]. Expression of the membrane protein Podocin, encoded by *NPHS2*, was detected on the plasma membrane of cells within the 3D-NPCs (Figure 6). It has to be noted that native UdRPCs express SIX2 [17], Nephrin, and Podocin (data not shown); therefore, it is further evidence in support of our generated 3D-NPCs.

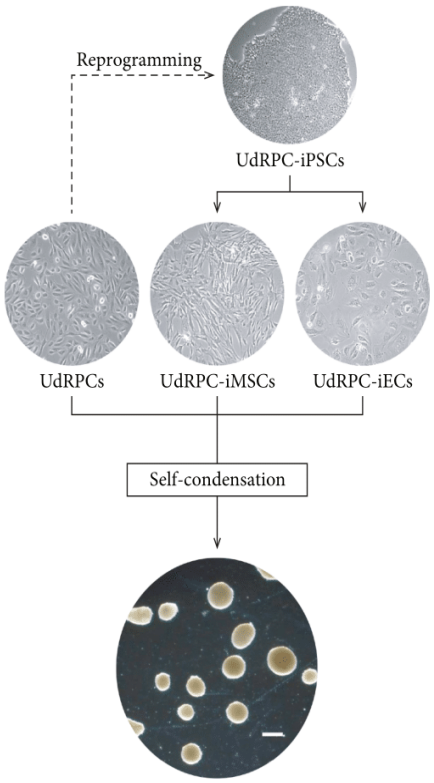


FIGURE 5: Overview of the formation of three-dimensional nephron progenitor cells (3D-NPCs). Isogenic 3D-NPCs were generated from three cell types namely SIX2-positive UdRPCs, UdRPC-iMSCs, and UdRPC-iECs of the same genetic background. The scale bar corresponds to a length of 500 μm.

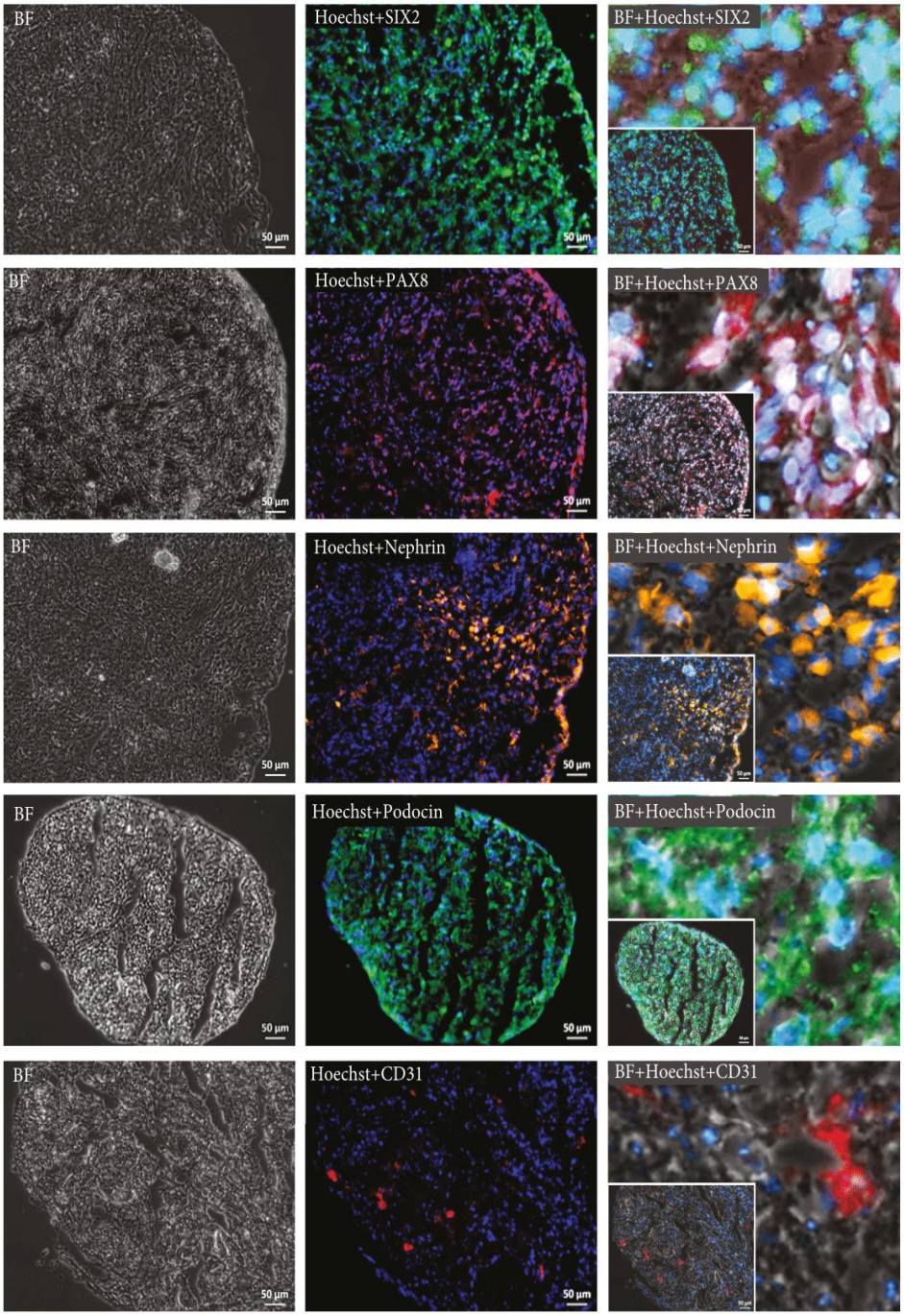
Furthermore, 3D-NPCs harbour endothelial cells which express the cell surface marker CD31 (Figure 6). CD31 is also known as PECAM-1, a glycoprotein, and besides being present on the cell surface of endothelial cells, CD31 can also be found on platelets and some leukocytes [35]. This protein is involved in the adhesion between the endothelial cells by intercellular junctions [35]. Expression of the MSC markers Vimentin and PDGFR-β was not uniformly distributed as seen for PAX8. Vimentin is a type III intermediate filament, which forms the cytoskeleton together with microtubules and actin filaments. This protein is important for the maintenance of cell and tissue integrity [36]. Vimentin was also found to contribute to epithelial to mesenchymal transition (EMT) by upregulating the expression of EMT-related genes [37]. PDGFR-β is a receptor protein for the mitogen PDGF [38] and is involved in the development of mesenchymal stem cells. As mentioned before, MSCs are essential for self-condensation of organoids. In this case, UdRPC-iMSCs might have been involved in the condensation process where the contractile force of the cytoskeleton leads to 3D formation [2]. The addition of UdRPC-iMSCs should also be beneficial for the vascularization of 3D-NPCs. MSCs are in particular known to secrete a variety of growth factors and cytokines, some of them with proangiogenic properties such as VEGF-A, interleukin- (IL-) 6, IL-8, HGF, and PDGF [19, 38, 39].

Additionally, the sections were also stained for the expression of the pluripotency-associated proteins TRA-1-81, SSEA4, and NANOG (Figure 6). We chose to analyse NANOG expression because the cytoplasmic variant is known to be expressed in the kidney [40].

3.6. The Generation of 3D Kidney Organoids. The generation of kidney organoids has advanced in recent years. Compared to the 2D approach to cultivate renal tissues, 3D culture systems better mimic the *in vivo* configuration. Most protocols are based on the use of human pluripotent stem cells (ESCs and iPSCs) differentiated via formation of the intermediate mesoderm into renal structures [14, 41]. Alternatively, kidney tissues have been generated with a two-step protocol, starting with the formation of pluripotent stem cell-derived embryoid bodies followed by chemical-induced differentiation to kidney cell lineages including podocytes, cells of proximal and distal tubules, and collecting ducts [42]. In order to capture the complexity of the kidney organ, multicellular kidney spheroids from a coculture of PSCs, MSCs, and HUVECs driven by mesenchymal cell condensation were engineered by Takebe et al. [26] and Takahashi et al. [38]. Upon transplantation into mice, an *in vivo* environment, connection to the donor vasculature and self-organization into functional tissues fulfilling organ functions such as urine production were observed [26]. Moreover, instead of pluripotent stem cells, murine and human primary kidney cells isolated from biopsies have been described for the generation of three-dimensional renal structures *in vitro* [43, 44]. As renal development is completed before birth, isolation of human NPCs however is difficult. Several groups have worked on optimizing this isolation process as well as the *in vitro* cultivation conditions. Methods for the isolation of human NPCs from the human fetal kidney as well as long-term 3D culture of isolated fetal NPCs with retained nephrogenic potential have been described [45, 46]. With a similar nephrogenic potential as primary NPCs, our novel approach for the generation of 3D-NPCs was based on the use of UdRPCs in combination with isogenic UdRPC-iMSCs and UdRPC-iECs. As urine is an excretion product, isolation of UdRPCs is noninvasive, cost-effective, and indefinite [3]. Moreover, they can be isolated from every donor regardless of age, gender, and health condition. Additionally, even though these cells have moderate telomerase activity, they do not form teratomas or tumors [3, 5].

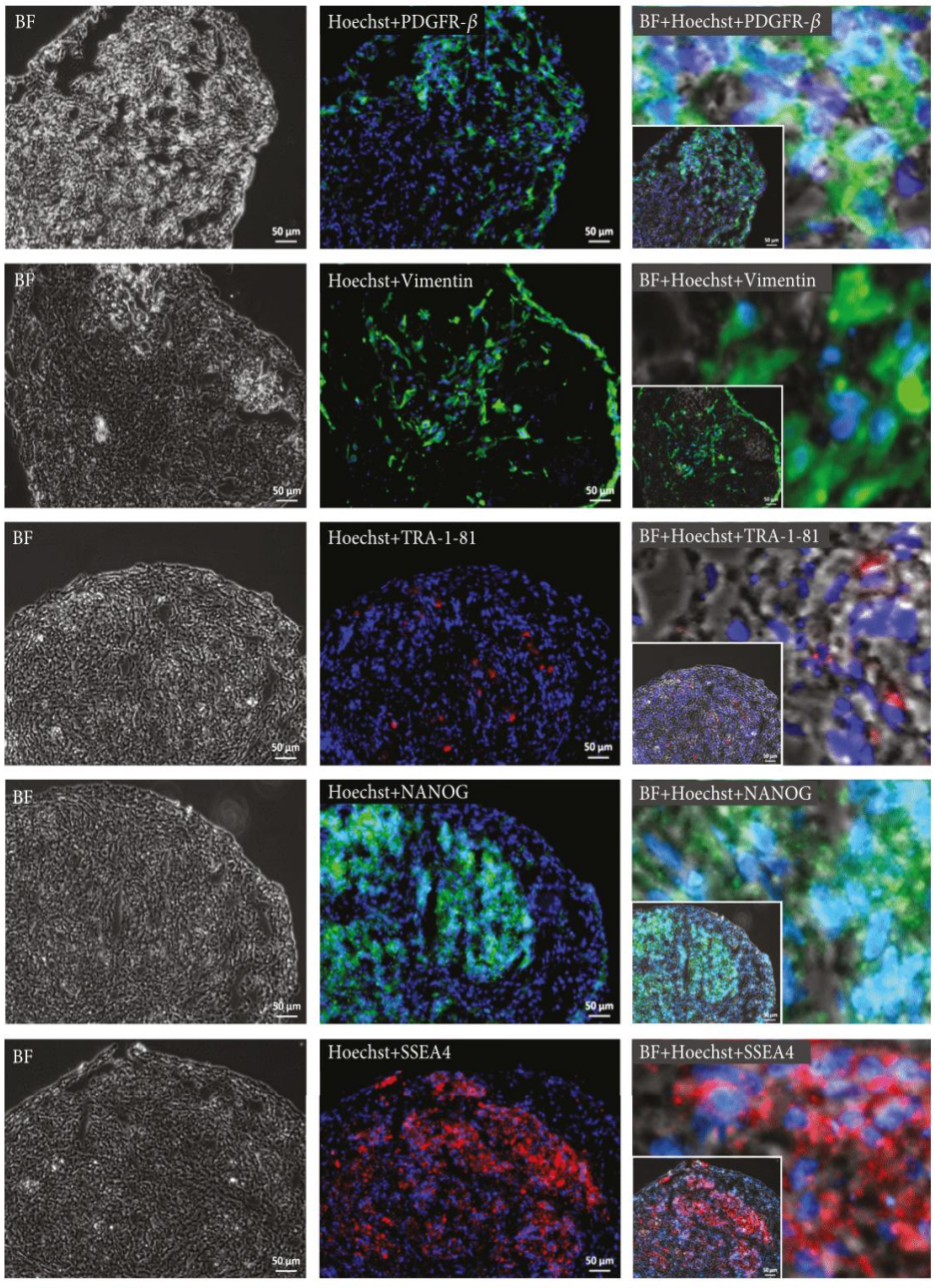
4. Conclusion

Summarizing our study, heterotypic 3D-NPCs were generated by combining UdRPCs, UdRPC-iPSC-derived UdRPC-iMSCs, and UdRPC-iECs originating from the same genetic background, hence isogenic. An immunofluorescence-based analysis demonstrated the expression of the renal progenitor markers (SIX2 and PAX8), the glomerular marker (Nephrin



(a)

FIGURE 6: Continued.



(b)

FIGURE 6: Expression of kidney markers (SIX2, PAX8, Nephrin, and Podocin), endothelial marker (CD31), mesenchymal markers (PDGFR-β and Vimentin), and pluripotency-associated markers (TRA-1-81, NANOG, and SSEA4) in 3D-NPCs. Cell nuclei were stained with Hoechst. Zoom-in pictures show the subcellular localization of the respective proteins. Pictures were taken under 20x magnification. The scale bars represent a length of 50 μm.

and Podocin), and the endothelial marker (CD31) as well as the mesenchymal markers (Vimentin and PDGFR-β). 3D-NPCs have renal progenitor characteristics and therefore have the potential to generate several cell types of the kidney lineage. As the 3D-NPCs arose from isogenic cell types, inducing the differentiation of renal cell types with subsequent organoid formation could lead to future use in cell replacement therapies, drug screening, and nephrotoxicity studies as well as kidney-associated disease modelling.

Data Availability

The photo and plot data used to support the findings of this study are included within the article and in the supplementary files.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

We thank Martina Bohndorf for technical support and Katharina Raba for technical advice on FACS analyses. Prof. Dr. James Adjaye acknowledges support from the Medical Faculty of the Heinrich-Heine-Universität, Düsseldorf. We thank Prof. Richard Oreffo, Southampton University, UK, for providing the fetal MSCs used in this study.

Supplementary Materials

Immunophenotype of fetal MSCs and native UdRPCs. Expression of MSC cell surface markers CD73, CD90, and CD105 and hematopoietic markers CD14, CD20, CD34, and CD45 was analysed. (A) Fetal MSCs. (B) Native UdRPCs. Histograms of IgG control are displayed in orange, and histograms of MSC markers are displayed in blue ($n = 2$). *(Supplementary Materials)*

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3.2 The Nephrotoxin Puromycin Aminonucleoside Induces Injury in Kidney Organoids Differentiated from Induced Pluripotent Stem Cells

Lisa Nguyen, Wasco Wruck, Lars Erichsen, Nina Graffmann, James Adjaye

Cells 11 (2022), 635

Abstract:
Kidney diseases, including acute kidney injury (AKI) and chronic kidney disease (CKD), which can progress to end stage renal disease (ESRD), are a worldwide health burden. Organ transplantation or kidney dialysis are the only effective available therapeutic tools. Therefore, in vitro models of kidney diseases and the development of prospective therapeutic options are urgently needed. Within the kidney, the glomeruli are involved in blood filtration and waste excretion and are easily affected by changing cellular conditions. Puromycin aminonucleoside (PAN) is a nephrotoxin, which can be employed to induce acute glomerular damage and to model glomerular disease. For this reason, we generated kidney organoids from three iPSC lines and treated these with PAN in order to induce kidney injury. Morphological observations revealed the disruption of glomerular and tubular structures within the kidney organoids upon PAN treatment, which were confirmed by transcriptome analyses. Subsequent analyses revealed an upregulation of immune response as well as inflammatory and cell-death-related processes. We conclude that the treatment of iPSC-derived kidney organoids with PAN induces kidney injury mediated by an intertwined network of inflammation, cytoskeletal re-arrangement, DNA damage, apoptosis and cell death. Furthermore, urine-stem-cell-derived kidney organoids can be used to model kidney-associated diseases and drug discovery.

Authors Contribution: 60 %

L.N. designed and performed experiments, analysed the data, wrote and edited the manuscript.

Status: Published in *Cells* (doi.org/10.3390/cells11040635).

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Article

The Nephrotoxin Puromycin Aminonucleoside Induces Injury in Kidney Organoids Differentiated from Induced Pluripotent Stem Cells

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Keywords: urine cells; iPSCs; organoids; puromycin aminonucleoside; AKI; inflammation; apoptosis; DNA damage; RAAS

Citation: Nguyen, L.; Wruck, W.; Erichsen, L.; Graffmann, N.; Adjaye, J. The Nephrotoxin Puromycin Aminonucleoside Induces Injury in Kidney Organoids Differentiated from Induced Pluripotent Stem Cells. *Cells* **2022**, *11*, 635. https://doi.org/10.3390/cells11040635

Academic Editor: Alexander E. Kalyuzhny

Received: 28 October 2021
Accepted: 9 February 2022
Published: 11 February 2022
Corrected: 21 June 2022

1. Introduction

Kidney diseases such as acute kidney injury (AKI) and chronic kidney disease (CKD) are a worldwide health problem. While AKI describes a sudden loss of renal function and can be caused by diverse conditions such as sepsis, nephrotoxicity or ischemia-reperfusion injury (IRI) [1], CKD is defined as a gradual deprivation of kidney function over a time period of more than three months [2]. Major causes of CKD include diabetes and hypertension as well as oxidative stress and inflammation [2,3]. The progression of CKD leads to end-stage renal disease (ESRD) requiring dialysis or organ transplantation [4]. Besides CKD and AKI, various conditions such as diabetes, hypertension and obesity can lead to the emergence of ESRD [5].

Hypertension may result in damage of the glomeruli in the kidney, as an increased hydraulic pressure disrupts the delicate glomerular capillary system [6]. The glomerulus is an organ compartment, essential for blood filtration and the excretion of toxins. The glomerular barrier consists of a basement membrane, endothelial cells and intertwined podocytic foot processes [5]. Part of the filtration barrier is formed by slit diaphragms, assembled by podocyte-associated proteins nephrin, podocin, synaptopodin, CD-2-associated protein (CD2AP) and the zonula occludens protein-1 (ZO-1) [7]. In many cases, the main

target of various kidney diseases, including minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS), are glomerular podocytes [8]. Moreover, various nephrotoxic substances lead to podocyte injury [5,7].

One such nephrotoxic substance is puromycin aminonucleoside (PAN), a purine antagonist, which is classified as an antibiotic, antineoplastic and antimetabolite substance [7]. It inhibits RNA synthesis and is known to induce acute glomerular damage. Interestingly, other than rats, monkeys and humans, no other species is affected by PAN nephrotoxicity [7]. It was first shown in 1990 that PAN affects and alters the ultrastructure of rat glomerular podocytes of kidney slices, leading to a decrease in or loss of microvilli, flattening of the podocyte cell bodies and the emergence of membranous blebbing [9].

To date, human models of kidney diseases are sparse. As most primary cells lose their functionality and viability in vitro, numerous kidney diseases were modelled in rodents [10,11]. However, as murine models do not accurately recapitulate the human disease conditions, the knowledge gained from this research cannot be completely extrapolated to human kidney-associated diseases [10].

Besides improving rodent models, the establishment of pluripotent stem-cell-based kidney cells in 2D or 3D organoids holds great potential for gaining further insights into disease mechanisms and potential therapies [12]. Recent studies have suggested using primary kidney cells for the generation of kidney organoids as an alternative to pluripotent stem cells [13–15]. Kidney organoids are composed of a variety of kidney cell types and fulfil organ functions to a certain degree, which make them the best option for modelling kidney diseases. Approaches to model podocyte injury and possible therapies were made by harnessing PAN treatment on iPSC-derived podocytes and kidney organoids [5].

In this study, we applied puromycin aminonucleoside for the induction of renal damage in our kidney organoids to model kidney injury, and we then used transcriptome-based analyses to identify affected pathways and gene ontologies such as DNA damage and inflammation.

2. Materials and Methods

2.1. Cell Cultivation and Formation of Kidney Organoids

Integration-free iPSC lines used for the generation of kidney organoids were urine-cell-derived—UM51 and UF21 [16,17]—and foreskin-fibroblast-derived—B4 [18]. The cells were reprogrammed with episomal plasmids.

Cells were maintained in mTeSR1 medium (Stem Cell) with daily medium change. Single-cell splitting was carried out at 70–80% confluence. The iPSCs were incubated in 0.5% EDTA/PBS for 5–10 min, and the total cell number was determined by counting in a Neubauer Counting Chamber. Approximately 0.2–0.5 $\times$ 10^5 cells per well were seeded into a low-attachment, 96-well plate (Thermo Fisher, Darmstadt, Germany). The plate was centrifuged at 300$\times$ *g* for 3 min. After 3–5 days, iPSCs clustered to small, round and dense cell aggregates, which were transferred to non-adherent, bacterial 92 $\times$ 16 mm Petri-dishes and placed in a shaking incubator at 37 °C, 5% CO_2, normoxia and 60 rpm. Undifferentiated iPSC spheroids were maintained in basal spheroid medium (BSM) (see Table S1).

The protocols for differentiating iPSC spheroids towards kidney were adapted from Low et al. [19] with slight modifications. The induction of primitive streak was started by culturing the cells for 4 days in basal differentiation medium (BDM) (see Table S2) supplemented with 10 µM CHIR99021 (Tebu-bio, Offenbach, Germany), which is a GSK3 inhibitor and WNT pathway activator. The medium was changed to BDM without additional supplements to achieve the second stage of intermediate mesoderm for 3 days. The emergence of nephron progenitor cells was induced by incubating the cells with BDM supplemented with 3 µM CHIR99021 and 50 ng/mL FGF9 (peprotech, Cranbury, NJ, USA) for 2 days. Over the course of 5 days, the spheroids were fed with BDM plus 50 ng/mL FGF9 to induce nephrogenesis. Starting from D14, a supplementation with 1 µM of CHIR99021 was applied. Patterning was carried out for 6 days with BDM supplemented with 1 µM of CHIR99021. Kidney organoids were then maintained in unsupplemented BDM until further use. Four

independent organoid batches in biological duplicates (see Table 1) were generated. The names of the kidney organoid batches are composed of their origin, the donors' gender and the replicate number (see Table 1). The FFK1/2 organoids were derived from foetal foreskin and were only named after their cellular origin and the replicate number without the donors' gender (see Table 1). The organoids were used for kidney injury modelling by induction with the cytotoxic substance puromycin aminonucleoside (PAN) (Sigma-Aldrich, Taufkirchen, Germany). In previous works, we determined the ideal PAN concentration for our kidney organoids (not shown). We tested the concentrations 10, 50 and 100 µg/mL PAN and selected the concentration of 50 µg/mL for subsequent experiments. To induce kidney damages, kidney spheroids were treated with 50 µg/mL PAN for 48 h.

Table 1. Representation of naming for all kidney organoid batches.

Description	Gender and Ethnicity	Age	Abbreviation
iPSC spheroids derived from urine cell UM51 iPSCs	Male, African	51	iPSC spheroids (SPH)
Kidney organoids derived from urine cell UM51 iPSCs, biological replicate 1	Male, African	51	UM51 kidney organoids_1 (UMK1)
Kidney organoids derived from urine cell UM51 iPSCs, biological replicate 2	Male, African	51	UM51 kidney organoids_2 (UMK2)
Kidney organoids derived from foetal foreskin (FF) iPSCs, biological replicate 1	Male, Caucasian	foetal	FF kidney organoids_1 (FFK1)
Kidney organoids derived from foetal foreskin (FF) iPSCs, biological replicate 2	Male, Caucasian	foetal	FF kidney organoids_2 (FFK2)
Kidney organoids derived from urine cell UF21 iPSCs, biological replicate 1	Female, Caucasian	21	UF21 kidney organoids_1 (UFK1)
Kidney organoids derived from urine cell UF21 iPSCs, biological replicate 2	Female, Caucasian	21	UF21 kidney organoids_2 (UFK2)

2.2. Cryosectioning

Kidney organoids were fixed in 4% Formaldehyde (Polysciences, Warrington, FL, USA) prior to embedding. Dehydration was achieved by washing the cells with distinct concentrations of sucrose solutions. Thereafter, the organoids were placed in moulds filled with TissueTek O.C.T Compound (Sakura Finetek, Umkirch, Germany). 2-methylbutan (Carl Roth, Karlsruhe, Germany) and dry ice were used to snap-freeze the organoids. Sections of 10 µm thickness were prepared with a Cryostat (CM1850, Leica, Nussloch, Germany).

2.3. Western Blotting

Total protein from kidney organoids, treated with 50 µg/mL PAN and the specific untreated control, and the undifferentiated iPSC spheroids was extracted with RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Roche, Mannheim, Germany). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher). Approximately 20 µg of the protein lysates were separated in a 4–12% SDS-PAGE and the proteins transferred to a nitrocellulose membrane by wet blotting. Membranes were then stained with anti-P53, anti-cleaved Caspase 3 and anti-γH2A.X, and compatible secondary antibodies were used subsequently (for dilutions, see Table S3). Beta-actin was used to normalize protein expression. Protein bands were visualized with ECL Western Blotting Detection Reagents (Cytiva, Freiburg, Germany) and detected in

a UV chamber. Band intensity was quantified in the software *Fiji: Image J* (version 1.52a, National Institues of Health, Bethesda, MD, USA) and was normalized to beta-actin levels.

2.4. Immunocytochemistry

Frozen sections were thawed to room temperature. TissueTek was washed off with PBS. Organoid sections were encircled with a hydrophobic pen and were incubated in 0.5% Triton/PBS to permeabilize the cells within the sections. Unspecific binding sites were blocked with 3% BSA for 1 h. The primary antibody solution was incubated overnight at 4 °C (see Table S3). After thoroughly washing off the primary antibodies, 1 h incubation with secondary antibodies was conducted. Nuclei were stained with Hoechst. Stained sections were analysed using a Zeiss fluorescence microscope (LSM 700). Particular staining regions were observed under a Zeiss confocal microscope (LSM 700).

2.5. Immunohistochemistry and Histology

Proximal tubules within the kidney organoid sections were detected by immunohistochemistry via glycoprotein Lotustetragonolobus lectin (LTL) (Vectorlabs, Burlingame, CA, USA). The histochemistry procedure was conducted following the manufacturer's instruction manual. Streptavidin Alexa488 antibody (R&D Systems, Bleiswijk, Netherlands) was used to detect LTL.

Histological images of kidney organoids were prepared with Haematoxylin and Eosin staining. A standard protocol was followed for the histological staining. Tissue structures were imaged under a light microscope.

2.6. Fluorometric Renin Assay

The conditioned media of kidney organoids 48 h after PAN treatment and the specific untreated control were used for the detection of Renin concentration employing the Renin assay kit (Abcam). Samples were prepared in technical duplicates. The fluorometric assay was conducted following the manufacturer's instructions manual. Fluorescence intensity was measured using the micro plate reader infinite M1000 Pro (Tecan, Grödig/Salzburg, Austria) at Ex/Em = 540/590 nm. The renin standards were plotted in a standard curve and were subsequently used for the determination of renin concentration in the conditioned media of kidney organoids.

2.7. In Vitro Dextran Uptake Assay

A 100 µg/mL concentration of Alexa Fluor™ 647-coupled dextran (10.000 MW; Thermo Fisher) was applied to kidney organoids and iPSC spheroids for 4 h. Thereafter, the medium was replenished, and live organoids were visualized under a fluorescence microscope. Kidney organoids and iPSC spheroids were then cultivated for 24 h without dextran and were visualized under a fluorescence microscope.

2.8. qRT-PCR

RNA was isolated from 3-4 kidney spheroids treated with and without PAN as well as the undifferentiated iPSC spheroids using the QIAgen RNeasy Micro Kit. RNA extraction was conducted following the manufacturer's protocol. In brief, kidney spheroids were lysed in 350 µL of RLT buffer. Supernatant was mixed with an equal volume of 70% ethanol and centrifuged through a gDNA eliminator column. After several washing steps, the RNA was solved in 25 µL of RNAse-free water. A total of 500 ng RNA was used as an input for cDNA synthesis, and qRT-PCR based on SYBR Green was conducted. The primer sequences are presented in Table S4.

2.9. Analysis of Gene Expression Data

Duplicates of spheroids and kidney organoids treated with PAN as well as untreated samples were hybridized at the BMFZ (Biomedizinisches Forschungszentrum) core facility of the Heinrich-Heine University (Düsseldorf, Germany) on the Affymetrix Human

Clariom S assay. Raw data (CEL files) delivered from the core facility were read into the R/Bioconductor environment for follow-up-processing. The Bioconductor package oligo [20] was employed to correct data for background signals and to normalize data via the Robust Multi-array Average (RMA) method. Via the VennDiagram package [21], Venn diagrams were generated for the dissection of genes annotated uniquely to microarray probesets. Probesets were considered expressed when their detection-*p*-value was below a threshold of 0.05. The detection-*p*-value was calculated by a statistic over designated background probesets on the microarray, as described in Graffmann et al. [22]. With the R hierarchical clustering function *hclust*, a clustering dendrogram of genes with a coefficient of variation greater than 0.1 was produced using Pearson correlation as the similarity measure and complete linkage agglomeration. Heatmaps and associated clustering analyses were generated with the function *heatmap.2* from the R gplots package [23]. Genes that were expressed in at least one of the experiments were marked with an asterisk (p-value < 0.05).

2.10. Over-Representation Analysis of Pathways and Gene Ontologies (GOs)

The test associated with the hypergeometric distribution which is implemented in R was employed to calculate the over-representation of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways [24], which had been downloaded from the KEGG website in July 2020. GOs were analysed for over-representation via the R package Gostats [25].

2.11. Metascape Analysis

Gene enrichment analyses of differential GO/KEGG terms, biological processes, etc. comparing UMK1_con and UMK1_PAN were performed using the software metascape (http://metascape.org, [26]). Exclusive gene-sets of UMK1_con and UMK1_PAN based on Venn analysis were used as data sources. The metascape software applied hierarchical clustering to display calculated significant GO terms into a tree, which was spread into term clusters with a 0.3 kappa score as a threshold. The top enrichment clusters were represented as heatmaps with a colour scale ranging from grey to dark orange. Statistical significance was hereby displayed in dark orange and lack of enrichment in a grey colour.

2.12. Kidney-Associated Cytokine Assay

Cell culture supernatants of untreated kidney organoids and kidney organoids treated with PAN (2 days after treatment) were kept for cytokine array. Relative expression levels of specific human kidney-associated and urinary proteins were determined using the Human Kidney Biomarker Array Kit from R&D Systems. The cytokine assay was implemented as recommended by the manufacturer. In brief, membranes were blocked for 1 h on a rocking platform. Prepared samples were incubated in the Detection Antibody Cocktail for 1 h at room temperature. Thereafter, the antibody–sample mixtures were pipetted onto the membranes and were incubated overnight at 2–8 °C on a rocking platform. The membranes were washed thoroughly and Streptavidin-HRP was added onto the membranes, which were incubated for 30 min at room temperature. ECL detection reagent was used to detect the spots on the membrane.

2.13. Image and Data Analysis of the Kidney Cytokine Assay

Untreated organoids and organoids treated with PAN, which had been subjected to analysis with the kidney cytokine assay Human Kidney Biomarker Array Kit (R&D Systems) and scanned, were image-analysed with ImageJ [27]. The Microarray Profile plugin by Bob Dougherty and Wayne Rasband (https://www.optinav.info/MicroArray_Profile.htm) was employed to localize and quantify all spots on the array. As read-out, the integrated density generated by the Microarray profile plugin function Measure RT was used. In R/Bioconductor [28], the data resulting from the quantification was normalized with the Robust Spline Normalization from the Bioconductor lumi-package [29].

3. Results

3.1. Kidney Organoids Possess Structured Lobes with Distinct Tubular and Basement Membrane Structures

Kidney organoids of three iPS cell lines in duplicates were generated in approximately 20 days (Figure 1a; Table 1). In contrast to the round-shaped iPSC spheroids, kidney organoids acquired a lobular morphology (Figure 1b). H&E staining of kidney organoid sections revealed structures morphologically similar to renal tubules and glomeruli (Figure 1c; Figure S1a). The tubule-like structures are marked with an asterisk and glomeruli framed with a dashed square (Figure 1c; Figure S1a). The presence of tubule-like structures was also detected by LTL and α-Actinin 4 (ACTN4) staining (Figure 1d). A structure at the rear end, which was negative for LTL but positive for ACTN4, was morphologically similar to a glomerulus and is marked with an arrow (Figure 1d). Additionally, the tubule-like structures were positive for an antibody against the Organic Cation Transporter 2 (OCT2), which marks the tubular plasma membranes (highlighted with a dotted line) (Figure 1d). The functional activity of the kidney organoids was shown with a dextran uptake assay (Figure 1e). A strong fluorescent signal was detected in kidney organoids incubated with dextran for a 4 h pulse (Figure 1e). In comparison, no signal was detected in dextran-treated iPSC spheroids (Figure 1e). After a 24 h chase, kidney organoids still showed a fluorescent signal (Figure 1e).

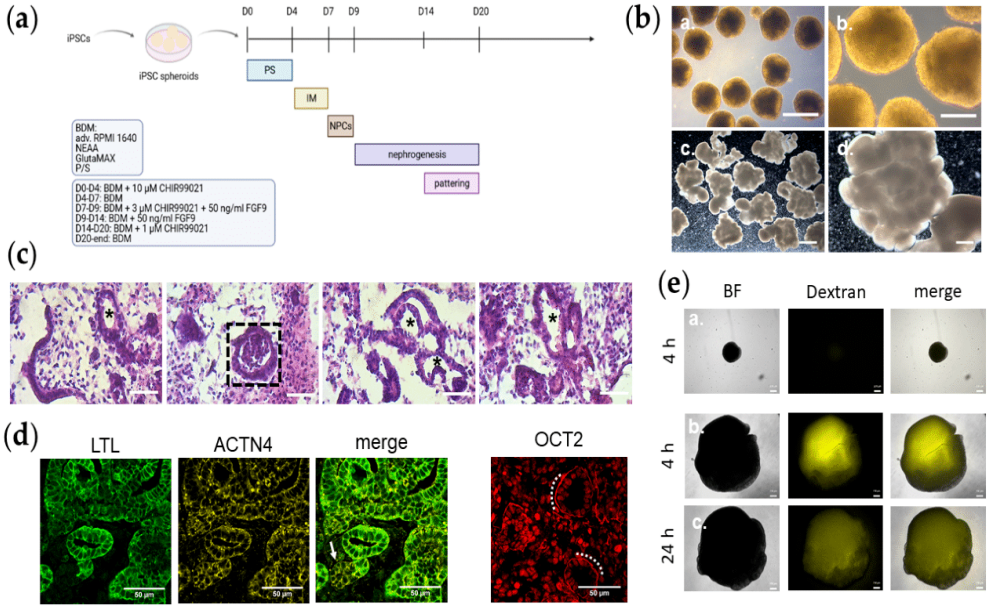

Figure 1. Lobular kidney organoids contain distinct kidney structures. (**a**) Schematic depiction of the protocol for generating kidney organoids. (**b**) Overview of iPSC spheroids at D8 after generation (a.,b.) and kidney organoids UMK2 at D21 (c.,d.) with binocular. (a.) 4× magnification under light microscope. Scale bar depicts 500 µm. (b.) 10× magnification under light microscope. Scale bar depicts 200 µm. (c.) 1× magnification. Scale bar depicts 2000 µm. (d.) 4× magnification. Scale bar depicts 2000 µm. (**c**) Morphology of organoid section via histological H&E staining. A typical glomerulus-like structure is depicted by a dashed rectangle. Tubule-like structures are marked with an asterisk. Scale bar depicts 50 µm. (**d**) Confocal pictures of glomerular (ACTN4, yellow) and tubular (LTL, green) structures in UMK1 sections. Nuclear OCT2 (*POU2F2*) (red) is expressed in UMK1 sections. A glomerulus-like structure was marked with an arrow. Scale bar depicts 50 µm. (**e**) Monitoring of iPSC spheroids and kidney organoids in a dextran uptake assay. (a.) iPSC spheroids treated with dextran after 4 h pulse. (b.) kidney organoids treated with dextran after 4 h pulse. (c.) kidney organoids treated with dextran after 24 h chase. Scale bar depicts 200 µm.

3.2. iPSC Spheroids Lose Pluripotent Gene Expression and Gain Kidney-Associated Genes during Differentiation

Global gene expression was investigated employing RNA Microarray Analysis. RNA of UMK1 kidney organoids, PAN-induced (PAN) and untreated control (con) were prepared in technical duplicates. Along with the aforementioned samples, RNA of iPSC spheroids (SPH) of the same genetic background as UMK1 was also analysed. A cluster dendrogram demonstrated similarities between UMK1_con and UMK1_PAN, whereas the duplicates of SPH clustered separately (Figure 2a). In order to control for successful differentiation into the kidney, we compared the gene-sets between SPH and UMK1_con. This revealed a total common gene-set of 15332 genes (Figure 2b). In total, 500 genes were exclusively expressed in SPH, and 310 exclusive genes were expressed in UMK1_con (Figure 2b). The common gene-set included upregulated and kidney-related GO terms such as "urogenital system development", "mesonephric epithelium development", "ureteric bud development", "renal tubule development" and "nephron epithelium development" (see Supplementary File S1). A heatmap analysis displays the expression of pluripotency-associated genes in UMK1_con and SPH (Figure 2c). The genes *SOX2*, *FGF2*, *DNMT3B*, *CER1*, *GREM1* and *POU5F1* were expressed in SPH, whereas expression of the genes *BMP4*, *TGFB1 and INHBA* was observed in UMK1_con (Figure 2c).

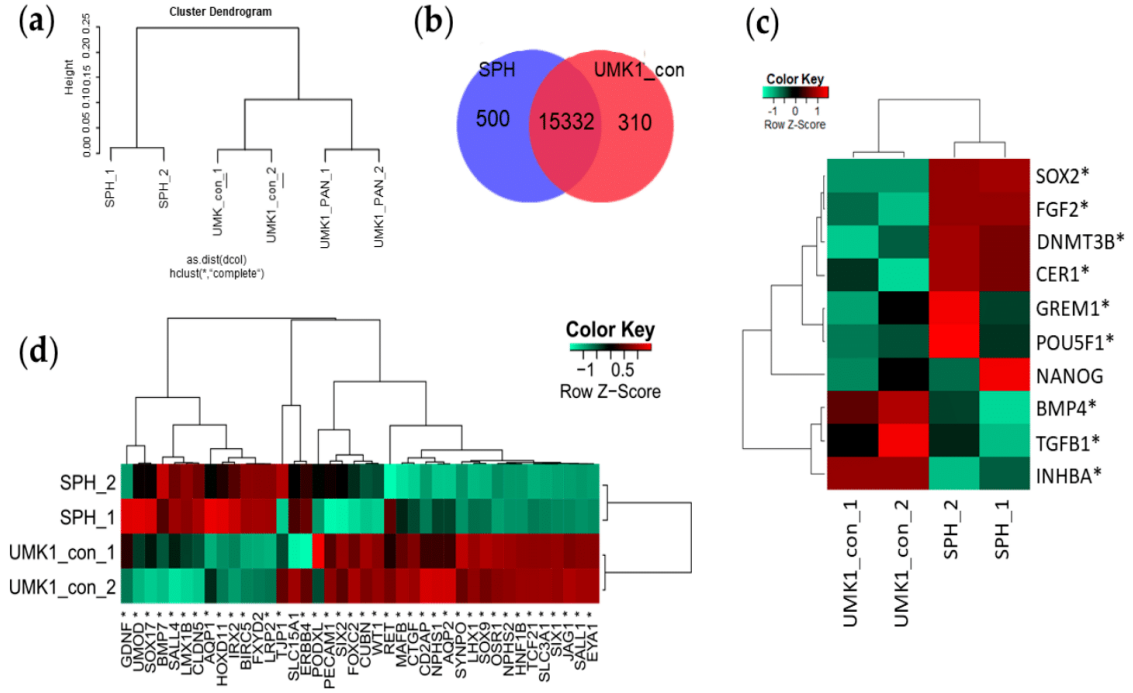

Figure 2. Comparative analysis of gene expression in iPSC spheroids and kidney organoids. (**a**) Similarities between spheroids and non-treated and PAN-treated organoids are shown in the cluster dendrogram. Control and PAN-treated UMK1 cluster together, and iPSC spheroids cluster separately. (**b**) The common gene-sets between spheroids and untreated kidney organoids consists of 15,332 genes. In total, 500 genes are exclusively expressed in spheroids and 310 genes in control organoids. (**c**) Expression of pluripotency-associated genes in UMK1_con and SPH depicted in a Pearson heatmap. (**d**) Expression of kidney-associated genes in UMK1_con and SPH depicted in a Pearson heatmap.

The differentiation of iPSC spheroid towards kidney organoids was confirmed with a heatmap analysis composed of genes of early and late nephrogenesis (Figure 2d). Genes typical of early kidney development—*GDNF*, *SOX17*, *BMP7*, *SALL4*, *LMX1B* and *HOXD11*—

were expressed in SPH, while podocyte-associated genes—*NPHS1*, *NPHS2*, *PECAM1*, *PODXL* and *SYNPO*—as well as renal-tubule-related genes—*AQP2* and *CUBN*—were expressed in UMK1_con (Figure 2d).

3.3. PAN Negatively Affects Podocytes and Partly Tubular Cells

The kidney organoids sustained a high proliferation rate during the entire differentiation process. Immunofluorescence-based analysis of organoid sections detected proliferating KI67-positive cells, which were found to be reduced in organoid sections treated with PAN (Figure 3a).

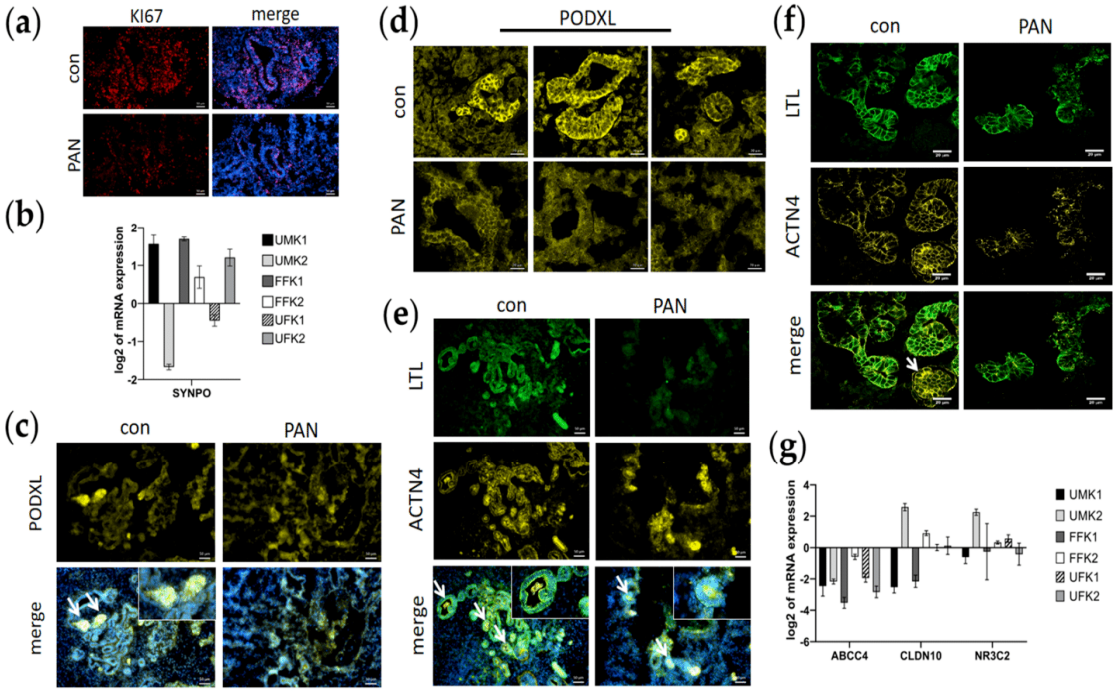

Figure 3. PAN leads to less defined glomerular and tubular structures. (**a**) PAN induction leads to a decrease in the number of highly proliferative KI67+ cells (red). Nuclei were stained with Hoechst33342 (blue). Scale bar depicts 50 µm. (**b**) Expression of the podocyte marker *SYNPO* is upregulated (UMK1, FFK1, FFK2, UFK2) after PAN treatment. Error bars depict standard error. (**c**) PODXL+ glomeruli (yellow) are less defined after PAN treatment. Glomeruli are marked with a white arrow. Nuclei were stained with Hoechst33342 (blue). Scale bar depicts 50 µm. (**d**) Confocal microscopy pictures of PODXL+ glomeruli with and without PAN. Scale bar depicts 20 µm. (**e**) LTL+ proximal tubules (green) and ACTN4+ glomeruli (yellow) are less defined after PAN treatment. Glomeruli are marked with a white arrow. Nuclei were stained with Hoechst33342 (blue). Scale bar depicts 50 µm. (**f**) Comparative confocal pictures of ACTN4 (yellow) and LTL-stained (green) organoid sections treated with and without PAN. Scale bar depicts 20 µm. Glomeruli are marked with a white arrow. (**g**) Expression of the tubular markers *ABCC4*, *CLDN10* and *NR3C2* are downregulated by PAN. Error bars depict standard error.

Gene expression analysis revealed the upregulated expression of the podocyte-associated gene *SYNPO* in UMK1, FFK1, FFK2 and UFK2 kidney organoids (Figure 3). The podocyte marker PODXL was expressed by glomerular-like cells at the rear ends of the tubule-like structures (Figure 3; Figure S1b). Treatment with PAN resulted in a reduction in PODXL-expressing cells, and glomerular-like structures appeared to be less defined (Figure 3). The blurred boundaries of glomerular-like structures induced by PAN were closely observed by confocal microscopy (Figure 3). Additionally, tubular-like structures were found to be LTL-

positive, and their rear ends were positive for ACTN4 (Figure 3). Reduced numbers of LTL^+ and $ACTN4^+$ cells were observed in PAN-treated organoid sections (Figure 3). In addition, the expression of the tubular markers—*ABCC4*, *CLDN10* and *NR3C2*—was downregulated in all organoid batches except for UMK2 (Figure 3). PAN treatment was found to negatively affect glomerular-like cells and partially affect tubular-like cells as well.

3.4. Transcriptome Analysis Reveal Kidney-Related GO Terms Are Expressed in Urine-Stem-Cell-Derived iPSC Spheroids and Kidney Organoids

A Venn diagram comparison between UMK1_con and UMK1_PAN revealed a common gene-set of 15344, 298 exclusive genes in UMK1_con and an exclusive gene-set of 215 in UMK1_PAN (Figure 4). The top ten GO biological pathways of the exclusive UMK1_PAN gene-set are further described in Table S5 (see Supplementary File S2).

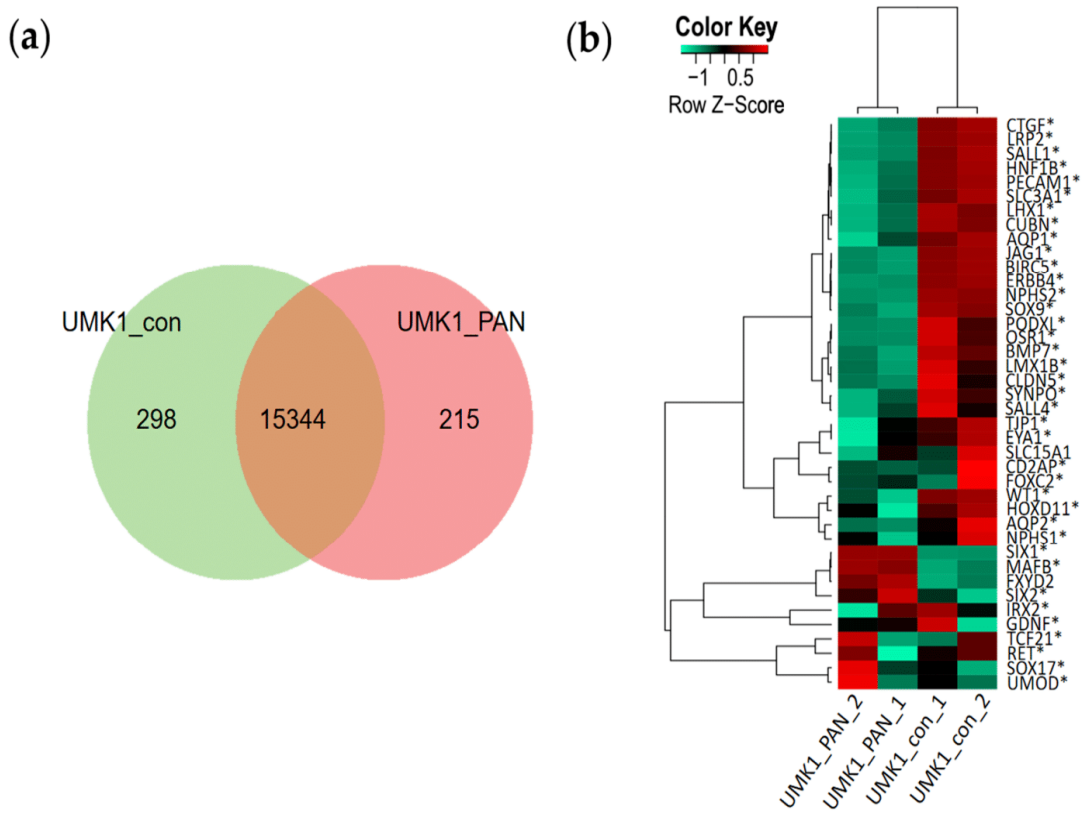

Figure 4. Transcriptome analysis of UMK1 with and without PAN treatment. (**a**) The common gene-sets between UMK1_con and UMK1_PAN consisted of 15344 genes. Exclusively expressed in UMK1_con and UMK1_PAN are 298 and 215 genes, respectively. (**b**) Expression of kidney-associated genes in UMK1_con and UMK1_PAN is displayed in a Pearson heatmap.

The expression of genes associated with nephrogenesis was compared between UMK1_con and UMK1_PAN with a Pearson heatmap (Figure 4b). Expression of the podocyte-associated genes *NPHS1*, *NPHS2*, *PECAM1*, *PODXL* and *SYNPO* as well as the tubular genes *SLC3A1*, *AQP1*, *CUBN* and *CLDN5* was observed in UMK1_con but was downregulated in UMK1_PAN (Figure 4b).

3.5. PAN Induces DNA Damage in Kidney Organoids

DNA damage in PAN-induced kidney organoids was evaluated by immunofluorescence and Western blot analysis. The DNA damage marker γH2A.X was expressed in both

untreated and PAN-treated kidney organoid sections (Figure 5a). Interestingly, quantitative Western blot analysis revealed a three-fold increase in γH2A.X protein expression levels in PAN-induced UMK1 compared to an untreated control (Figure 5a). DNA damage induced by PAN was also observed by the detection of total P53 protein, which was expressed at higher levels in PAN-treated UMK1 than in the controls (Figure 5b). Additionally, a higher amount of cleaved Caspase 3 protein was observed upon PAN treatment (Figure 5b). We could also observe higher amounts of γH2A.X, P53 and cleaved Caspase 3 protein in the other PAN-treated kidney organoid batches—UMK2, FFK1, FFK2, UFK1 and UFK2 (Figure S1c). However, in the UFK2 organoids, we observed reduced levels of γH2A.X and cleaved CASP3 after PAN treatment (Figure S1c). Further indicators of cell damage were also observed in upregulated GO enrichment clusters such as "p53 transcriptional gene network", "autophagy", "TRAIL-activated apoptotic signalling pathway" and "negative regulation of cell cycle" (Figure 5c).

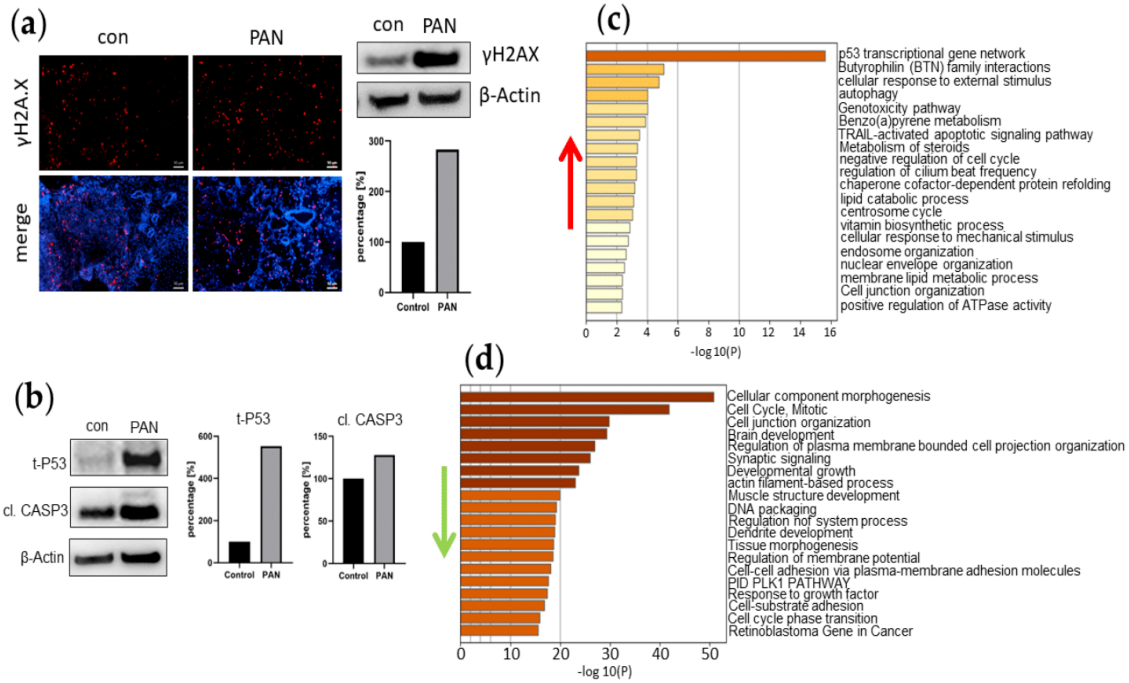

Figure 5. PAN treatment induces DNA damage in kidney organoids. (**a**) Elevated expression of γH2A.X (red) in PAN-treated kidney organoids UMK1. Nuclei were stained with Hoechst33342 (blue). Scale bar depicts 50 µm. (**b**) Elevated expression of t-P53 and cleaved Caspase 3 in PAN-treated kidney organoids UMK1. (**c**) Upregulated enrichment clusters include cell damage. (**d**) Cell-cycle-related enrichment clusters were downregulated by PAN treatment.

Additionally, other downregulated cell-cycle-related GO enrichment clusters included "mitotic cell cycle" and "developmental growth" (Figure 5d). Besides other effects caused by PAN, the substance is known to damage podocytes, which particularly affects the podocyte membranes. With regard to this, we observed the downregulation of the GO terms "cell junction organization", "regulation of plasma membrane bounded cell projection organization", "actin-filament-based process" and "cell–cell adhesion via plasma-membrane adhesion molecules" (Figure 5d). Similarly, a KEGG pathway analysis revealed the downregulation of genes associated with "cell cycle" (Table S6; Supplementary File S3), as well as upregulated genes within the "P53 signalling pathway" (Table S7; Supplementary File S4). Treating kidney organoids UMK1 with PAN was associated with the regulation of various biological pathways. We especially concentrated on the 215 genes solely expressed by the

PAN-treated UMK1 organoids as seen in the Venn analysis (Figure 4). With a detection p-value below the threshold of 0.05, we focused on the top 10 expressed GO biological pathways, which included: "lipoxygenase pathway", "inflammatory response", "establishment of skin barrier", "hepoxilin biosynthetic process", "regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains", "lactate transmembrane transport", "response to biotic stimulus", "establishment of localization", "multicellular organismal water homeostasis" and "regulation of cytokine production" (Table S5). Interestingly, pathways involved in inflammation and immune responses were significantly regulated in kidney organoids treated with PAN. We could also confirm the transcriptome data by qRT-PCR, which demonstrated an upregulation of genes associated with the lipoxygenase pathway (*ALOX12B*) and P53 signalling pathway (*SESN2*, *FAS*) and a downregulation of cell-cycle-related genes (*CCNB2*, *PLK1* and *BUB1*) (Figure S2a).

3.6. Inflammation-Associated Processes Are Elevated in PAN-Induced Kidney Organoids

As the transcriptome analysis revealed indications that PAN induces inflammation and acute immune responses, we analysed the expression of markers of the myeloid cell lineage via Pearson heatmap analysis (Figure 6a). Various pro-inflammatory –CXC- and –CC-chemokines as well as DPP4—an established marker of the proximal tubular compartment—were expressed in UMK1_PAN but not in UMK1_con (*CXCR4*, *DPP4*, *CCL20*, *CCL2*, *CXCL5*, *CXCR3*, *CXCL1* and *CXCL6*) (Figure 6a). In contrast, anti-inflammatory –CXC- and –CC- chemokines were primarily expressed in UMK1_con and not UMK1_PAN (*CX3CL1*, *CXCL17*, *CCR2*, CXCL2 and *CXCL3*) (Figure 6a).

The expression of pro-inflammatory cytokines—*IL8* and *IL6*—was upregulated in all organoid batches, except for UMK1, after PAN treatment compared to their specific controls (Figure 6b). Cytokine secretion of PAN-induced UMK1 was additionally analysed using a "Human Kidney Biomarker array" and compared to an untreated control. A cluster dendrogram based on the cytokine array data showed clustering of the duplicates control versus PAN treatment (Figure 6c). Upon PAN treatment, the concentrations of ADIPOQ, ANPEP, ANXA5, DPP4, EGF, EGFR and IL1RN increased significantly (Figure 6d). In contrast, CXCL1, CCL2, MMP9, THBS1, PLAU and VCAM1 were secreted at lower levels compared to untreated controls (Figure 6d).

The exclusive gene-sets of UMK1_con (298 genes) and UMK1_PAN (215 genes) from the Venn analysis in Figure 4 were subjected to metascape-based analysis (Figure 5e,f and Figure 6). When comparing the enriched gene clusters of UMK1_con and UMK1_PAN, the gene clusters "regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains" and "synthesis of 12-eicosatetraenoic acid derivates" emerged in UMK1_PAN (Figure 6e). These fatty acids are involved in inflammatory processes and thus implying that PAN induces inflammatory responses in the kidney organoids. The analysis of non-redundant enrichment clusters additionally supported the observation that PAN treatment induces inflammation and immune response as the enrichment clusters comprised "synthesis of 12-eicosatetraenoic acid derivates" and "regulation of cytokine production involved in immune response" (Figure 6f).

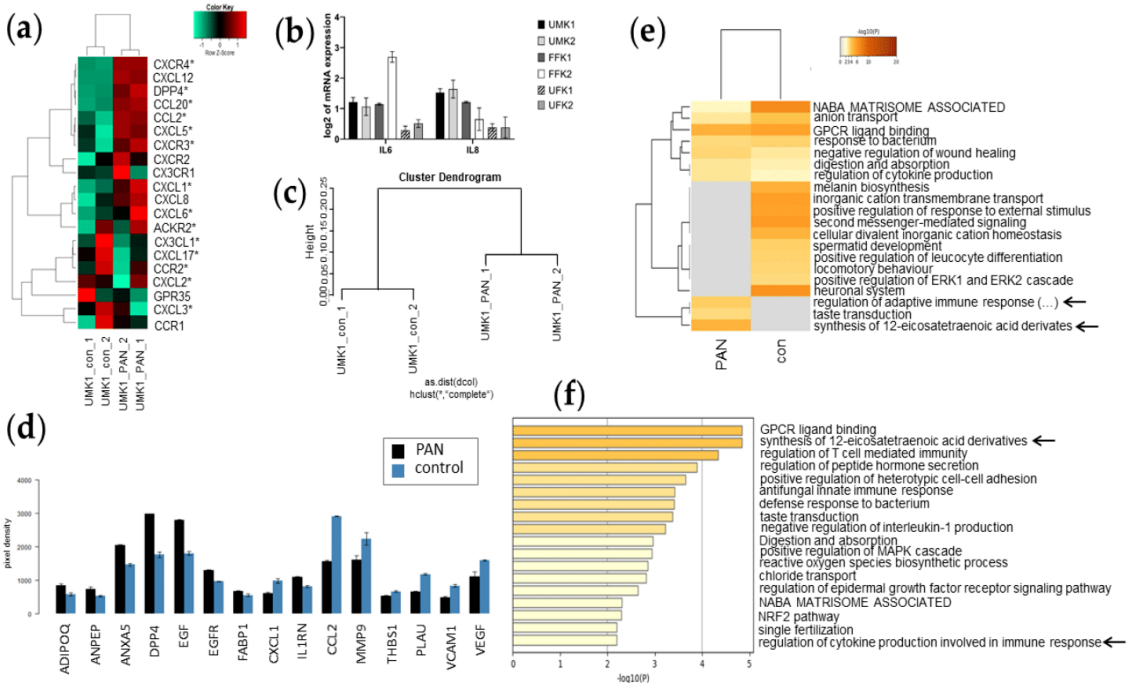

Figure 6. Inflammation-associated gene expression and cytokine secretion in untreated and PAN-treated kidney organoids. (**a**) Pearson heatmap of the expression of immune-related genes in control and PAN treatment. (**b**) Expression of IL-6 and IL-8 is elevated in PAN-induced kidney organoids as measured by qRT-PCR. Error bars depict standard error. (**c**) Cluster dendrogram with the technical duplicates of untreated kidney organoids clustering together and the PAN-treated kidney organoids cluster separately. (**d**) Cytokine array data comparing expression between untreated control and PAN treatment. (**e**) Metascape-generated heatmap comparing UMK1_PAN and UMK1_con included inflammation- and immune-response-related GOs. Subjected gene-sets are based on the Venn analysis in Figure 4. (**f**) Bar graph of non-redundant enrichment clusters after PAN treatment.

3.7. PAN Activates the Renin–Aldosterone–Angiotensin System (RAAS)

Kidney injury, especially glomerular damage, is associated with altered blood pressure. We examined this by investigating the renin–aldosterone–angiotensin System (RAAS). The transcriptome data revealed PAN-induced downregulation of the renin-secretion-associated genes—*PAC1*, *AQP1*, *Cav*, *IP3R*, *PLC*, *sGC*, *PDE1*, *PDE3* and *Cn* (Figure 7a). The secretion of renin by the kidney organoids was measured using a commercially available renin assay kit. An increase in renin secretion after PAN treatment was observed in the organoid batches UMK1, UMK2 and UFK2, whereas statistical significance with respect to the control was only found in UMK2 and UFK2 (α-value ≤ 0.05) (Figure 7b). No significant difference was observed between control and PAN treatment in FFK1, FFK2 and UFK1 organoids (Figure 7b). The mRNA expression of the key genes of the RAAS pathways—*AGT* and *AGTR1*—was upregulated in almost all kidney organoids (Figure 7c). AGT was downregulated in PAN-treated UFK2 organoids and *AGTR1* was downregulated in PAN-treated UFK1 organoids (Figure 7c). In the Pearson's heatmap of genes involved in RAAS, we could survey a section in which the genes *MME*, *NR3C2*, *AGTR2*, *ACE* and *AGT* were mostly expressed in UMK1_con (Figure 7d). On the other hand, the genes *ATP6AP2*, *PRCP*, *KLK2 and PREP* were mainly expressed in UMK1_PAN (Figure 7d).

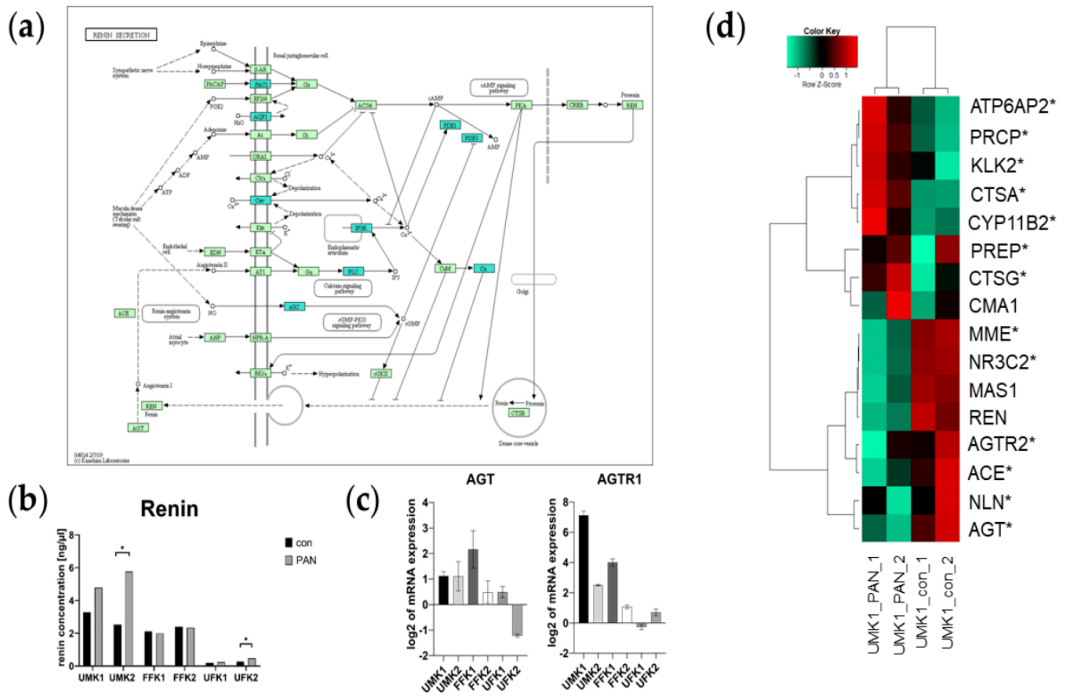

Figure 7. PAN induction affects the RAAS in kidney organoids. (**a**) PAN induces downregulation of genes of the KEGG pathway renin secretion. Downregulated genes are marked in blue. (**b**) Renin concentration (ng/µL) in conditioned media of untreated and PAN-treated kidney organoids. Significance is determined by α-value ≤ 0.05. (**c**) Expression of RAAS-associated AGT and AGTR1 is upregulated in PAN-induced kidney organoids. Error bars depict the standard error. (**d**) Pearson's heatmap depicting the expression of RAAS-associated genes in untreated and PAN-treated kidney organoids.

4. Discussion

In this study, we demonstrated the successful generation of kidney organoids. We used three iPS cell lines of distinct genetic background in technical duplicates, of which two were derived from urine-derived renal progenitor cells [16,17] and one from foetal foreskin fibroblast cells [18] (see Table 1). Kidney damage was modelled by stimulating kidney organoids for 48 h with 50 µg/mL of the nephrotoxic substance puromycin aminonucleoside (PAN).

In our previous studies, we observed that urine-derived renal progenitor cells (UdRPCs) are MSCs and additionally could be differentiated into tubular- and podocyte-like cells [17,30]. Interestingly, we observed that UdRPC-derived iPSCs spheroids (SPH) and untreated kidney organoids (UMK1_con) shared a common set of 15332 expressed genes, while they clustered distinctly. From this common gene-set, we observed upregulated genes associated with kidney-related GO terms such as "urogenital system development", "mesonephric epithelium development", "ureteric bud development", "renal tubule development" and "nephron epithelium development". Additionally, our heatmap analysis revealed that a set of genes associated with kidney development was expressed in both untreated kidney organoids as well as iPSC spheroids (e.g., *CUBN*, *NPHS1*, *NPHS2* and *SYNPO*). Additional and deeper investigations regarding the potential of UdRPCs is beyond the scope of the current study and should be conducted in the future.

While comparing kidney organoids with iPSC spheroids, we observed that kidney organoids had a more lobular morphology in comparison to the typical round morphology of iPSC spheroids. Moreover, H&E stainings as well as immunofluorescence-based stainings showed the emergence of tubular- as well as glomerular-like structures within

our kidney organoids. Previous studies demonstrated the ability of proximal tubules to take up dextrans within hPSC-derived kidney organoids [19,31]. Therefore, the functionality of our kidney organoids was evaluated with a dextran uptake assay. Endocytosis of dextran was demonstrated in our kidney organoids, whereas iPSC spheroids did not take up dextran. As previously mentioned, the kidney organoids are composed of LTL- and OCT2-positive tubular- and glomeruli-like structures, which express PODXL and ACTN4. PODXL is expressed on the podocyte cell surface [32] and is involved in the formation of the slit diaphragm, while ACTN4 co- localizes with cytoskeleton filaments within the foot processes [33]. Stimulation with the nephrotoxic purine antagonist PAN interestingly led to the upregulation of *SYNPO* expression. However, our transcriptome data suggest PAN-induced podocyte damage, as we found the increased expression of podocyte-associated factors such as *NPHS2* and *PODXL* in UMK1_con and reduced expression in UMK1_PAN. Corroborating our findings, Luimula et al. [34] and Lee et al. [35] observed reduced NEPHRIN expression in regions of foot processes effacement after PAN injection in rats. *NPHS1* mutation or reduced NEPHRIN expression was found to be involved in various glomerular diseases [35,36]. A lack of filtration of proteins due to a defect in the slit diaphragm results in proteinuria [37], which can eventually lead to reversible podocyte detachment from the glomerular basement membrane (GBM) [7]. Downregulated enrichment clusters "cell junction organization", "regulation of plasma membrane bounded cell projection organization", "actin-filament-based process" and "cell–cell adhesion via plasma-membrane adhesion molecules" imply that PAN treatment interferes with cell–cell contact. Based on these findings, we concluded that our PAN-treated kidney organoids can be used to model glomerular-associated diseases.

The kidney organoids contained a high number of $KI67^{+}$ -proliferative cells, which were reduced by PAN induction. KI67 expression is found in cells during the interphase of the cell cycle but is absent in the G0 phase [37]. Adult kidney cells have a slow proliferation rate [38]. Especially glomerular cells, such as podocytes, are in the postmitotic G0 phase [38]. Therefore, we suspect that our kidney organoids are rather immature and similar to findings of previous studies, where hPSC-derived kidney organoids resemble human foetal kidneys [31,39,40]. Besides the reduction in KI67-positive cells, we observed upregulation in the enrichment terms "negative regulation of cell cycle" and downregulation of "mitotic cell cycle" and "developmental growth "in PAN-treated kidney organoids. In addition, the levels of t-P53 protein increased in all PAN-treated organoid batches, and an enrichment of the KEGG pathway "p53 transcriptional gene network" was observed. Therefore, we assumed a progression from interphase to G0 resulting in cell cycle arrest in our PAN-treated kidney organoids.

DNA damage triggered by PAN treatment might be the reason for the observed cell cycle arrest. Even though we did not observe a difference in the level of γH2AX between the control and PAN-treated UMK1 in the IF-based staining, we found a 2-fold higher amount of protein in PAN-treated UMK1 than in the untreated control. A higher level of γH2AX was also observed in the other organoid batches—UMK2, FFK1, FFK2 and UFK1. PAN treatment induced increased apoptosis in the kidney spheroids—UMK1, UMK2, FFK1, FFK2 and UFK1—which was detected by increased levels of cleaved Caspase 3 in PAN-treated kidney organoids. This finding was also observed by Kang et al. [8], where they detected active Caspase 3 in PAN-induced podocytes [8]. In addition, they found autophagy was induced by PAN prior to apoptosis [8]. Similar to their observation, in our kidney organoid model, enriched clusters of "autophagy" and "TRAIL-activated apoptotic signalling pathway" were upregulated upon PAN treatment. Interestingly, less γH2AX and cleaved Caspase 3 was found in PAN-treated UFK3 organoids. This finding can be attributed to the variability in batches of organoids. There are manifold reasons for a high variability between organoids, depending on the employed cell lines or the heterogeneity of clones and the genotypes [12]. As the other organoid batches showed the same tendencies regarding increased levels of γH2AX and cleaved Caspase 3, we conclude

that PAN treatment results in DNA damage and the activation of cell-death-associated processes.

Besides the induction of DNA damage, we also observed the upregulation of inflammatory processes upon PAN treatment. The pro-inflammatory regulators *IL-6* and *IL-8* as well as the expression of other pro-inflammatory genes such as *CXCR4*, *CXCL12*, *DPP4*, *CCL20*, *CCL2*, *CXCL5*, *CXCR3*, *CXCR2*, *CX3CR1*, *CXCL1*, *CXCL8* and *CXCL6* were upregulated in PAN-treated UMK1. From the results of the cytokine array, we observed the secretion of the reno-protective cytokines ADIPOQ, Diannexin (ANXA5), EGF and EGFR. These cytokines are known to protect against renal ischemia and reperfusion injury as well as to promote recovery after AKI [41,42]. Moreover, ADIPOQ levels are used as a prognostic tool for ESRD [42]. We therefore propose that PAN treatment causes renal inflammation and as a consequence, the activation of protective and anti-inflammatory reactions within cells. On the other hand, previous studies have demonstrated that tubular epithelial cells express the pro-inflammatory Interleukin-1α, which is involved in immune response [43]. This cytokine induces local inflammation via chemokine secretion, subsequently attracting neutrophils, macrophages and lymphocytes [43]. Signalling is dependent on the IL-1 receptor, which can be blocked by the anti-inflammatory antagonist IL1RN [43,44]. This led us to the assumption that the higher amounts of IL1RN in the secretome of PAN-treated kidney organoids functioned as a counteraction to the induced inflammation. Likewise, our transcriptome data suggest a cytokine-induced immune reaction in PAN-treated kidney organoids as the non-redundant enrichment cluster "regulation of cytokine production involved in immune response" was unveiled.

Moreover, the untreated kidney organoids primarily expressed anti-inflammatory genes such as *CX3CL1*, *CXCL17*, *CCR2*, *CXCL2*, *GPR35*, *CXCL3* and *CCR1*, which might imply a form of protection against inflammatory processes in the control kidney organoids UMK1_con.

An inflammation response upon PAN treatment was additionally detected in the metascape-based analysis with the enrichment clusters "synthesis of 12-eicosatetraenoic acid derivates" and "regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains". Moreover, we found "synthesis of 12-eicosatetraenoic acid derivates" to be the top non-redundant enrichment cluster in UMK1_PAN. Eicosatetraenoic acids are metabolites from the lipoxygenase pathway, which we observed as one of the top GO biological pathways within the 215 exclusive genes of the PAN-treated UMK1 organoids. Eicosanoids are synthesized from polyunsaturated fatty acids such as arachidonic acids [45,46]. Lipoxygenases synthesize 5-, 12-, or 15-hydroxyeicosatetraenoic (HETE) acids from arachidonic acids [47]. They are involved in various metabolic processes such as cellular inflammation [45,46]. It was recently discovered that lipoxygenases play a role in kidney damage of diabetic nephropathy and their synthesised products induce the synthesis of stimulants involved in kidney fibrosis [47]. One of the lipoxygenases is the 5-lipoxygenase (*ALOX5*), which is involved in inflammatory diseases [48]. Previous reports suggest that inhibiting 5-lipoxygenase led to a reduction in renal fibrosis and CKD progression [48]. Additionally, the synthesis product 5-HETE stimulates T-cell production, one of the major immune cells [47]. In addition, it has been described that 12-lipoxygenase and 15-lipoxygenase synthesize 12-HETE and 15-HETE, which contribute to the overexpression of pro-inflammatory cytokines in macrophages [47]. We detected the elevated expression of 12-lipoxygenase in PAN-treated kidney organoids and together with the findings of our transcriptome data, it suggests that PAN stimulation leads to an increase in inflammation and other injury-associated processes. We conclude that PAN induction of pro-inflammatory processes in kidney organoids can be considered for modelling inflammation-associated acute kidney injury and associated diseases.

Due to their inherent transport activity, proximal tubules are susceptible to damage by kidney injury, renal ischemia and nephrotoxicity [49]. LTL staining detected proximal tubules in our kidney organoids, and we observed these to be partially affected by PAN

induction. Structurally, tubules of PAN-induced kidney organoids had partly disrupted cell membranes, and tubular-associated genes such as *ABCC4*, *CLDN10* and *NR3C2* were downregulated. Additionally, our transcriptome data revealed the low-level expression of tubule-associated genes such as *CUBN* and *AQP1* in PAN-treated UMK1 organoids compared to the control. Similar observations of tubular damage and stimulated glomerular nephropathy were made in rats when PAN was administered chronically [50]. Additionally, PAN-treated kidney organoids secreted significantly higher amounts of FABP, ANPEP (CD13) and DPP4 (CD26), which are associated with the proximal tubule compartment.

Within the proximal tubular compartment, L-FABP is capable of binding to lipid peroxide and protects from oxidative stress [51]. However, tubular damage results in the secretion of L-FABP into urine [51]. The proteins ANPEP and DPP4 are expressed in the apical brush border epithelium of proximal tubules and are shed into urine upon increased tubular stress, caused by various renal injuries [52]. This may lead to an impairment of the glomerular filtration rate and thus a shedding of glomerular proteins [52]. The higher amounts of proteins in urine eventually exceed the capacity of proximal tubules to reabsorb proteins, which further adds up to proteinuria [52]. Since the aforementioned proteins are cumulatively secreted into urine upon renal injuries, this was an indication that our kidney organoids can be used for kidney injury models, specifically the proximal tubular compartment.

We observed that PAN had an effect on the renin–angiotensin–aldosterone system (RAAS). The RAAS has a key role in the maintenance of blood pressure and body fluid homoeostasis. In the kidney, the RAAS is essential for normal development, and complications can lead to congenital anomalies of the kidney and the urinary tract (CAKUT) [53]. A heatmap analysis revealed the expression of *ATP6AP2*, *PCRP*, *KLK2* and *PREP* in our PAN-treated, but not in the untreated kidney organoids. Upon pathological conditions such as renal dysfunction, previous studies observed the activation of pro-inflammatory and pro-fibrotic molecules by ATP6AP2 [54]. Similarly, KLK2 plays a major role in inflammatory kidney diseases, where it is involved in various physiological processes [55]. On the other hand, PCRP and PREP have reno-protective properties [56,57]. While PRCP degrades ANGII to ANG (1–7) [56], studies in hypertensive rats revealed that PREP synthesizes anti-fibrotic molecules, which also decrease the infiltration of inflammatory cells [57].

In the untreated kidney organoids, we observed the expression of key components of the RAAS such as *AGT*, *ACE* and *AGTR2*, as well as *MMP* and *NR3C2*. MMP acts together with ACE2 for the production of ANG (1-7) to counteract against overproduced ANGII, which finally prevents hypertension and organ damage [58,59]. Even though NR3C2 is involved in the regulation of fluid, electrolytes and blood pressure homoeostasis [60], an over-activation can cause organ injury induced by inflammatory and fibrotic processes [61,62].

Interestingly, KEGG pathway analysis revealed the downregulation of renin-secretion-associated genes, mainly involved in the calcium and cGMP-PKG signalling pathways. Renin secretion is directly linked to cAMP formation, which is regulated by intracellular Ca^{2+} concentration [63]. High levels of Ca^{2+} correlate with decreased renin secretion [63]. Additionally, high levels of cGMP were also found to negatively affect renin metabolism [64]. The results of the renin secretion assay as well as the KEGG analysis revealed decreased calcium signalling and increased renin secretion in the PAN-treated kidney organoids. Previous studies suggest that increased renin and angiotensinogen (AGT) levels lead to an increased ANGII concentration, thereby stimulating hypertension and organ damage [65]. We made a similar observation, as we measured an upregulation of AGT expression in PAN-induced kidney organoids. Additionally, we demonstrated the upregulated expression of the ANGII receptor *AGTR1*. Besides its role in the regulation of blood pressure, AGTR1 is associated with various pathological conditions such as hypertension and diabetic nephropathy [66]. In conclusion, PAN treatment induced upregulated renin secretion, which subsequently increased ANGII and AGTR1 expression and which may lead to the emergence of pathological conditions such as high blood pressure under certain circumstances.

5. Conclusions

This study has revealed that human kidney organoids treated with puromycin aminonucleoside hold promise in the study of various pathological kidney conditions, which affect the glomerular and tubular regions. PAN induces immune response such as inflammation, DNA damage, apoptosis and cell death. Furthermore, PAN activated the RAAS pathway, therefore demonstrating the relevance in pro-inflammatory and reno-protective processes as well as the emergence of pathological kidney conditions such as ANGII-induced hypertension over time. In the future prospect, a deeper understanding of kidney-related disease mechanisms by harnessing kidney organoid models can be beneficial for novel drug discovery and development.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells11040635/s1, Table S1: Composition of basal spheroid medium, Table S2: Composition of basal differentiation medium, Table S3: List of utilized antibodies, Table S4: List of used qRT-PCR primers, Table S5: Top ten GO biological pathways expressed in PAN-treated kidney organoids, Table S6: Top ten downregulated biological pathways and processes of KEGG analysis, Table S7: Top ten upregulated biological pathways and processes of KEGG analysis, Figure S1: Effects of PAN on kidney organoids detected by H&E staining, immunofluorescence staining and Western blotting, Figure S2:Confirmation of transcriptome data and details of the cytokine array data. Supplementary File S1: Complete list of GO terms based on the common gene-set between SPH and UMK1_con_. Supplementary File S2: Complete list of regulated GO biological pathways in UMK1_PAN exclusive gene-set. Supplementary File S3: Complete list of downregulated KEGG pathways in the common gene-set of UMK1_con and UMK1_PAN. Supplementary File S4: Complete list of upregulated KEGG pathways in the common gene-set of UMK1_con and UMK1_PAN.

Author Contributions: L.N. designed and performed experiments, analysed the data, wrote and edited the manuscript. N.G. and L.E. assisted in experimental design, performed experiments, analysed data and edited the manuscript. W.W. performed the bioinformatic analysis, data curation, helped with the figures and edited the manuscript. J.A. conceptualized the work, wrote and edited the manuscript, ordinated the study, acquired funding and supervised the study. All authors have read and agreed to the published version of the manuscript.

Funding: J.A. acknowledges the medical faculty of Heinrich Heine University for financial support.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Heinrich-Heine University, Germany (protocol code: 5704 and date of approval: 22 February 2017).

Data Availability Statement: All microarray data will be available at NCBI GEO under the accession number GSE186823 when the manuscript is accepted.

Conflicts of Interest: The authors declare no conflict of interest.

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3.3 JNK signalling regulates self-renewal of proliferative urine-derived renal progenitor cells via inhibition of ferroptosis

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BioRiV (2022)

Abstract:
With a global increase in chronic kidney disease patients, alternatives to dialysis and organ transplantation are needed. Stem cell-based therapies could be one possibility to treat chronic kidney disease. Here, we used multipotent urine-derived renal progenitor cells (UdRPCs) to study nephrogenesis. UdRPCs treated with the JNK inhibitor- AEG3482, displayed decreased proliferation and downregulated transcription of cell cycle-associated genes as well as the kidney progenitor markers -SIX2, CITED1, and SALL1. In addition, levels of activated SMAD2/3, which is associated with the maintenance of self-renewal in UdRPCs, were decreased. JNK inhibition resulted in less efficient oxidative phosphorylation and more lipid peroxidation via ferroptosis- an iron-dependent non-apoptotic cell death pathway linked to various forms of kidney disease. Our study reveals the importance of JNK signalling in maintaining self-renewal as well as protection against ferroptosis in SIX2-positive UdRPCs. We propose that UdRPCs can be used for modelling ferroptosis-induced kidney diseases.

Author Contribution: 60%

L.N. designed and executed experiments, analysed data, wrote and edited the manuscript.

Status: Submitted to *iScience* Journal (under revision).
Pre-print available in the open access pre-print repository *BioRiV*
(doi: 10.1101/2022.08.24.505101).

JNK signalling regulates self-renewal of proliferative urine-derived renal progenitor cells via inhibition of ferroptosis

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Summary:

With a global increase in chronic kidney disease patients, alternatives to dialysis and organ transplantation are needed. Stem cell-based therapies could be one possibility to treat chronic kidney disease. Here, we used multipotent urine-derived renal progenitor cells (UdRPCs) to study nephrogenesis. UdRPCs treated with the JNK inhibitor- AEG3482, displayed decreased proliferation and downregulated transcription of cell cycle-associated genes as well as the kidney progenitor markers -SIX2, CITED1, and SALL1. In addition, levels of activated SMAD2/3, which is associated with the maintenance of self-renewal in UdRPCs, were decreased. JNK inhibition resulted in less efficient oxidative phosphorylation and more lipid peroxidation via ferroptosis- an iron-dependent non-apoptotic cell death pathway linked to various forms of kidney disease. Our study reveals the importance of JNK signalling in maintaining self-renewal as well as protection against ferroptosis in SIX2-positive UdRPCs. We propose that UdRPCs can be used for modelling ferroptosis-induced kidney diseases.

JNK signalling, urine stem cells, self-renewal, ferroptosis, cell death, mitochondrial metabolism

Introduction:

Due to the increasing numbers of patient mortality due to kidney-associated diseases, new medical options besides the conventional dialysis and organ transplantation are needed. However, shortage of donor organs and immune compatibility restrict these therapeutic approaches. A future therapy option may be cell replacement therapies with pluripotent stem cells (PSCs)-derived cellular products; however, these have drawbacks that include ethical concerns and tumorigenicity. Counter measurements to the use of PSCs are the cost-effective and non-invasive urine cells. Cell types isolated from urine include urine stem cells, podocytes, and proximal tubule epithelial cells (Oliveira Arcolino et al., 2015). Highly interesting for research and medical purposes is the subpopulation of multipotent urine stem cells (USCs) which display typical MSC characteristics (Pavathuparambil Abdul Manaph et al., 2018; Rahman et al., 2020; Sato et al., 2019; Zhang et al., 2014). Besides expressing kidney-specific markers such as Podocin and Synaptopodin (Pavathuparambil Abdul Manaph et al., 2018; Sato et al., 2019), USCs express the renal progenitor marker sine oculis homeobox homolog 2 (SIX2). Based on SIX2 expression we conveniently coined the term urine-derived renal progenitor cells (UdRPCs) (Rahman et al., 2020). Presumably, UdRPCs originate from the upper urinary tract of the kidney, which was indicated by the presence of Y-Chromosome in these cells, isolated from the urine of a female patient who received a kidney transplant from a male donor (Bharadwaj et al., 2013).

The proliferative potential of UdRPCs and their ability to differentiate into various kidney cell types, such as podocytes (Erichsen et al., 2022) and tubular cells (unpublished) make them a promising tool for kidney regeneration experiments. Results from our earlier study, suggested the maintenance of self-renewal of UdRPCs via FGF and TGFβ signalling with modulations on JNK signalling (Rahman et al., 2020). JNK is part of the mitogen-activated protein kinase (MAPK) family and each pathway is a sequential activation of MAPKKKs (Javelaud and Mauviel, 2005). This pathway is involved in various processes such as proliferation, survival as well as stress and inflammation-induced apoptosis and is also linked to acute and chronic kidney diseases (Awazu, 2017; Engel et al., 1999; Smith et al., 2021; Tournier et al., 2000). In nephron progenitor cells JNK signalling plays a major role in maintaining the progenitor fate.

In the last decades, the cellular process of ferroptosis was discovered to play a role in the emergence of kidney diseases (Tang and Xiao, 2020). Ferroptosis was first described by Dixon *et al.* in 2012 as an iron-dependent form of non-apoptotic cell death. It is characterized by

Results:

High concentrations of JNK inhibitor induce cell death

First, optimal concentrations of the JNK inhibitor AEG3482 were tested on UM51 cells. Tested concentrations were 10 µM, 50 µM and 100 µM. The treatment was conducted for 2 to 5 days with daily medium change. The UM51 cells tolerated a concentration of 10 µM without any morphological changes and were cultivated for up to 5 days before stopping the treatment due to high cell confluency (Figure S1A). A concentration of 50 µM led to a morphology change from the typical rice grain-like to a more flattened appearance with partial detachment and indistinctive cell membrane (Figure S1A). The highest dose of 100 µM caused complete cell detachment and eventually cell death after 48 h (Figure S1A). UF21 cells treated with 50 µM and 100 µM JNK inhibitor could be kept for 72 h before the cells died (Figure S1B). As the concentrations of 50 µM and 100 µM were highly stressful to the cells, therefore, a concentration of 10 µM AEG3482 was used for all following experiments.

JNK inhibition leads to decreased proliferation and loss of progenitor state

Cell morphology changes in UM51, UM27 and UF21 cells after JNK inhibition with AEG3482 were observed after 72 h Figure 1A). In all three cell cultures, JNK inhibition resulted in lower confluency compared to their respective controls. The proliferation capacity of the UdRPCs with and without JNK inhibition at 72 h was analysed by immunofluorescence-based staining for KI67 (Figure 1B). Reduced numbers of KI67$^+$-proliferative cells were observed under JNK inhibition, which was confirmed by a proliferation assay displaying the ratio of Ki67$^+$ cells against the total cell number (Figure 1*B-C*). An unpaired t-test analysis confirmed a highly significant downregulation of KI67$^+$ UM51 cells ($p < 0.001$) and significant downregulation in UM27 cells ($p < 0.05$), while there was no significant difference between control and JNK inhibition in UF21 cells (Figure 1*C*). mRNA expression of progenitor- and cell cycle-related genes was analysed via semi-quantitative real-time PCR. The progenitor markers SIX2, SALL1 and VCAM1 as well as KI67 were downregulated in JNK-inhibited UdRPCs (Figure 1D). A heatmap depicted the expression of *CCND2, SMAD4, CDC14B, HDAC1, CCNH, WEE1, TFDP2* and *RBX1* in the controls, but down-

regulated in JNK-inhibited UM51 cells in the time points 24 h, 72 h and 120 h Figure 1E).

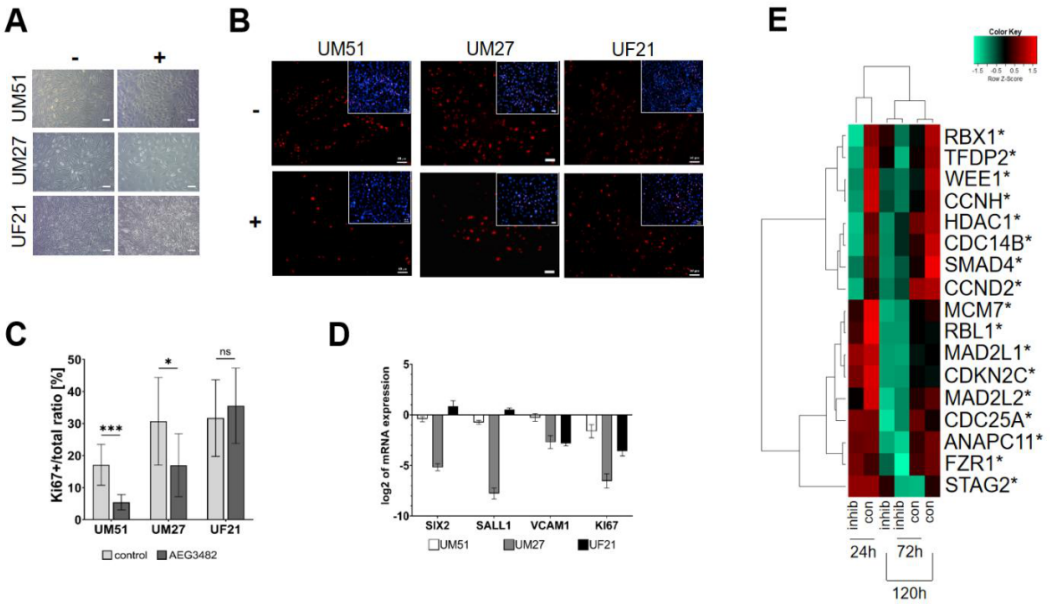

Figure 1. Inhibition of JNK reduces the proliferation of UdRPCs. A) Morphology of the three UdRPCs UM51, UM27 and UF21 with or without AEG3482 treatment after 72 h. Scale bars represent 100 µm. B) Ki67 expression in UdRPCs treated with or without AEG3482 for 72 h. Scale bars represent 50 µm. C) Ki67 proliferation assay for JNK-inhibited UdRPCs (n=10; * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). D) mRNA expression of nephron progenitor marker SIX2, SALL1, VCAM1 and Ki67. Mean values were normalized to the housekeeping gene RPL37A. Error bars indicate SEM. E) Gene expression of cell cycle-related genes for the time points 24 h, 72 h and 120 h depicted in a Pearson's heatmap.

Cell cycle-related processes are regulated by JNK inhibition

Microarray-based gene expression profiles of UM51 cells treated with JNK inhibitor for adjusted time span and their specific controls were analysed via transcriptome analysis. A comparison of gene expression between control and JNK inhibition were displayed in Venn diagrams for the time points 24 h, 72 h and 120 h (det p >0.05). Green colour represents the control, while red denotes JNK inhibition (Figure 2A-C). Complete gene lists for all time points 24 h, 72 h and 120 h are listed in Table S2-4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using exclusive gene-sets pertinent to control and AEG3482 treatment. Significantly downregulated in all three time points (24 h, 72 h, 120 h) was the KEGG pathway cell cycle (ratio >1.5) (Table S5). We were able to pinpoint 6 genes common in all three time points, which we found to be regulated by JNK *(BUB1, CCNA2, CCNB2, CCND2, MCM7, PLK1)* (Table S5).

Additionally, Metascape-generated enrichment analyses were processed based on the exclusive gene sets in JNK inhibition and control of all three time points (0.3 kappa score). Representative terms of enrichment clusters related to cell cycle and cell division (Table S6) with the highest P-values in the UM51 data sets of each time point were represented by heatmaps (Figure 2A-C). Notably, the GO BP terms related to cell cycle lacked enrichment in the JNK inhibitions of 24 h, 72 h and 120 h compared to high enrichment in the specific controls (marked with an arrow in (Figure 2A-C).

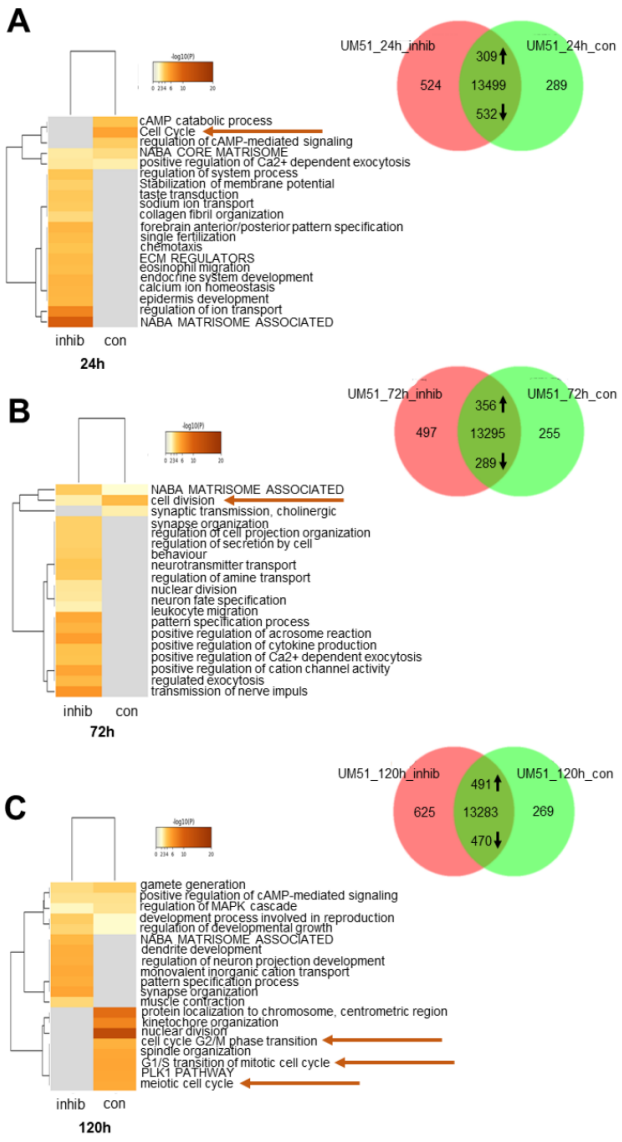

Figure 2. JNK signaling is associated with cell cycle processes in UdRPCs. A) Representative enrichment clusters for control and JNK-inhibition after 24 h depicted in a heatmap and cell cycle-related processes marked with an arrow. Venn diagram of control and JNK-inhibited UM51 cells for the time point 24 h. B) Representative enrichment clusters for control and JNK-inhibition after 72 h depicted in a heatmap and cell cycle-related processes marked with an arrow. Venn diagram of control and JNK-inhibited UM51 cells for the time point 72 h. C) Representative enrichment clusters for control and JNK-inhibition after 120 h depicted in a heatmap and cell cycle-related processes marked with an arrow. Venn diagram of control and JNK-inhibited UM51 cells for the time point 120 h.

The downstream target phospho-cJUN is downregulated by JNK inhibition

Next, expression of JNK and downstream target cJUN and its phosphorylated form before and after JNK inhibition was examined (Figure 3A-B). Successful inhibition of JNK phosphorylation by JNK inhibitor AEG3482 was confirmed in all three cell cultures (Figure 3A). Interestingly, the level of p-JNK in treated UF21 cells was less reduced than in the other two cell lines (Figure 3A). The protein level of non-phosphorylated t-JNK was not affected by JNK inhibition (Figure 3A). JNK inhibition resulted in a reduced phosphorylation of downstream target cJUN in UM51 and UM27, but not in UF21 cells (Figure 3B). Immunofluorescence-based analysis revealed a lack of differential expression of cJUN and phosphorylated cJUN in untreated and treated UdRPCs (Figure 3C).

As our previous study demonstrated the relevance of TGFβ-signalling in the maintenance of self-renewal, protein expression of SMAD2/3 and SMAD1/5 under influence of JNK inhibition was studied. Interestingly, the level of phosphorylated SMAD1/5 and SMAD2 decreased after JNK inhibition in UM51 and UM27 but not in UF21 cells (Figure 3D). Protein levels of total-SMAD2/3 and total SMAD1 were reduced in all three cell cultures (Figure 3D).

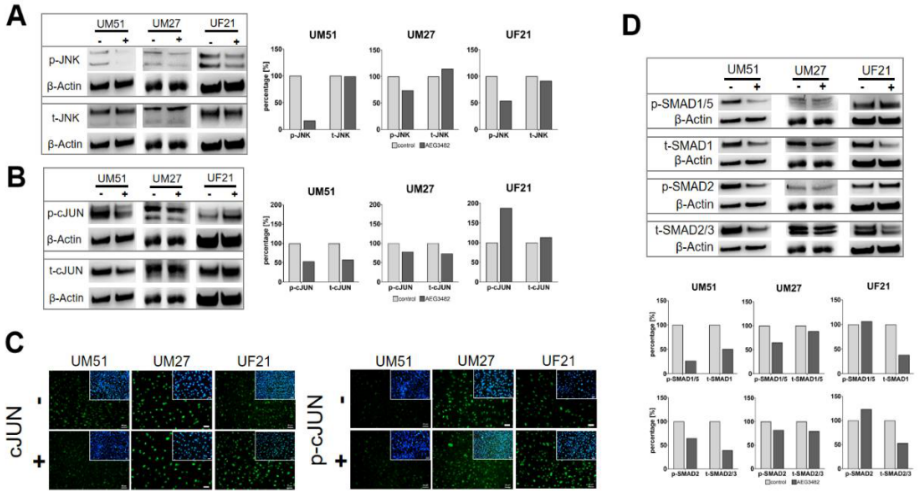

Figure 3. Inhibition of JNK signaling affects the downstream target cJUN and SMAD proteins. A) Protein expression of p- and t-JNK in UdRPCs with or without JNK inhibition. B) Protein expression of p- and t-cJUN in UdRPCs with or without JNK inihibition. C) Immunofluorescence stainings of cJUN and p-cJUN in UdRPCs with or without JNK inhibition. Scale bars represent 50 µm. D) Protein expression of p-/t-SMAD1/5 and p-/t-SMAD2/3 in UdRPCs with or without JNK inhibition.

UdRPCs are more susceptible to ferroptosis upon JNK inhibition

KEGG pathway analysis revealed significant upregulation of numerous KEGG pathways including ferroptosis and glutathione metabolism in JNK-inhibited UM51 cells at all time points (ratio <0.67) (Table S5B*)*. Following the results of the KEGG analysis, we decided to carry out an in-depth analysis of the ferroptosis pathway. Since lipid peroxidation is a major hallmark of ferroptotic cell death, the accumulation of lipid peroxides was measured in JNK-inhibited UdRPCs (Figure 4A). The cells exhibited a significant increase in lipid peroxidation, indicating increased sensitivity to ferroptosis. Treatment with the ferroptosis inhibitor Liproxstatin-1 protected the cells from lipid peroxidation (Figure S1C). Additionally, we could also observe a significant increase in lipid peroxidation when FGF signalling was inhibited (Figure S1D). JNK inhibition led to increased expression of gene sets involved in iron and glutathione metabolism (Figure 4B-C). Interestingly, protein expression of the key mediator of the removal of lipid peroxides, GPX4, did not show any difference between control and JNK-inhibition (Figure 4D). Analysis of the mRNA expression of genes related to the glutathione and iron metabolism, confirmed the findings of the heatmap analyses, as the genes *GLC* and *GLCM* and *HMOX1*, *SLC11A2* and *TFR1* were upregulated upon treatment (Figure 4E-F).

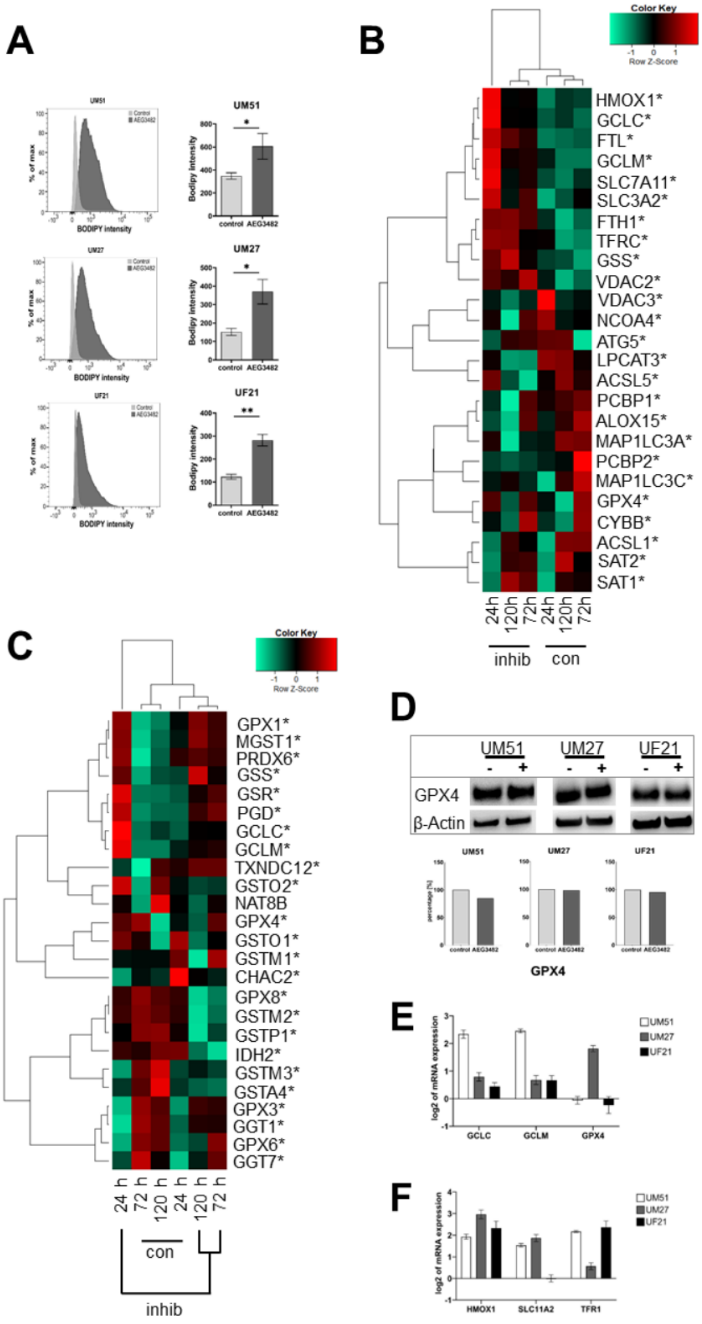

Figure 4. Inhibition of JNK signalling increases lipid peroxidation. A) Representative histograms of measured fluorescence intensities after BODIPY staining and the respective bar plots of mean fluorescence intensity of control or JNK-inhibited UdRPCs (n=5; * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). Error bars indicate SEM. B) Gene expression of iron metabolism-related genes in UM51 cells for the time points 24 h, 72 h and 120 h depicted in a Pearson's heatmap. C) Pearson's heatmap depicting gene expression of glutathione metabolism-related genes in UM51 cells for the time points 24 h, 72 h and 120 h. D) Protein expression of GPX4 in UdRPCs with or without JNK inhibition. E) mRNA expression of glutathione metabolism-related markers GCLC, GCLM, GPX4. Error bars indicate SEM. F) mRNA expression of iron metabolism-related markers HMOX1, SLC11A2 and TFR1. Mean values were normalized to the housekeeping gene RPL37A. Error bars indicate SEM.

JNK inhibition partially disrupts the mitochondrial membrane potential and reduces respiration

Oxidative Phosphorylation in JNK-inhibited and control UdRPCs was evaluated by measuring the oxygen consumption rate (OCR) via Seahorse Agilent Mito Stress test. The graphs in Figure 5A show basal respiration, the maximal respiratory capacity and the spare respiratory capacity in UdRPCs treated with the JNK inhibitor. The data obtained demonstrate a significant reduction in basal respiration, maximal respiration and spare respiratory capacity in UM51, UM27 and UF21 cells (Figure 5A).

Reduction of oxidative phosphorylation and SRC are indicators of defective mitochondrial function. Therefore, we investigated the influence of JNK inhibition on mitochondrial membrane potential in UdRPCs via TMRM staining (Figure 5B). We observed a significant reduction of mitochondrial membrane potential in UM51 and UM27 compared to the respective controls (Figure 5C). UF21 cells showed no change in mitochondrial membrane potential upon JNK inhibition (Figure 5C).

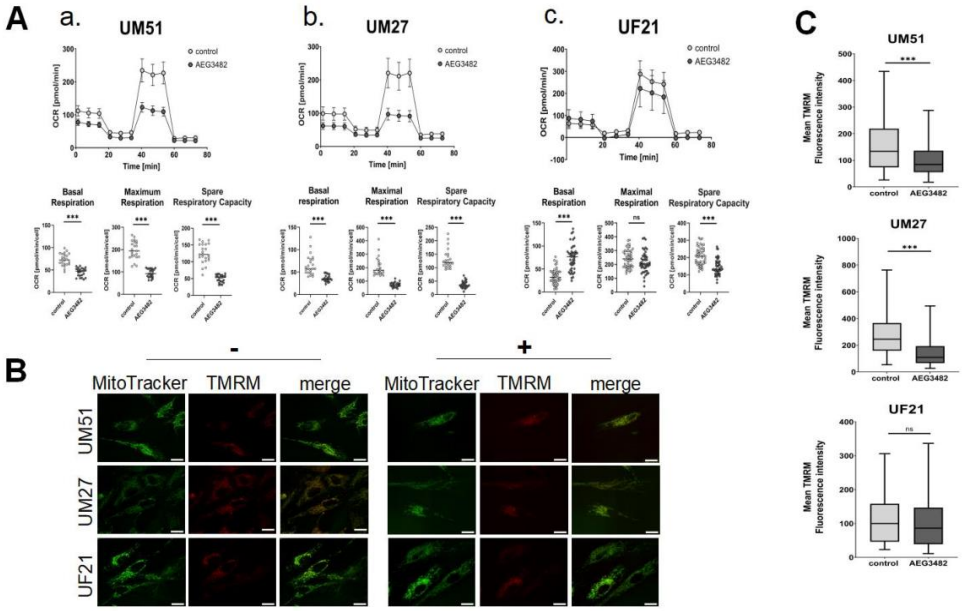

Figure 5. JNK signaling regulates mitochondrial respiration via membrane potential. A) Measurement of OCR in real time in UdRPCs. Basal respiration, maximal respiration and spare respiratory capacity are depicted (* ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). Error bars indicate SD. **a.** OCR in UM51 (n=1; N=23). **b.** OCR in UM27 (n=1; N=23). **c.** OCR in UF21 (n= 1; N= 46). B) Fluorescence images of MitoTracker Green and TMRM stainings in UdRPCs UM51, UM27 and UF21 with and without JNK inhibition. Scale bars depict 10 µm. C) Measurement of mean TMRM fluorescence signal intensity in UdRPCs * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). Outliers were removed. Error bars indicate SEM. **a.** Mean TMRM signal in UM51 is depicted (n=2; ctrl. N= 1; + JNK inhibitor N= 2). **b.** Mean TMRM signal in UM27 is depicted (n=2; ctrl. N= 1; + JNK inhibitor N= 2). Error bars indicate SEM. **c.** Mean TMRM signal in UF21 is depicted (n=2; ctrl. N= 1; + JNK inhibitor N= 2).

Discussion:
JNK inhibition decreases the proliferation and leads to loss of progenitor character

In this study, JNK signalling was inhibited using the small molecule inhibitor AEG3482 on three UdRPC cultures, UM51, UM27 and UF21. We discovered the importance of JNK for the proliferative potential of the self-renewing urine-derived renal progenitor pool.
Different concentrations of the inhibitor were tested beforehand, and the final concentration of 10 µM AEG3482 was used further. Inhibition of the active JNK enzyme was demonstrated by Western Blot analysis. The inhibitor did not completely block the phosphorylation of JNK, which would explain why the active form of the direct downstream target cJUN was still observed (Javelaud and Mauviel, 2005). Interestingly, in UF21 cells, an inhibitor concentration of 10 µM resulted in higher p-cJUN protein levels of treated than in non-treated cells. Besides the observation that UF21 cells could tolerate higher concentrations of the JNK inhibitor AEG3482 for longer periods, adaptation or insensitivity to the 10 µM concentration of the inhibitor might be an explanation for higher p-cJUN levels.

Even though the cell morphology of the three UdRPC cultures did not change upon JNK inhibition, we observed lower cell density. We therefore assumed a reduced proliferation rate in the AEG3482-treated cells. Comparable results were found in a study on the role of JNK signalling in proliferative nephron progenitors of a mouse model (Blank et al., 2009). While it was demonstrated that BMP7 signalling enhanced the proliferation of murine $SIX2^+$ nephron progenitor cells, JNK inhibition disrupted this effect (Blank et al., 2009). It has been shown that BMP7-induced JNK signalling increases the proliferative capacity of $SIX2^+$ nephron progenitor cells in the mouse model. As UdRPCs share similar characteristics with nephron progenitor cells, a similar outcome regarding the proliferation is very likely. To test the effect of JNK inhibition on the proliferative capacity of UdRPCs, we conducted a follow-up proliferation assay using KI67 as marker. We could detect statistically significant reduction of proliferation in UdRPCs, UM51 and UM27, treated with JNK inhibitor after 72 h. The proliferation rate of JNK-inhibited UF21 cells was not significantly reduced, which might be caused by higher inhibitor tolerance as exhibited before. Moreover, gene expression of KI67 and gene ontologies related to cell cycle were downregulated in JNK-inhibited UdRPCs. In our study, genes of KEGG annotated cell cycle-related GO term (*BUB1, CCNA2, CCNB2, CCND2, MCM7, PLK1)* were downregulated by JNK inhibition. The identified common genes are mainly involved in the cell cycle phase transitions, indicating

interruption of the cell cycle and subsequent reduction of proliferation. Additionally, a heatmap analysis revealed absence of cell cycle-related genes (*CCND2*, *SMAD4*, *CDC14B*, *HDAC1*, *CCNH*, *WEE1*, *TFDP2*, *RBX1*) upon JNK inhibition and expression in all controls as well as sustained expression in JNK-inhibited UM51 after 24 h. We observed a downregulation of the progenitor marker *SIX2*, *SALL1* and *VCAM1* (*CD106*) after AEG3482 treatment. Besides the effect of JNK on the proliferative capacity of UdRPCs, reduced expression of the genes *SIX2*, *SALL1* and *CD106* indicate a loss of progenitor status. Muthukrishnan *et al.* confirmed the Tak1-Jnk-Jun pathway maintained the numbers murine nephron progenitors by keeping the cells in a proliferative state (Muthukrishnan et al., 2015). Therefore, our results signify that JNK signalling is involved in cell cycle progression, proliferation and maintenance of the progenitor state of UdPRCs.

In vivo, NPC self-renewal is sustained by the interaction of growth factors, signalling pathways and metabolic pathways within the nephron progenitor niche and changes can induce differentiation (Liu et al., 2017; Oxburgh and Rosen, 2017). In our previous work, we have observed that FGF-induced TGFβ/BMP signalling determines the cell fate of urine-derived renal progenitor cells, since we have demonstrated downregulation of p-SMAD2/3 and upregulation of p-SMAD1/5/8 in differentiated UdRPCs upon activation of WNT signalling (Rahman et al., 2020). We hypothesized that active SMAD2 is necessary to maintain the progenitor state of UdRPCs. In contrast to our previous findings, we observed downregulation of both, p-SMAD2 and p-SMAD1/5, upon JNK inhibition. This indicates an interconnection between JNK and SMAD signalling, which is unexpected as SMAD signalling is commonly believed to be independent from JNK signalling (Zhang, 2009). Based on our findings from previous works (Rahman et al., 2020), the decreased expression of p-SMAD2/3 implies the loss of nephron progenitor state due to JNK inhibition. Other signalling pathways such as the BMP7-induced and JNK-independent Smad1/5/8 pathway were also observed to be contributing to the maintenance of the renal progenitor pool in the murine model (Tomita et al., 2013). Tomita *et al.* (2013) were able to demonstrate that an inhibition of BMP7-Smad signalling leads to the differentiation of nephron progenitor cells and thus they proposed an important role of BMP7-Smad signalling for the maintenance of the renal progenitor cells and the determination of final nephron numbers (Tomita et al., 2013). Similar to the mentioned observations in the mouse model, phosphorylated SMAD1/5/8 was decreased in JNK-inhibited UdRPCs UM51 and UM27, indicating the loss of the progenitor state.

Active JNK signalling protects urine-derived renal progenitor cells against ferroptosis

In this study, we have shown that the inhibition of JNK induces ferroptosis. Ferroptosis is characterized by the iron-dependent formation of lipid peroxides leading to cell death. The FACS-based measurement of lipid peroxides confirmed that JNK inhibition significantly increased sensitivity to ferroptosis. Moreover, addition of a ferroptosis inhibitor (Liproxstatin-1) protected the cells from accumulating lipid peroxides. FGF signaling regulates the maintenance of self-renewal in NPCs and UdRPCs (Barak et al., 2012; Rahman et al., 2020). Inhibition of FGF signaling via FGFR inhibitor SU-5402 increased the level of lipid peroxides significantly. A recent publication described the role of FGF21 in the suppression of iron overload-induced ferroptosis in liver (Wu et al., 2021). Similarly, FGF signaling may have protective properties against ferroptosis in UdRPCs, however this warrants further investigation beyond the scope of the current study.

KEGG pathway analysis revealed a significant upregulation of genes associated with the GO term ferroptosis and glutathione metabolism upon JNK inhibition. In our heatmap analyses, we observed clustered expression of ferroptosis-related genes in the JNK-inhibited samples, which are mainly involved in iron and glutathione metabolism. In line with these results, we demonstrated upregulated mRNA expression of glutathione metabolism-related genes *GCLC, GCLM,* and *SLC7A11* (*cystine-glutamate antiporter* X_c^-) as well as iron metabolism-related genes *HMOX1*, *SLC11A2* and *TFR1*. Increased expression of regulators of the iron metabolism involved in the iron (Fe^{2+}) release from cellular storage and influx of iron can be one of the inducers of ferroptosis (Chen et al., 2020). An accumulation of free iron (Fe^{2+}) catalyzes the Fenton reaction, which generates hydroxyl radicals, thus leading to peroxidation of polyunsaturated fatty acids (Latunde-Dada, 2017). Heme oxygenase 1 (HO-1, encoded by *HMOX-1*) could contribute to increased iron levels by liberation of iron during heme degradation (Kwon et al., 2015). In contrast, other publications describe that HO-1 suppresses ferroptosis. Adedoyin *et al.* demonstrated increased expression of HO-1 in renal proximal tubular cells resulted in alleviation of ferroptosis (Adedoyin et al., 2018). Moreover, HO-1 is a marker for active NRF2, a transcription factor activating the expression of several genes encoding anti-ferroptotic proteins, including GPX4 (Nishizawa et al., 2022).

Interestingly, we could not observe a change in the protein level of GPX4 post JNK inhibition. However, upregulated transcript levels of genes encoding proteins important for GSH biosynthesis indicate that the cell is limited in GSH, the essential cofactor for GPX4 activity. High

levels of GSH and increased amounts of GPX4 are usually negative regulators of ferroptosis (Sharma et al., 2021), while the opposite induces this form of cell death (Berndt and Lillig, 2017). Ferroptosis-was discovered to be one of the causes in the induction of kidney diseases such as acute kidney injury, I/R injury, Clear Cell Renal Cell Carcinoma and Adrenocortical Carcinomas (Galluzzi et al., 2018). Therefore, we assume that UdRPCs treated with the JNK inhibitor AEG3482 may represent an easily available model for studying ferroptosis-induced kidney diseases in the near future.

JNK signaling is involved in the metabolic activity associated with the maintenance of self-renewal in UdRPCs

The role of mitochondria in ferroptosis is not understood so far (Jiang et al., 2021). Mitochondria could promote ferroptosis via formation of peroxides by altered electron transfer during oxidative phosphorylation but could also be a target of ferroptosis by inducing damage to mitochondrial membranes leading also to dysfunctional energy metabolism. Since JNK inhibition is accompanied by ferroptosis, we decided to study the respiratory function of mitochondria in JNK-inhibited UdRPCs. Therefore, Mito Stress test was performed to determine the influence of JNK inhibition on mitochondrial respiration in UdRPCs. We observed a significant reduction of mitochondrial respiration in UdRPCs upon JNK inhibition. In particular, measurement of the spare respiratory capacity (SRC) is an indicator of cellular health, since it represents the cell's ability to react to increased energy demand or stress. Therefore, significant reduction of the SRC in all three UdRPCs cultures demonstrates a loss of metabolic capacity of mitochondria upon inhibition of JNK signaling.

Oxidative phosphorylation is crucially dependent on the membrane potential generated by the electron transport chain (ETC) in the inner mitochondrial membrane (Perry et al., 2011). Thus, malfunction of the ETC in mitochondria is usually accompanied with the loss of mitochondrial membrane potential (van der Stel et al., 2022). We performed TMRM staining to investigate if there is a link between the reduction OCR and a rupture of mitochondrial membrane potential caused by JNK inhibition. With TMRM staining, we could indeed observe a reduction in mitochondrial membrane potential upon JNK inhibition. Overall, there is multiple evidence that mitochondrial dysfunction is increased by JNK pathway inhibition in UdRPCs.

JNK signaling is involved in proliferation processes, consequently this pathway also regulates

cellular respiration. Xie, Sun *et al.* demonstrated that inhibiting the JNK signaling pathway in hematopoietic stem cells results in a reduction in the expression of genes related oxidative phosphorylation (Xie et al., 2022). Our data indicates that inhibition of JNK signaling pathway leads to mitochondria impairment and ferroptosis. One of the hallmarks for iron-dependent cell death include morphological changes of the mitochondria such as a blistered cell membrane, reduction in size and loss of mitochondria cristae (Galluzzi et al., 2018; Li et al., 2020). Since mitochondrial complexes involved in oxidative phosphorylation are localized in the inner mitochondrial membrane, ferroptosis-related damage of mitochondria may be an explanation for the rupture of mitochondrial membrane and thus to an impaired oxidative phosphorylation (Nolfi-Donegan et al., 2020). Moreover, mitochondrial dysfunction via mitochondrial ROS production activates mitochondrial JNK signalling, which promotes Bax-dependent apoptosis (Chambers and LoGrasso, 2011; Niizuma et al., 2010). Based on this finding, JNK inhibition in our study could lead to an inhibition of Bax-dependent apoptosis, which simultaneously enhances ferroptosis.

Conclusion:

In this study, we demonstrated the importance of JNK signaling for the maintenance of self-renewal and the proliferation capacity SIX2-positive in urine-derived renal progenitor cells. Pathway inhibition led to the emergence of ferroptosis-induced cell death in UdRPCs and was accompanied by disrupted mitochondrial membrane potential and overall reduced oxidative phosphorylation. Therefore, we propose the use of JNK-inhibited UdRPCs as model for ferroptosis-induced kidney diseases such as acute kidney injury.

Acknowledgements: J.A. acknowledges the medical faculty of Heinrich Heine University for financial support.

J.A., C.B., and A.S.R. acknowledge that this work is partly funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 417677437/GRK2578.

Author Contributions: L.N. designed and performed experiments, analysed the data, wrote and edited the manuscript. M.W. and L.T. assisted in experimental design, performed experiments,

analysed data, wrote and edited the manuscript. W.W. prepared the formal analysis, data curation, helped with the figures and edited the manuscript. A.S.R. supervised oxidative phosphorylation-related experiments and edited the manuscript. C.B. supervised ferroptosis-related experiments and edited the manuscript. J.A. conceptualized the work, wrote and edited the manuscript, had the project administration, acquired funding and supervised the study. All authors have read and agreed to the published version of the manuscript.

Declaration of interests: The authors declare no competing interests.

Figure Titles and Legends

Figure 1. Inhibition of JNK reduces the proliferation of UdRPCs. A) Morphology of the three UdRPCs UM51, UM27 and UF21 with or without AEG3482 treatment after 72 h. Scale bars represent 100 µm. B) Ki67 expression in UdRPCs treated with or without AEG3482 for 72 h. Scale bars represent 50 µm. C) Ki67 proliferation assay for JNK-inhibited UdRPCs (n=10; * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). D) mRNA expression of nephron progenitor marker *SIX2*, *SALL1*, *VCAM1* and *Ki67*. Mean values were normalized to the housekeeping gene *RPL37A*. Error bars indicate SEM. E) Gene expression of cell cycle-related genes for the time points 24 h, 72 h and 120 h depicted in a Pearson's heatmap.

Figure 2. JNK signaling is associated with cell cycle processes in UdRPCs. A) Representative enrichment clusters for control and JNK-inhibition after 24 h depicted in a heatmap and cell cycle-related processes marked with an arrow. Venn diagram of control and JNK-inhibited UM51 cells for the time point 24 h. B) Representative enrichment clusters for control and JNK-inhibition after 72 h depicted in a heatmap and cell cycle-related processes marked with an arrow. Venn diagram of control and JNK-inhibited UM51 cells for the time point 72 h. C) Representative enrichment clusters for control and JNK-inhibition after 120 h depicted in a heatmap and cell cycle-related processes marked with an arrow. Venn diagram of control and JNK-inhibited UM51 cells for the time point 120 h.

Figure 3. Inhibition of JNK signaling affects the downstream target cJUN and SMAD proteins. A) Protein expression of p- and t-JNK in UdRPCs with or without JNK inhibition. B) Protein expression of p- and t-cJUN in UdRPCs with or without JNK inihibition. C) Immunofluorescence stainings of cJUN and p-cJUN in UdRPCs with or without JNK inhibition. Scale bars represent 50 µm. D) Protein expression of p-/t-SMAD1/5 and p-/t-SMAD2/3 in UdRPCs with or without JNK inhibition.

Figure 4. Inhibition of JNK signalling increases lipid peroxidation. A) Representative histograms of measured fluorescence intensities after BODIPY staining and the respective bar plots of mean fluorescence intensity of control or JNK-inhibited UdRPCs (n=5; * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). Error bars indicate SEM. B) Gene expression of iron metabolism-related genes in UM51 cells for the time points 24 h, 72 h and 120 h depicted in a Pearson's heatmap. C) Pearson's heatmap depicting gene expression of glutathione metabolism-related genes in UM51 cells for the time points 24 h, 72 h and 120 h. D) Protein expression of GPX4 in UdRPCs with or without JNK inhibition. E) mRNA expression of glutathione metabolism-related markers GCLC, GCLM, GPX4. Error bars indicate SEM. F) mRNA expression of iron metabolism-related markers *HMOX1*, *SLC11A2* and *TFR1*. Mean values were normalized to the housekeeping gene *RPL37A*. Error bars indicate SEM.

Figure 5. JNK signaling regulates mitochondrial respiration via membrane potential. A) Measurement of OCR in real time in UdRPCs. Basal respiration, maximal respiration and spare respiratory capacity are depicted (* ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). Error bars indicate SD. **a.** OCR in UM51 (n=1; N=23). **b.** OCR in UM27 (n=1; N=23). **c.** OCR in UF21 (n= 1; N= 46). B) Fluorescence images of MitoTracker Green and TMRM stainings in UdRPCs UM51, UM27 and UF21 with and without JNK inhibition. Scale bars depict 10 µm. C) Measurement of mean TMRM fluorescence signal intensity in UdRPCs * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). Outliers were removed. Error bars indicate SEM. **a.** Mean TMRM signal in UM51 is depicted (n=2; ctrl. N= 1; + JNK inhibitor N= 2). **b.** Mean TMRM signal in UM27 is depicted (n=2; ctrl. N= 1; + JNK inhibitor N= 2). Error bars indicate SEM. **c.** Mean TMRM signal in UF21 is depicted (n=2; ctrl. N= 1; + JNK inhibitor N= 2).

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, James Adjaye (James.Adjaye@med.uni-duesseldorf.de).

Methods

Cell Culture

For this study, three distinct UdRPC lines UM51, UM27 and UF21 were used. Their names describe the donor's gender (UM-urine male; UF- urine female) and age. The cells were cultivated on 0.2 % gelatine-coated plates and were maintained in proliferation medium supplemented with 5 ng/ml bFGF (Peprotech) every second day (Rahman et al., 2020). The optimal concentration of the JNK inhibitor AEG3482 (TOCRIS) was determined by titration of different concentrations on UM51 cells. The concentrations 10 µM, 50 µM and 100 µM AEG3482 inhibitor were applied to UM51 cells for 48 h to 120 h with daily medium change. A concentration of 10 µM AEG3482 was adopted for subsequent experiments. At 80 % confluency, cells were trypsinized with TrypLE (Life Technologies) and were seeded on gelatine-coated plates. The treatment with 10 µM AEG3482 inhibitor was started at 60-70 % confluency and was maintained for 24 h, 72 h and 120 h. In parallel, untreated cells were kept as control for the same time points and were cultivated in proliferation medium with daily changes of medium. Like UM51 cells, the cell lines UM27 and UF21 were treated for 72 h with 10 µM JNK inhibitor AEG3482 with daily medium changes.

Proliferation assay

After JNK inhibition, cells were fixed with 4 % paraformaldehyde (Polysciences) and stained with the antibody anti-mouse KI67, 1:200 (CST). Randomly chosen pictures were taken from each well and KI67-positive cells as well as total cell numbers were counted (N=10). The ratio of KI67-positive/total cell number was calculated and statistical analysis was processed in Graphpad Prism software (Dotmatics). *P*-values were calculated with an unpaired t-test (two-tailed). (* ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001).

Immunofluorescence

Cells were fixed with 4 % paraformaldehyde and were subsequently permeabilized with 0.5 % Triton X-100/PBS (Sigma-Aldrich) for 15 min. Prior to incubation with the primary antibodies, blocking with 3 % BSA (Sigma-Aldrich) for 1 h at room temperature was performed. Primary

antibodies were diluted as following: anti-mouse KI67 (1:200), anti-rabbit cJUN (1:400) (CST), anti-rabbit phospho-cJUN (1:800) (CST). The plates were then incubated at 4°C overnight. Labelled cells were detected with the secondary antibodies- anti-rabbit Alexa Fluor™ 488 (1:500) (Thermofisher) and anti-mouse Alexa Fluor™ 555 (1:500) (Thermofisher). Nuclei were stained with Hoechst (1:5000) (Thermofisher). Pictures were taken under fluorescence microscope (LSM700; Zeiss) and processed with ZenBlue 2012 Software Version 1.1.2.0 (Zeiss) and Image J (NIH).

Western Blotting

Total protein was extracted by lysing the cells with RIPA Buffer (Sigma-Aldrich) containing phosphatase and protease inhibitors (Sigma-Aldrich). Protein was quantified with the Pierce's BCA assay kit from Thermo Scientific. Electrophoresis was run with a protein input of 20 µg. Proteins were bound with antibodies including anti-rabbit phospho-SAPK/JNK (1:1000) (CST), anti-rabbit SAPK/JNK (1:1000) (CST), anti-rabbit cJUN (1:1000) (CST), anti-rabbit phospho-cJUN (1:1000) (CST), anti-rabbit SMAD2/3 (1:1000) (CST), anti-rabbit phospho-SMAD2 (CST) (1:1000), anti-rabbit SMAD1 (1:1000) (CST), anti-rabbit phospho-SMAD1/5 (1:1000) (CST), anti-rabbit GPX4 (1:1000) (CST) and anti-mouse β-actin (1:5000) (CST). Enhanced chemiluminescent (ECL) horseradish-peroxidase (HRP) detection technique was used to detect the specific proteins (Life Technologies).

Flow cytometry-based measurement of lipid peroxidation

Untreated and JNK-inhibited cells were cultivated for 72 h with daily medium change. For inhibition of ferroptosis, cells were additionally treated with 100 nM Liproxstatin-1 (Lip-1) (Sigma-Aldrich) 1h before JNK inhibition via AEG3482. Cells were washed twice with PBS and stained with 1 µM BODIPY™ 581/591 C11 (Invitrogen) for 15 min at 37 °C and 5 % CO_2. Subsequently, cells were washed twice with PBS and harvested with trypsin. The samples were centrifuged for 10 minutes at 700 g and resuspended in 300 µl MACS buffer (0.5 % BSA, 2 mM EDTA, PBS). Lipid peroxidation was detected by measuring the fluorescence intensity of BODIPY 581/591 C11 using BD FACS Canto 2 (BD Biosciences). In each case, $3x10^4$ events were measured, and the data evaluated using FlowJo software (BD Biosciences). Statistical significance was calculated

according to unpaired t-test (two-tailed) using GraphPad Prism software (n=5; * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001).

Seahorse XF Cell Mito Stress Test

Optimal seeding density and FCCP concentration without toxicity response were tested before starting the Seahorse XF Cell Mito Stress Test assay (Agilent). A cell density of $4x10^3$ cells per well and a FCCP concentration of 2 µM was determined and used for the following assays. Cells of the three UdRPC lines UM51, UM27 and UF21 were seeded on gelatine-coated Seahorse XF Cell Culture Microplates. For mitochondrial oxygen consumption rate (OCR) measurements of JNK pathway inhibition, cells were treated with or without JNK inhibitor AEG3482 for 72 h. Following the manufacturer's protocol, XF sensor cartridges were hydrated with Seahorse XF Calibrant at 37°C in a hypoxic incubator overnight. The medium was changed to phenol-free Seahorse XF DMEM (10 mM glucose, 1mM pyruvate and 2 mM L-glutamine (all from Sigma Aldrich). The OCR was measured in a Seahorse XFe96 Flux Analyser with Seahorse Wave 2.4 software (Agilent). In 3 cycles of 3 min mixing and 3 min recording, 1 µM Oligomycin, 2 µM FCCP and 0.5 µM Antimycin/Rotenone AA was injected and OCR was measured. The OCR of UF21, UM51 and UM27 was measured with one biological replicate (UM51 and UM27: N=23; UF21: N=46). The data was normalized to the cell number and statistical significance was calculated with an unpaired t-test (* ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001).

TMRM Staining

Cells were seeded on MatTek glass bottom dishes for microscopy followed by a cultivation time of 72 h with or without AEG3482 and daily media change. Cells were then stained with Tetramethylrhodamin (TMRM) and MitoTracker™Green FM (Invitrogen) for 30 min. Briefly, cells were washed thrice with 1 x PBS and were covered in Opti-MEM™(Gibco) without phenol red containing 10 µM HEPES buffer for buffering oxidation of media. Fluorescence imaging of UdRPCs was acquired at Nikon Ti2 inverted confocal microscope, coupled with UltraVIEW®VoX spinning disc laser system (PerkinElmer) equipped with a 63-x oil objective (N.A. 1.2). Imaging was performed in a chamber at 37 °C. For analysis of the fluorescence images, background correction was performed by manually defining a region of interest (ROI) in the background of the image

and subtraction of the mean fluorescence signal in all images. For each individual cell, a ROI was manually defined for measurement of TMRM fluorescence intensity. Background correction and TMRM intensity measurement of 50 cells per sample was performed with Volocity® Software for spinning disk microscopy. High variations in fluorescence intensities were cleaned by an outlier test ROUT (Q = 1%). Cleaned data was used for statistical and graphical analysis, performed in GraphPad Prism software. Statistical significance was calculated with an unpaired t-test (n=2; control: N= 1; JNK inhibition: N= 2; * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001).

Quantitative RT-PCR

RNA was isolated with the DIrectzol RNA MiniPrep kit (Biozol) according to the manufacturer's protocol. Subsequently, 500 ng RNA was transcribed to cDNA using MultiScribe Reverse Transcriptase (Life Technologies). Quantitative PCRs were performed in technical triplicates using POWER SYBR Green Master Mix (Life Technologies). Primer sequences are listed in *Table S1* (purchased from MWG). Mean values were normalized to the housekeeping gene *RPL37A*, compared to an untreated control for the specific time point and calculated by the 2−ΔΔCt method.

Gene expression analysis

Total RNA of UdRPCs treated with the JNK inhibitor AEG3482 and untreated cells was hybridized onto microarrays of type Affymetrix Human Clariom S assay at the BMFZ (Biomedizinisches Forschungszentrum) core facility of the Heinrich-Heine University, Düsseldorf. The R/Bioconductor environment (Gentleman et al., 2004) was employed to process the Affymetrix microarray data. The data was background-corrected via the Bioconductor package oligo (Carvalho and Irizarry, 2010) and normalized applying the Robust Multi-array Average (RMA) method. The packages VennDiagram (Chen and Boutros, 2011) and gplots (Warnes et al., 2005) were used to generate Venn diagrams of the numbers of genes expressed in the control or JNK-inhibition conditions. A gene was considered expressed when the detection p-value - determined as described in Nguyen *et al.* (2022) was below the threshold of 0.05 (Nguyen et al., 2022). Hierarchical clustering was analyzed via (i) the R function *hclust* parametrized with Pearson correlation as similarity measure and complete linkage as agglomeration method in dendrograms,

(ii) the R function *heatmap.2* from the gplots package (Warnes et al., 2005) and also parametrized with Pearson correlation as similarity measure and additionally with colour-scaling per row/gene in heatmaps.

Gene ontology (GO) and pathway analysis

Upregulated genes were calculated by the criteria: ratio between the JNK-inhibited state and control greater than 1.5 and detection-p-value in the JNK-inhibited state below the threshold of 0.05, down-regulated genes analogously by the criteria: ratio between the JNK-inhibited state and control less than 0.67 and detection-p-value in the control state below the threshold of 0.05. From the resulting gene sets over-represented GOs were determined via the Bioconductor package GOstats (Falcon and Gentleman, 2007). KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al., 2017) pathways were analyzed for over-representation based on associations between pathways and genes downloaded from the KEGG database in July 2020. For each of the KEGG pathways the hypergeometric test was applied to the sets of up- and downregulated genes, which could be annotated to that pathway. Furthermore, genes from KEGG pathways as well as gene sets found by single cell sequencing analysis of fetal kidney development by Lindström *et al.* (2018) were employed to generate heatmaps for cluster analysis as described above (Lindström et al., 2018).

Metascape Analysis

Gene enrichment analyses of differential GO/KEGG terms and biological processes between JNK-inhibited UdRPCs and untreated controls were performed using metascape (http://metascape.org; (Zhou et al., 2019)). Exclusive gene sets of JNK inhibition and control of each time point (24 h, 72 h, 120 h) were used as data source. The metascape software applied hierarchical clustering to display calculated significant GO terms into a tree, which was spread into term clusters with a 0.3 kappa score as a threshold. The top enrichment clusters were represented as heatmaps with a color scale ranging from gray to dark orange. Statistical significance was hereby displayed in dark orange and lack of enrichment in gray color.

Data Availability

Microarray raw data have been deposited at NCBI GEO and are publicly available as of the date of publication. Accession number is listed in the key resources table. All data reported in this paper will be shared by the lead contact upon request.

Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

Supplemental Information

Figure S1. Inhibition of JNK and FGF induces stress in UdRPCs (Related to Figure 1 and 4). A) Morphological analysis of cellular stress and cell death in UM51 treated with 50 µM and 100 µM AEG3482 inhibitor. Scale bars represent 100 µm. B) Morphological analysis of cellular stress and cell death in UF21 treated with 50 µM and 100 µM AEG3482 inhibitor. Scale bars represent 100 µm. C) Representative histograms of measured fluorescence intensities after BODIPY staining and the respective bar plots of mean fluorescence intensity of control, JNK inhibition and JNK inhibition+Lip-1 (n=5; * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). Error bars indicate SEM. D) Representative histograms of measured fluorescence intensities after BODIPY staining and the respective bar plots of mean fluorescence intensity of control or FGF-inhibited UdRPCs (n=5; * ρ-value < 0.05, ** ρ-value < 0.01, *** ρ-value < 0.001). Error bars indicate SEM.

Table S1: List of qRT-PCR primers (Related to Figure 1 and 4).

Table S2: Lists of exclusive and common genes of the Venn analysis for 24h (Related to Figure 2).

Table S3: Lists of exclusive and common genes of the Venn analysis for 72h (Related to Figure 2).

Table S4: Lists of exclusive and common genes of the Venn analysis for 120h (Related to Figure 2).

Table S5: GO terms of down- and up- regulated genes from KEGG analysis for the time points 24 h, 72 h and 120 h after JNK inhibition (Related to Figure 2).

Table S6: Downregulated cell cycle-related gene lists of Metascape analysis for the time points 24 h, 72 h and 120 h after JNK inhibition (Related to Figure 2).

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4. Discussion

Kidney diseases are a worldwide health burden, and the mortality has increased tremendously in the last few years. Kidney diseases describe the state of the kidneys when renal functions are restricted or lost. The most common conditions, associated with kidney diseases, are referred to as acute kidney injury (AKI) and chronic kidney disease (CKD). Manifold reasons such as renal ischemia, medications and surgeries can cause AKI, while hypertension and diabetes are the main causes of CKD (Makris & Spanou, 2016; Webster et al., 2017). AKI is associated with an increased mortality, high risk for progression to CKD and other health complications including an increased risk for cardiac infarcts and strokes (Centers for Disease Control and Prevention, 2021; Lewington et al., 2013). In 2017, between 700-800 million people suffered from chronic kidney disease, whereas disease prevalence in people of older age, female sex and certain ethnicities were statistically higher (Carney, 2020; Kovesdy, 2022). Replacement therapies such as organ transplantation and hemo-dialysis are the current treatment options to prevent the progression to end-stage renal disease, but come with complications including low donor organ availability, immune suppression, and a general reduction of life quality for the patients. In 2017, 2.6 million patients have died from CKD and cardiovascular disease resulting from impaired kidney function (Bikbov et al., 2020). It is estimated that until 2030, restricted access to renal replacement therapies will lead to a premature death of approximately 2.3-7.1 million patients (Bikbov et al., 2020). Therefore, finding cellular alternatives to restore kidney functions is the overall aim in the field of regenerative medicine. A step closer to this goal was achieved by the invention of cellular reprogramming, a process to reset the cell's potential from a somatic cell back to a pluripotent stem cell, a state of self-renewal and increased differentiation potential (Takahashi et al., 2007). While cells used for the reprogramming often come from skin biopsies, urine has become a cell source with easier access. In the last years, cells isolated from urine were extensively studied, whereas urine-derived stem cells gained attention due to their potential use in regenerative medicine and studying kidney diseases. Current cell models for researching kidney pathologies include immortalized tubular cells with possible chromosomal aberrations and primary proximal tubular epithelial cells (PTECs), which rapidly lose their phenotype *in vitro* (Barnett & Cummings, 2018; Faria et al., 2019). The function of re-absorption makes tubular cells the main target of drugs and other toxic

substances. Thus, novel medications must be tested for their nephrotoxicity, before these compounds can be approved for the clinical use. Urine-derived cells have developed to a promising tool for drug screenings and disease modelling. Similar to mesenchymal stem cells, urine stem cells are capable to adhere to plastic, have a multi-lineage differentiation potential and express mesenchymal surface markers while hematopoietic stem cell markers are absent. Since these cells also express markers typical for nephron progenitor cells, we have previously used the term urine-derived renal progenitor cells (UdRPCs) for better description (Rahman et al., 2018). Since UdRPCs share characteristics with pluripotent stem cells, such as the expression of pluripotency-associated cell surface markers, reprogramming of urine cells to induced pluripotent stem cells has been proved to be efficient. Moreover, the high differentiation potential of iPSCs has become a source for the generation of three-dimensional cell models, called organoids, which mimic the variability of cell types, histological structure, and functions of the target organ. Although tissues and organs of the human body are well-studied, these *in vitro* models can give deeper insights into early developmental and metabolic processes, which cannot be studied otherwise because of ethical concerns regarding research on human embryos. Besides drug toxicity testing, the organoid technology can be utilized for the development of novel regenerative therapies and studying the pathophysiology of various diseases.

The overall aim of this thesis was directed to understand cellular processes in the kidney and improve the current possibilities to model kidney diseases *in vitro*. Three-dimensional kidney models were established using urine-derived renal progenitor cells in co-culture with mesenchymal and endothelial cells and another approach utilized induced pluripotent stem cells, which were induced with a nephrotoxin to model kidney injury. Additionally, signalling mechanisms of the JNK pathway and the involvement with self-renewal and the maintenance of progenitor state in urine-derived renal progenitor cells were studied.

4.1 Generation of an isogenic 3D kidney model based on three cell types

Recently, the organoid technology emerged as an alternative for conventional cell layer models by resembling the *in vivo* organ more closely. Pluripotent stem cells can develop into all organ tissues, while adult stem cells mainly generate a single epithelial layer of a specific organ and are more useful for toxicological studies. One adult stem cell population can be found in urine, referred to as urine-derived renal progenitor cells (UdRPCs), which are promising for regenerative medicine. The multipotent stem cells have a high differentiation potential to various kidney cells. To exploit the full potential of the easily available cell source, this work aimed to establish a 3D cell model based on urine stem cell with an improved structural complexity by co-culture with two additional cell types, mesenchymal stem cells and endothelial cells.

A three-dimensional nephron progenitor model based on three cell types from an isogenic background was established. Current cell therapies in regenerative medicine are predominantly based on allogenic donor cells, causing subsequent problems such as immune rejection. Cells from the same genetic background can be useful for personalized medicine such as cell replacement therapies, drug screenings and disease modelling. The co-culture of various cell types should support the recreation of a more complex environment for better mimicking of the *in vivo* conditions in the kidney. Previously shown by Takebe *et al.* (2015), mesenchymal stem cells are the driving force of the self-condensation process, while endothelial cells support oxygen and nutrient supply via vascularization. iPSCs, which were previously reprogrammed from UdRPCs, were differentiated to mesenchymal and endothelial cells, which were referred to as iMSCs and iECs. Characterization of iMSCs revealed the expression of mesenchymal cell surface marker CD73, CD90, CD105 and absence of hematopoietic stem cell marker CD14, CD20, CD34 and CD45. Additionally, they had a tri-lineage differentiation potential to adipocytes, chondrocytes and osteoblasts and expressed the MSC markers, α-SMA, Vimentin and PDGFR-β. The differentiated iECs expressed endothelial marker CD31 like human umbilical cord endothelial cells. The co-culture of the three cell types resulted in self-condensed, three-dimensional cell aggregates. The cell aggregates in this study were referred to as 3D-NPCs, as they shared characteristics of nephron progenitor cells and pluripotent stem cells. The round-shaped 3D-NPCs expressed markers associated with nephron progenitor cells such as SIX2 and PAX8 and pluripotency-related genes such as TRA-1-81, TRA-1-60 and SSEA4. The presence of glomerular epithelial

cells, such as podocytes, was demonstrated by the expression of podocyte-associated genes *NPHS1* and *NPHS2*. Moreover, integration of the $CD31^+$-endothelial cells into the cell aggregate was observed, while the iMSCs were mainly detected in the borders of the 3D-NPCs via expression of Vimentin and PDGFR-β. Nonetheless, nephron structures including glomeruli and tubules were not detected in 3D-NPCs.

Due to their high differentiation potential, UdRPCs are of peculiar interest for regenerative research. Urine-derived renal progenitor cells can be easily differentiated into various kidney cell types including podocytes and tubular cells (Erichsen et al., 2022; unpublished). The mentioned cell types are crucial for the correct function of the filtration apparatus and are affected by many kidney diseases and nephrotoxic substances. Urine-derived stem cells represent an accessible cell source for the generation of kidney cells, as they are primed to differentiate into cells of the renal lineage. However, as 2D cultures are less similar to the *in vivo* system, 3D cell cultures are a closer representation for toxicological studies. Demonstrated by the findings in chapter 3.1, not all three-dimensional kidney cell aggregates, even though they expressed nephron progenitor and pluripotency-associated genes, were capable to recapitulate kidney-specific structures. Due to the adult stem cell nature of UdRPCs, resulting organoids are more epithelial and less complex, which could not be compensated by the addition of iMSCs and iECs. Moreover, the absence of renal structures could also be attributed to the cultivation medium, which supported the progenitor state of UdRPCs, but did not induce differentiation towards glomerular and tubular cells. Interestingly, other studies previously demonstrated the generation of tubular organoids from urine stem cells with extracellular matrices, which recapitulated tubular morphology and expressed tubule-specific genes (Guo et al., 2020; Schutgens et al., 2019; G. Sun et al., 2020). Moreover, these USC-derived tubular organoids were sensitive to nephrotoxic substances and showed increased expression of the kidney injury markers (Guo et al., 2020; G. Sun et al., 2020). Since previous studies demonstrated USC-derived organoids are susceptible to nephrotoxic compounds, autologous 3D-NPCs as described in chapter 3.1 can be used in a similar manner for personalized medicine such as drug screenings and nephrotoxicity tests, and also hold promise to personalized disease modelling.

With this work, renal cell aggregates consisting of three cell types, namely UdRPCs, iMSCs and iECs from an isogenic background were established. Characteristics of renal progenitor cells

make 3D-NPCs a potential tool for the generation of various cell types of the renal lineage and could be further used for disease modelling, nephrotoxicity tests and drugs screenings.

4.2 Modelling acute kidney injury in iPSC-derived kidney organoids with the nephrotoxin PAN

The increasing number of patients suffering from kidney diseases make studying the pathophysiology of kidney syndromes, as well as the development of effective therapies an urgent concern. Kidney diseases can be modelled with 3D cellular models, called kidney organoids, which can recapitulate the renal structure and functions. Incidence of AKI is often associated with damages on the renal filtration apparatus, which can be provoked by continuous administration of medications or other toxins. Therefore, the lack of specific nephron structures makes 3D-NPCs as described in chapter 3.1 unsuitable for modelling renal damage. Thus, the aim was to establish an iPSC-based kidney organoid recapitulating renal structure and function and can be induced for kidney injury.

In this study, kidney organoids with self-organized kidney structures were derived from three human iPS cell lines. Characterization of the generated kidney organoids revealed upregulation of genes associated with kidney-related GOs, the presence of kidney-specific markers of glomeruli and tubules, such as ACNT4 and LTL, as well as functionality of proximal tubules via dextran endocytosis assay. Kidney injury was induced with 50 µg/ml of the nephrotoxin puromycin aminonucleoside (PAN) for 48 h. Structural disruption of glomerular regions was shown in the organoids, which was supported by the downregulation of genes related to cell-cell contact and the glomerular cell fate. Loss of slit diaphragm proteins due to external insults is associated with dysregulated filtration and proteinuria, which finally leads to glomerular diseases (Kawachi & Fukusumi, 2020). Disruption of tubular cell membranes and downregulation of tubule-related genes such as *ABCC4*, *CLDN10* and *NR3C2* was observed in PAN-induced kidney organoids. Moreover, administration of PAN led to a significant secretion of proteins such as FABP, ANPEP and DPP4, which are associated with the proximal tubular compartment. High amounts of proteins associated with the proximal tubules shed into urine are often used as urinary markers for AKI (Gerber et al., 2016). Renal ischemia, drug toxicity or hypertension can lead to tubular damages and inefficient reabsorption, followed by a cascade of pathological consequences, including proteinuria (Priante et al., 2019). *In vivo*, ischemic conditions activate the secretion of pro-inflammatory cytokines by tubular epithelial

cells to attract immune cells (Stein et al., 2021). Our PAN-induced kidney organoids secreted significant amounts of pro-inflammatory cytokines and as consequence to the inflammation, anti-inflammatory and reno-protective cytokines were activated. Even though presence of immune cells within the PAN-treated kidney organoids was not observed, part of the general immune response was PAN-activated shown by regulation of pro-and anti-inflammatory processes. Additionally, activated RAAS signalling contributed to the inflammatory processes within the PAN-treated kidney organoids. Upregulated renin secretion subsequently increases the *AGT* and *AGTR1* expression and can lead to the development of high blood pressure, which can cause pathological kidney conditions. Other processes regulated by PAN and associated with renal injury included DNA damage and cell death.

Since stress mechanisms were activated in the kidney injury model of chapter 3.2, PAN-treated organoids are a prospective tool to screen for potential drugs to treat acute kidney injury. Kidney injury is not the only pathological kidney condition, which can be modelled in organoids. However, the organoid technology has two major drawbacks, which restricts the possibilities to model kidney diseases *in vitro*. *In vivo*, the immune system reacts to pathogenic stimuli or diseases via immune cells and the production of antibodies, often coupled with the activation of inflammatory processes (Fudenberg, 1976). However, most kidney organoid models lack immune cells. Even though immune cells of the hematopoietic line originate in the same germ layer as the kidney, iPSC-generated kidney organoids are challenged by the absence of immune cells (Anastassova-Kristeva, 2003). Previous studies have successfully demonstrated the derivation of immune cells from human pluripotent stem cells, such as lymphocytes including NK cells and T-cells (Nianias & Themeli, 2019) and thymic epithelial cells, which supported T cell differentiation in immune-suppressed mice (Parent et al., 2013). Alternatively, isolated primary immune cells were utilized for the generation of immune organoids, such as thymus and lymph nodes organoids, which can be used for further studies on immune tissues as well as immunological diseases (Fan et al., 2015b; Purwada et al., 2015; Ye et al., 2020). These organoids also supported the re-population of immune cells in thymus-deficient mice (Fan et al., 2015a). Therefore, a combination of kidney organoids with isolated primary immune cells or PSC-derived immune cells could improve the system for closer resemblance to the *in vivo* situation and for better *in vitro* recapitulation of immunological diseases. Besides the lack of immune system, diseases of vascular origin are difficult to model as most organoid models lack a proper vasculature (W.-Y. Chen et al., 2021). Previous studies

have demonstrated an improvement of vascularization and further maturation of kidney organoids by transplanting them into mice (Bantounas et al., 2018; van den Berg et al., 2018). Similar effects were observed, when organoids were transplanted into chicken chorioallantoic membrane or coelomic cavity of chicken embryos (Garreta et al., 2019; Koning et al., 2022). *In vitro* engineering approaches include the use of microfluidic systems, based on the self-organized formation of vessels or 3D-printed scaffolds, which are populated by ECs and form a pre-defined vasculature (Whisler et al., 2014; Y. S. Zhang et al., 2016). Moreover, the composition of extracellular matrices has to be carefully orchestrated in order to suffice organoids and the engineered vasculature alike (Homan et al., 2019). Enhancing vascularization within kidney organoids can improve the tissue maturity and ideally, kidney disease models using these organoids have a greater resemblance to the *in vivo* situation.

In summary, we have successfully generated self-organized and functional kidney organoids from iPSCs and kidney injury was modelled through the external introduction of the nephrotoxin puromycin aminonucleoside. A cascade of cellular events including inflammation, DNA damage and cell death associated with kidney injury followed the administration of the toxin.

4.3 JNK signalling regulates self-renewal in UdRPCs by inhibiting ferroptosis

Studies on urine-derived stem cells have increased in the recent years due to their easy access and high differentiation potential. Moreover, these cells share characteristics with nephron progenitor cells (NPCs). Within NPCs, the progenitor state is maintained by JNK signalling among other cellular signalling mechanisms. The JNK enzymes are members of the mitogen-activated protein kinase (MAPK) family (Javelaud & Mauviel, 2005). The MAPK signalling pathway regulates various cellular processes such as proliferation, differentiation, inflammation or apoptosis (Weston & Davis, 2007). JNK is activated by various upstream MAPKs, caused by external or internal stimuli (Smith et al., 2021; Weston & Davis, 2007). Due to their similarity to NPCs, effects of JNK signalling in UdRPCs in regards to self-renewal were studied.

Three UdRPC lines, namely UM51, UM27 and UF21, were treated with the JNK inhibitor AEG3482 for 72 h. A systematic inhibition of JNK signalling resulted in the reduction of cell proliferation on the transcriptional and translational level and loss of nephron progenitor

markers including SIX2, SALL1 and VCAM1. Moreover, a downregulation of the SMAD pathways upon JNK inhibition was observed. SMAD proteins mediate the signalling of TGF-β superfamily pathway, which is involved in various developmental and pathophysiological processes (Tzavlaki & Moustakas, 2020). The signalling cascade begins with the binding of ligands to transmembrane receptor serine/threonine kinases, namely type I or type II receptors, which activate SMAD proteins via phosphorylation. This results in the formation of complexes and a subsequent translocation into the nucleus, where transcription is initiated (Derynck & Zhang, 2003; Wrana & Attisano, 2000). The ligands of the TGF-β superfamily can be divided into activins, BMPs, GDFs, MIS, nodal and TGF-βs (Tzavlaki & Moustakas, 2020). Especially the BMPs and TGF-βs play a major role in UdRPCs. The BMP ligands bind to BMP receptors and subsequently activate SMAD1,5 and 8 while SMAD2 and 3 are phosphorylated by TGF-β and activin receptors (Wrana & Attisano, 2000). The activated SMADs further form complexes with SMAD4 and can then regulate gene expression. Signalling mediated by SMAD proteins is referred to as canonical TGF-β pathway, while non-canonical TGF-β signalling activates SMAD-independent pathways including MAPK/ERK, PI3K/Akt and RHO/ROCK (Q. Liu et al., 2022). Both SMAD pathways were previously shown to play a role in the self-renewal of nephron progenitor cells. In murine kidneys, a decrease of BMP-dependent Smad signalling led to the loss of nephron progenitor cells (Tomita et al., 2013). Moreover, our precedent study demonstrated that differentiation of UdRPCs is associated with a downregulation of p-SMAD2/3 (Rahman et al., 2020). Indicated by these findings, JNK signalling is a possible mediator of self-renewal and proliferation maintained by SMAD signalling. Interference with the JNK pathway furthermore led to a significant increase of lipid peroxidation in UdRPCs via ferroptosis. Ferroptosis is a non-apoptotic cell death, characterized by iron-dependent accumulation of lipid peroxides (Dixon et al., 2012). Transcriptome analysis revealed upregulated genes of the GO terms associated with iron and glutathione metabolism. A balance in iron and glutathione household protects the cells from damages inflicted by ROS, which can be caused by ferroptosis (Galluzzi et al., 2018). In the ferroptosis setting, high levels of free iron can trigger the Fenton reaction resulting in increased lipid peroxidation, which we observed in JNK-inhibited UdRPCs. Since glutathione is an essential cofactor for the removal of ROS, a high transcript level of proteins involved in the glutathione biosynthesis might imply a compensation process to counter the increased lipid peroxidation. As lipid peroxidation is a major characteristic to ferroptosis, inhibiting the process via the ferroptosis inhibitor

Liproxstatin-1, protected the JNK-induced UdRPCs from ferroptosis. Lipid peroxides furthermore damaged the mitochondria and impaired their function. JNK inhibition resulted in the reduction of the spare respiratory capacity, an indicator for cellular health, which led to the loss of mitochondrial capacity to uphold normal respiration. Characteristic of ferroptosis is the blistering of mitochondria cristae, which may be a cause for the reduction of the mitochondrial membrane potential, an essential factor of oxidative phosphorylation. The JNK signalling pathway therefore inhibits the emergence of the non-apoptotic ferroptosis by maintaining the self-renewal and proliferation in urine-derived renal progenitor cells.

Understanding the signalling mechanisms in UdRPCs can significantly improve the cultivation methods in order to extend the time the cells can maintain the progenitor state *in vitro*. In general, the JNK pathway has varying roles during different developmental stages. In distinct stem cell populations, JNK participates in the maintenance of stemness, while differentiation is accompanied with downregulation of genes of the JNK pathway (Semba et al., 2020). Similarly, during renal development, activation of JNK is crucial for the proliferation and the maintenance of the nephron progenitor pool (Blank et al., 2009; Muthukrishnan et al., 2015). In the adult kidney, JNK signalling is mainly associated with the activation of inflammatory, fibrotic and cell death-related processes (Grynberg et al., 2017). In glomerular cells, JNK signalling is only active in case of pathological conditions such as glomerulonephritis or diabetic nephropathy (Nikolic-Paterson et al., 2021). JNK further induces other kidney diseases associated with inflammation and fibrosis such as ischemia/reperfusion injury (I/RI)-induced AKI. I/RI is often caused by surgeries or occurs in transplantation patients and can be divided into a phase of oxygen shortage and a subsequent re -oxygenation phase (Grynberg et al., 2021). The ischemic phase results in a depletion of ATP production and leads to pathological consequences including tubular damages, as the high oxygen and ATP demand of the tubular compartment cannot be met (Nieuwenhuijs-Moeke et al., 2020). Reactive oxygen species produced during the re-oxygenation phase additionally damage the tissue (Nieuwenhuijs-Moeke et al., 2020). High levels of JNK in tubular epithelial cells of murine I/RI models induced tubular necrosis and inflammation, which were prevented by the administration of JNK inhibitors (Nikolic-Paterson et al., 2021). Similarly, inflammation and cell death-processes activated JNK signalling in ferroptosis-induced I/RI AKI can also be reduced by an inhibition of JNK (Liang et al., 2022). Contrary to the previous studies, our investigation implies a mechanism in the JNK signalling pathway, which protects the early developmental stages of

the kidney from damages caused by ferroptosis. Besides further studies of the correlation between JNK signalling, ferroptosis and kidney diseases in the early kidney, UdRPCs represent a useful tool to decipher kidney diseases in general.

Summarizing, the findings of our study supported the hypothesis that JNK signalling is relevant for self-renewal of urine-derived renal progenitor cells in a similar manner as in nephron progenitor cells. Moreover, the JNK signalling pathway is also relevant for proper cell respiration in UdRPCs and inhibits cell death by ferroptosis. Application of the gained knowledge can improve the *in vitro* maintenance of UdRPCs for the potential use in cell therapies.

Conclusion

The current knowledge about kidney organogenesis is mostly based on research done in animal models. Interspecific differences between the human and the mouse model however restricted further understanding of this organ system. Alternatives have been discovered and rapidly developed in the last few years, including kidney-derived urine stem cells and the organoid technology. With this thesis, both options were utilized to gain a deeper understanding of the human kidney organ. The findings revealed that JNK signalling is involved in the maintenance of self-renewal of UdRPCs while inhibiting ferroptosis and maintaining a proper cell respiration. Moreover, UdRPCs represent a kidney-derived cell population, which can be utilized for the generation of 3D cellular models with a potential use in drug screenings and nephrotoxicity testings. Disease modelling was achieved by applying the nephrotoxin PAN in highly structural and functional kidney organoids derived from iPSCs resulting in activated stress mechanisms such as inflammation and cell death. In general, this thesis provided better understanding of the cellular processes within UdRPCs and deepened the knowledge about acute kidney injury.

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Appendix

I. Peer reviewed journal articles

i. The FGF, TGFβ and WNT axis Modulate Self-renewal of Human SIX2+ Urine Derived Renal Progenitor Cells

Rahman M.S, Wruck W, Spitzhorn L., **Nguyen L**, Bohndorf M, Martins S, Asar F, Ncube A, Erichsen L, Graffmann N, Adjaye J.

Sci Rep (2020) 10, 739

Abstract:

Human urine is a non-invasive source of renal stem cells with regeneration potential. Urine-derived renal progenitor cells were isolated from 10 individuals of both genders and distinct ages. These renal progenitors express pluripotency-associated proteins- TRA-1-60, TRA-1-81, SSEA4, C-KIT and CD133, as well as the renal stem cell markers -SIX2, CITED1, WT1, CD24 and CD106. The transcriptomes of all SIX2+ renal progenitors clustered together, and distinct from the human kidney biopsy-derived epithelial proximal cells (hREPCs). Stimulation of the urine-derived renal progenitor cells (UdRPCs) with the GSK3β-inhibitor (CHIR99021) induced differentiation. Transcriptome and KEGG pathway analysis revealed upregulation of WNT-associated genes- *AXIN2*, *JUN* and *NKD1*. Protein interaction network identified JUN- a downstream target of the WNT pathway in association with STAT3, ATF2 and MAPK1 as a putative negative regulator of self-renewal. Furthermore, like pluripotent stem cells, self-renewal is maintained by FGF2-driven TGFβ-SMAD2/3 pathway. The urine-derived renal progenitor cells and the data presented should lay the foundation for studying nephrogenesis in human.

Authors Contribution: 10 %

L.N. performed experiments.

Status: Published in *Scientific Reports* (doi.org/10.1038/s41598-020-57723-2).

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The FGF, TGFβ and WNT axis Modulate Self-renewal of Human SIX2+ Urine Derived Renal Progenitor Cells

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Human urine is a non-invasive source of renal stem cells with regeneration potential. Urine-derived renal progenitor cells were isolated from 10 individuals of both genders and distinct ages. These renal progenitors express pluripotency-associated proteins- TRA-1-60, TRA-1-81, SSEA4, C-KIT and CD133, as well as the renal stem cell markers -SIX2, CITED1, WT1, CD24 and CD106. The transcriptomes of all SIX2+ renal progenitors clustered together, and distinct from the human kidney biopsy-derived epithelial proximal cells (hREPCs). Stimulation of the urine-derived renal progenitor cells (UdRPCs) with the GSK3β-inhibitor (CHIR99021) induced differentiation. Transcriptome and KEGG pathway analysis revealed upregulation of WNT-associated genes- *AXIN2*, *JUN* and *NKD1*. Protein interaction network identified JUN- a downstream target of the WNT pathway in association with STAT3, ATF2 and MAPK1 as a putative negative regulator of self-renewal. Furthermore, like pluripotent stem cells, self-renewal is maintained by FGF2-driven TGFβ-SMAD2/3 pathway. The urine-derived renal progenitor cells and the data presented should lay the foundation for studying nephrogenesis in human.

According to the International Society of Nephrology, more than 850 million people worldwide are afflicted with kidney diseases[1], which raises the quest for alternative therapies to overcome the limitations associated with current treatments including transplantation and dialysis. One of the most promising options is the utilization of renal stem cells for treating of kidney diseases, disease modelling, and drug development[2,3]. Renal stem/progenitor cells are self-renewing, multipotent cells with the ability to generate various cell types of the kidney to maintain renal function[4]. These progenitors are in abundance during fetal kidney development in which the renal progenitor surface marker CD24 and stem cell self-renewal marker CD133 cells are required for primordial nephrogenesis[5,6]. However, in adults, CD24, CD133 (Prominin-1) and vascular cell adhesion molecule 1 (CD106)-positive renal progenitors are present in renal tubules and capsules[7]. Two progenitor cell populations can be distinguished based on the expression of CD106. For instance, $CD24^+CD133^+CD106^-$ progenitors are present in proximal tubules whereas $CD24^+CD133^+CD106^+$ cells are localized in the Bowman's capsule. The latter can differentiate into a variety of cell types of renal tissue such as podocytes and tubular epithelial cells[4–7].

Several groups have identified urine as a non-invasive and repetitive source of renal progenitor cells[8,9]. It has been estimated that each day approximately 2,000 to 7,000 cells composed of differentiated epithelial cells, bi-potential epithelial cells (transitional cells), multipotent mesenchymal stem cells, and glomerular parietal cells are flushed out from the renal tubular network and the upper urinary tract into urine[10–12]. A subpopulation of these urine-derived cells are renal stem/progenitor cells which express master renal markers such as Sine Oculis Homeobox Homolog 2 (SIX2), Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 1 (CITED1) and Wilms' Tumor 1 (WT1)[13–15] and CD24 and CD106[16]. Interestingly, these cells exhibit stem cell properties, i.e. expression of pluripotency-associated markers such as TRA-1-60, TRA-1-81, C-KIT (CD117), CD133 and SSEA4 and possess high proliferation capacity as they show telomerase activity. Further, they endow multi-differentiation potential and like bone marrow derived mesenchymal stem cells express

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Vimentin, CD105, CD90, CD73 and not the hematopoietic stem cell markers- CD14, CD31, CD34 and CD45[17,18]. Although, research interest on urine derived renal stem cells is gradually increasing but the mechanistic role of genetic factors in these cells *in vitro* regarding progenitor/differentiated status maintenance is not clear.

Studies in mice have shown that Odd-skipped related 1 (Osr1), Six2, Wnt, Cited1 and Wt1 are required to maintain renal progenitor cells during kidney organogenesis[19–25]. Additionally, signalling pathways such as Fgf, Tgfβ and Notch play major roles in renal stem cell maintenance and differentiation[26–29]. The transcription factor Osr1 is an early marker specific for the intermediate mesenchyme (IM); *Osr1* knockout mice lack renal structures due to the failure to form the IM[30]. The homeodomain transcriptional regulator Six2 is expressed in the cap mesenchyme (CM) originating from metanephric mesenchyme. Six2 positive populations can generate all cell types of the main body of the nephron[31]. Inactivation of Six2 results in premature and ectopic renal vesicles, leading to a reduced number of nephrons and to renal hypoplasia[32]. Mechanistically, Osr1 plays a crucial role in Six2-dependent maintenance of mouse nephron progenitors by antagonizing Wnt-directed differentiation, whereas Wt1 maintains self-renewal by modulating Fgf signals[22,23]. Cited1 has been reported to be co-expressed with a fraction of $Six2^+$ cells undergoing self-renewal and these can be differentiated in response to activated WNT signaling during kidney development[25]. Furthermore, it has been demonstrated in mice that Bmp7 promotes proliferation of nephron progenitor cells via a Jnk-dependent mechanism involving phosphorylation of Jun and Atf2[33].

To date, research related to transcriptional regulatory control of mammalian nephrogenesis has been limited to the mouse[19,26] or to transcriptome "snapshots" in human[13]. A recent study demonstrated conserved and divergent genes associated with human and mouse kidney organogenesis[34], thus further highlighting the need for primary human renal stem cell models to better dissect nephrogenesis at the molecular level. Furthermore, species differences need to be considered, for example, mammalian nephrons arise from a limited nephron progenitor pool through a reiterative inductive process extending over days (mouse) or weeks (human) of kidney development[35]. Human kidney development initiates around 4 weeks of gestation and ends around 34–37 weeks of gestation. At the anatomical level, human and mouse kidney development differ in timing, scale, and global features such as lobe formation and progenitor niche organization[34–36]. These are all further evidence in support of the need of a reliable and robust human renal cell culture model.

Expression of pluripotency-associated proteins has enabled rapid reprogramming of urine derived mesenchymal and epithelial cells into induced pluripotent stem cells (iPSCs)[37–41]. Differentiation protocols for generating kidney-associated cell types from human pluripotent stem cells have mimicked normal kidney development[28,42–44]. For example, WNT activation using a GSK3β inhibitor (CHIR99021), FGF9, Activin A, Retinoic acid (RA) and BMP7 as instructive signals have been employed to derive functional podocytes, proximal renal tubules, and glomeruli[29,45–49]. Despite these efforts and achievements, there will always be variabilities between differentiation protocols, the maturation state of the differentiated renal cells and genes associated with temporal maturation during human kidney organoids formation from human iPSCs[50,51]. We propose that using native renal stem cells isolated directly from urine will circumvent most of the shortfalls and deficiencies associated with human pluripotent stem cell-based models.

Here we provide for the first time the full characterisation of renal progenitors at the transcriptome, secretome and cellular level, which has led to the identification of a gene regulatory network and associated signalling pathways that maintain their self-renewal. We anticipate that our data will enhance our meagre understanding of the properties of urine-derived renal stem cells, and enable the generation of renal disease models *in vitro* and eventually kidney-associated regenerative therapies.

Results

Urine-derived renal progenitors express a subset of pluripotent stem cell-associated markers and possess features typical of bone marrow-derived MSC. Urine samples were collected from 10 healthy adult donors (4 males-UM and 6 females-UF) with ages ranging from 21 to 61 years, and of mixed ethnicity (3 Africans and 7 Caucasians) (Supplemental Table S1). Attached cells emerged from processed urine as isolated clusters after 7 days, thereafter these acquired a "rice grain" fibroblast-like morphology resembling MSCs (Fig. 1A, Supplemental Fig. S1A). A selection of distinct urine-derived renal stem cells populations (n = 4) were used to assay cell proliferation and growth. After 3 days in culture, the cells exited the lag phase and growth began in an exponential phase. Cells attained stationary phase at day 7 of subculture (Fig. 1B). All four populations-UM27, UM16, UM51 and UF45 showed similar proliferation and growth patterns.

Flow cytometry analysis revealed that approximately 98.9% of the cells express SSEA4, TRA-1–60 (11.3%) and TRA-1-81 (16.5%) (Fig. 1C). These data were confirmed by immunofluorescent-based staining of SSEA4 which also express the proliferation-associated stem cell markers- C-KIT and CD133 (Fig. 1D). In order to reveal the detailed methylation pattern of the 5′-regulatory region of the OCT4 gene in the UM51, we employed standard bisulfite sequencing. In total 330 Cytosine-phosphatidyl-Guanine-dinucleotides (CpG) upstream of the transcription-starting site (TSS) of the OCT4 gene were analysed. Within this 469 bp long region, a dense methylation pattern was observed in the UM51 cells, with 92.4% (305) of the CpG dinucleotides identified were methylated (Fig. 1E). In contrast, iPSCs derived from UM51 had 72.12% (207) of analysed CpGs were unmethylated (Supplemental Fig. S1B).

Urine-derived renal progenitors express the mesenchymal marker- Vimentin and not the epithelial marker-E-Cadherin (Fig. 1D, Supplemental Fig. S1C). Flow cytometry analysis of critical MSC cell surface markers were negative for the hematopoietic markers CD14, CD20, CD34, and CD45 and positive for CD73, CD90 and CD105 albeit at variable levels (Supplemental Fig. S1D). Typical of MSCs, urine-derived renal progenitor cells can also differentiate into osteocytes, chondrocytes, and adipocytes when cultured in the respective differentiation medium for 3 weeks (Fig. 1F, Supplemental Fig. S1E). Furthermore, employing a cytokine array (n = 2), a plethora of trophic factors such as IL8, GDF-15, SERPINE-1, Angiogenin, VEGF, and Thrombospondin-1 were detected,

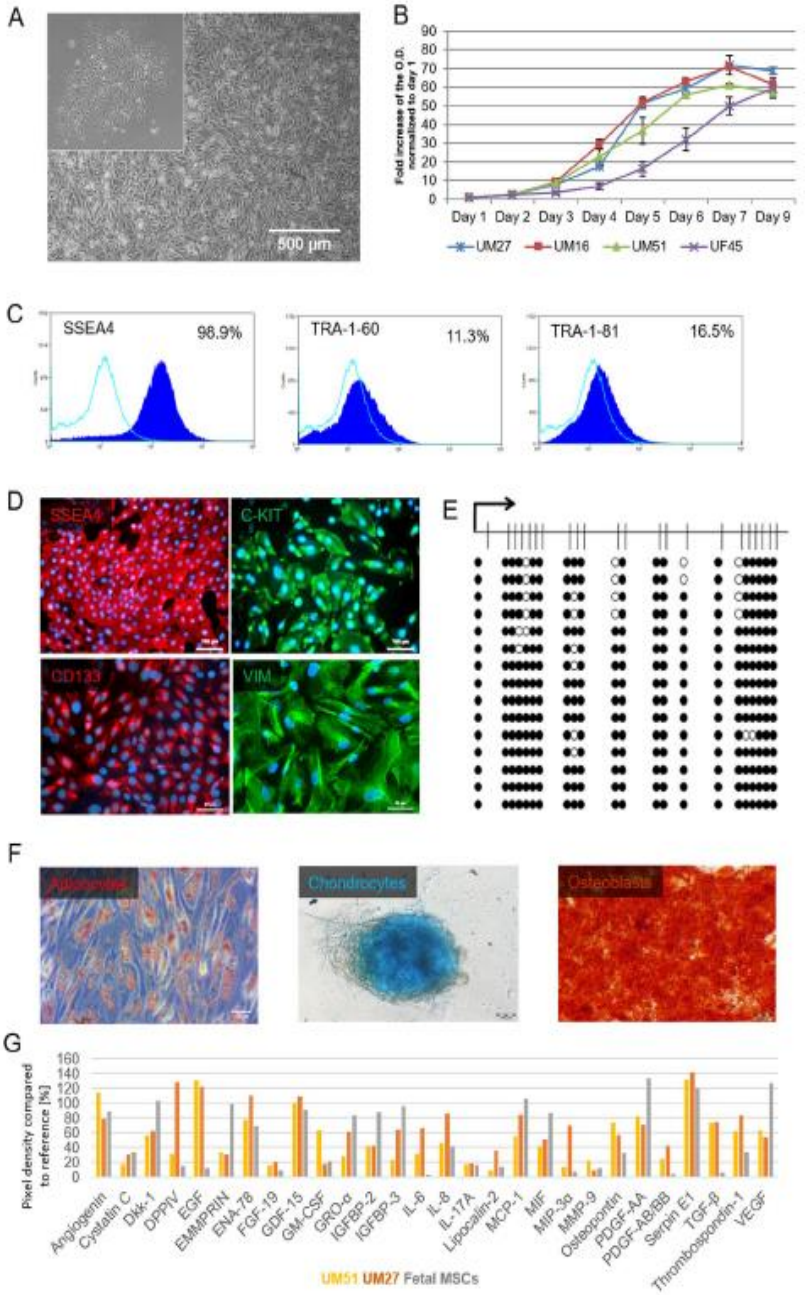

Figure 1. Propagation and characterisation of urine-derived renal progenitors. **(A)** Representative pictures of the "rice grain"-like appearance of the cells from the initial attachment to an elongated MSC-like morphology. **(B)** Growth curve analysis of selected urine-derived renal progenitors carried out using the Resazurin metabolic assay. Data are presented as means ± SEMs. **(C)** Immune-phenotyping for SSEA4, TRA-1-81 and TRA-1-60; and **(D)** immunofluorescence-based detection of the expression of pluripotency-associated stem cell- proteins SSEA4 (red), C-KIT (green), CD133 (red) and the mesenchymal-associated protein Vimentin (green); cell nuclei were stained using Hoechst/DAPI (scale bars: 100 µm and 50 µm). **(E)** Bisulfite sequencing of CpG island methylation patterns within the 5′- regulatory region of the OCT4 gene in UM51. Filled circles stand for methylated CpG dinucleotides. White circles stand for unmethylated CpGs. Arrows indicate the transcription start site. **(F)** *In vitro* Osteoblast, Chondrocyte and Adipocyte differentiation potential of urine-derived renal progenitors. **(G)** Cytokines secreted by urine-derived renal progenitors in culture media. Lists of significant GOs and KEGG pathways associated with the genes encoding the secreted cytokines are shown in Supplemental Fig. S1G.

To determine the variability of SIX2+cells between the progenitor cell preparations- UM27, UF31, UM51 and UF45 (n = 4) a flow cytometry analysis was performed. We observed approximately 95% SIX2+ cells in UM27, UF31 and UM51 whereas UF45 had 90% SIX2+ cells (Fig. 2B). In addition, to confirm the renal stemness status of the urine-derived progenitor cells a flow cytometry analysis was performed to evaluate expression of the renal progenitor markers CD24, CD106 and the self-renewal marker CD133 in the cell preparations- UM27, UF31, UM51 and UF45. CD24, CD133 and CD106 were variably expressed in the aforementioned cell preparations. For instance, 98% of the UF31 cell population was CD133+, 99% of the cells were positive for CD24 and 84% of the cells were CD106+. On the other hand, the UF45 sample displayed a different pattern for CD133 (68%), CD24 (70%) and CD106 (45%) positive cells, respectively (Fig. 2C). Bisulfite sequencing of a portion of the 5′-regulatory region of the SIX2 gene revealed methylation of only 1.9% of CpG dinucleotides (Fig. 2D). As, presence of albumin in urine is a mark of kidney cell functionality, and by the endo/exocytosis of albumin, kidney maintain the colloid osmotic pressure and transport biomolecules. We performed endocytosis assay and could show that urine-derived cells can transport Albumin (Fig. 2E). Furthermore, the CYP2D6 genotypes investigated were distinct between groups of individuals, thus reflecting potential diverse drug metabolizing activities. UM51 for example expresses the CYP2D6 *4/*17 genotype which confers an intermediate metabolizing activity whereas UF31 bears the CYP2D6* 1/*41 genotype with an ultra-rapid metabolizing activity. The other three individuals (UF21, UF45 and UM27) are endowed with normal drug metabolizing activity (Supplemental Table S1).

Comparative transcriptome analysis of urine-derived renal progenitors and kidney-biopsy derived renal epithelial proximal cells (hREPCs). A hierarchical clustering analysis comparing the transcriptomes of urine derived renal progenitors with the kidney biopsy-derived renal epithelial proximal cells (hREPCs) revealed that all urine derived renal progenitors samples clustered together as a common cell type distinct from hREPCs (Fig. 3A). Additionally, expression of renal progenitor surface markers *CD24*, *CD106* and *CD133* were detected in urine-derived renal progenitors whilst *PODXL* was not expressed (Fig. 3B). These renal progenitors are of mesenchymal origin expressing *VIM*, however a scatter plot comparison between UM51 with hREPCs shows similarity with a high Pearson correlation of 0.9575 (Fig. 3C). The epithelial character of hREPCs is reflected by *CDH1* expression. The comparison of expressed genes (det-$p < 0.05$) in renal progenitors (UM51) and hREPCs in a venn diagram revealed a common 12281 gene-set, whereas 566 are expressed exclusively in UM51 and 438 exclusively in hREPCs (Fig. 3D). The 10 most over-represented GO BP terms (biological processes) in the UM51 exclusive gene-set include triglyceride homeostasis, kidney development and urogenital system development, whereas the hREPCs exclusive gene set includes chloride transmembrane transport, anion transport and response to lipopolysaccharides (Fig. 3E). The common gene set consists of 874 up-regulated genes (ratio > 2) in UM51 (e.g. renal tubule development, urogenital system development and anterior/posterior pattern specification) and 1042 down-regulated genes (ratio < 0.5) in UM51 (e.g. cell division and cholesterol biosynthetic process) (Fig. 3F).

Comparative gene expression analysis of urine-derived and kidney biopsies-derived renal progenitor cells. Gene expression of urine-derived renal progenitors was compared to public available datasets GSE23911 in which nephron progenitor cells were derived from adult human renal cortical tissue[52]. Additionally, the comparison was extended by two further datasets GSE74450 and GSE75949 which contain data from fetal kidney biopsy derived nephron progenitor cells[53,54]. We could show that urine-derived renal progenitors have a high level of similarity to other human nephron progenitors at the transcriptome level. The resulting number of expressed genes were comparable: 12112 genes in urine-derived renal progenitors, 8446 genes in GSE23911, 10597 genes in GSE74450 and 13895 genes in GSE75949. In the Venn diagram analysis most genes were found in the intersection of all genesets (4411), followed by the intersection of urine-derived renal progenitors with the fetal kidney genesets from GSE74450 and GSE75949. Among the intersection with single genesets urine-derived renal progenitors had the highest overlap with the GSE75949 pointing at the highest similarity with this gene-set (Supplemental Fig. S2). A subset of genes expressed in common between urine-derived renal progenitors, GSE74450 and GSE75949, are associated with renal system development related GO's (BP) terms, thus confirming renal progenitor cell identity (Supplemental Table S5).

Confirmation of the renal origin of urine-derived progenitor cells and retention of renal-associated genes in urine-derived progenitors-iPSCs. A venn diagram-based comparison of gene expression (det-$p < 0.05$) in urine-derived renal progenitors and human foreskin fibroblasts (HFF) was carried out (Fig. 4A) in order to dissect common and distinct gene expression patterns. The majority of genes (11649) are expressed in common, 463 exclusively in urine-derived renal progenitors and 891 in fibroblasts. The 463 genes were further analysed for over-represented GOs and summarized as a GO network (Fig. 4B) with the tools REVIGO, and Cytoscape was used for the GO terms of the category BP. In addition to several developmental terms such as organ induction, regulation of embryonic development (high number of edges referring to similarity to many terms), specific renal-related terms including urogenital system development, mesenchymal cell proliferation involved in ureteric bud development and positive regulation of nephron tubule epithelial cell differentiation (marked with blue ellipse, intense red indicating higher significance) were identified. Interestingly, the non-canonical WNT signalling pathway, which plays a major role in kidney development, is also over-represented (orange ring-top left).

The dendrogram based on the global transcriptome analysis revealed a clear separation of urine-derived renal progenitors lines (n = 9) from the differentiated urine-derived renal progenitors (CHIR 99021 treated urine- derived renal progenitor cells, n = 3), urine derived renal progenitors-iPSCs (n = 4) and embryonic stem cells (H1 and H9) (Fig. 4C). Characterization of the derived urine derived renal progenitors-iPSCs is depicted in Supplemental Fig. S3. In the Venn diagram (Fig. 4D) we compared expressed genes (det-$p < 0.05$) in urine

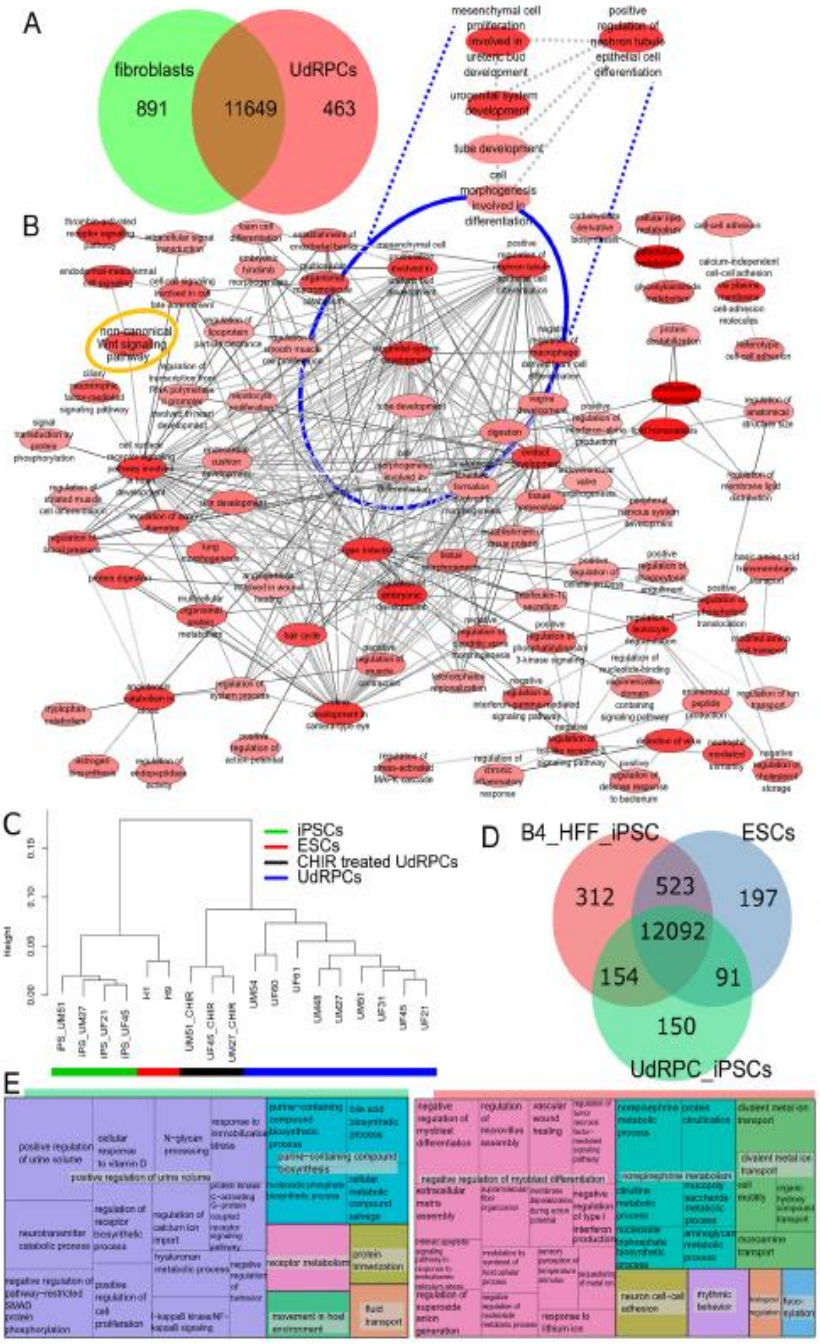

Figure 4. In-depth bioinformatic analysis of urine-derived renal progenitors and urine-derived renal progenitors derived-iPSCs. **(A)** Expressed genes (det-p < 0.05) in urine-derived renal progenitors (UdRPCs) and fibroblasts are compared in a venn diagram. Most genes are expressed in common (11649), 463 genes are expressed exclusively in urine-derived renal progenitors and 891 in fibroblasts. The subsets and urine-derived renal progenitors GOs are presented in supplemental_table_S4. **(B)** The gene ontology network was generated with the tools REVIGO and Cytoscape and summarizes the GO terms of category Biological Process (BP) over-represented in the 463 genes expressed exclusively in urine-derived renal progenitors. Several general developmental terms emerged, e.g. "organ induction". Specific renal-related terms including "urogenital system development" are marked with a blue ellipse. GOs are represented by the network nodes with the intensity of red indicating the significance of over-representation of a GO term. The edges refer to similarities between the GO terms. **(C)** The dendrogram shows a clear separation of urine-derived renal progenitors, differentiated urine-derived renal progenitors (CHIR treated UdRPCs of UM51, UM27, and UF45) (black bar), ESCs (H1 and

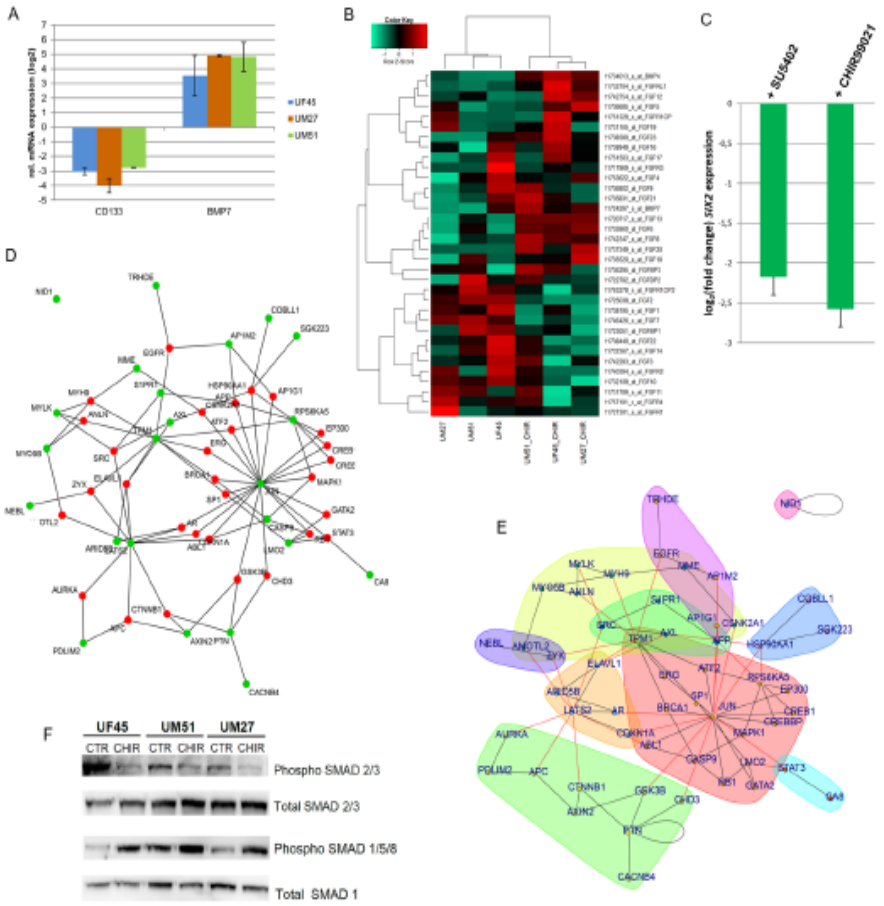

Figure 6. Regulation of self-renewal and differentiation in urine-derived renal progenitors. (**A**) Real-time PCR-based confirmation of down-regulation of *CD133* and activated expression of *BMP7* after CHIR stimulation. (**B**) Effect of CHIR99021 stimulation on FGF-signaling and BMP (BMP7 and BMP4) signaling. The heatmap depicts FGF signaling associated genes up and down regulated upon CHIR treatment of the urine-derived renal progenitors. (**C**) Downregulation of *SIX2* expression in differentiated urine-derived renal progenitors upon WNT stimulation using the GSK-3β-inhibitor CHIR99021 and blocking of FGF signaling using the FGF receptor inhibitor SU5402. (**D**) JUN is a major hub of protein interaction networks of urine-derived renal progenitors treated with CHIR. Based on the Biogrid database protein interaction networks were constructed from the set of the most highly regulated 40 genes either up- or down in the urine-derived renal progenitors treated with CHIR. The selected genes used to connect to the network with interactions from the Biogrid database are marked in green, genes added as Biogrid interactions are marked in red. Induction of WNT leading to GSK3B inhibition is reflected by the connection of GSK3B to JUN and to AXIN2 which is connected to CTNNB1 (β-catenin) – these all downstream targets of GSK3B in the WNT-signaling pathway. (**E**) Community clustering of the network identified several communities: JUN (red), GSK3B/AXIN2/CTNNB1 (green), LATS2 (yellow), EGFR (pink). Black lines refer to edges within a community, red lines to edges between different communities. (**F**) Western blot analysis of the phosphorylated levels of SMAD 2/3 and SMAD 1/5/8 in undifferentiated and differentiated UF45, UM51 and UM27.

pluripotency-associated factors, these renal progenitor cells do not express OCT4, SOX2 and NANOG- which are key pluripotency-regulating transcription factors[59,60]. Further evidence in support of the lack of OCT4 expression is our observed fully methylated CpG dinucleotides within the OCT4 promoter in the UM51 cells. Urine-derived renal progenitors are in fact bon-fide MSCs- i.e. they express VIM and not CDH1, adhere to plastic surfaces, express CD73, CD90 and CD105 and not the hematopoietic markers CD14, CD20, CD34, and CD45. Typical of MSCs, urine-derived renal progenitors can be differentiated into osteoblasts, chondrocytes and adipocytes[56,57,61]. They also secrete a plethora of cytokines and growth factors- such as EGF, GDF, PDGF and Serpin E1[62]. The multipotent features of urine-derived renal progenitors make these cells promising for studying nephrogenesis and in the future regenerative therapy of kidney-associated diseases.

Urine-derived renal progenitor cells express key renal progenitor-regulatory proteins SIX2, CITED1 and WT1 indicating they originate from the kidney as described from others[13,14,27,57,63]. Unmethylated CpG islands within

R/Bioconductor environment[86] and further processed with the package affy[87] using background-correction, logarithmic (base 2) transformation and normalization with the Robust Multi-array Average (RMA) method. For full details, please see supplemental materials and methods.

KEGG pathway, GO and network analysis. Gene ontology (GOs) terms were analysed within the Bioconductor environment employing the package GOstats[88]. GOs of category Biological Process (BP) were further summarized with the REVIGO tool[89] to generate treemaps populating the parameter for allowed similarity with tiny= 0.4. GO networks were generated from the REVIGO tool in xgmml format and imported into Cytoscape[90]. For full details, see supplemental materials and methods.

Activated WNT pathway associated protein interaction network. The network was constructed from the 20 most significantly up- and down down-regulated genes between CHIR99021 treatment and untreated controls. Genes were ranked by the limma-p-value and passed the criteria: detection p-value < 0.05 for the dedicated condition, ratio < 0.75 or ratio > 1.33, limma-p-value < 0.05. The resulting 40 genes were marked as green nodes in the network. Interacting proteins containing at least one protein coded by the 40 genes were retrieved from BioGrid version 3.4.161[91]. The plot of the interactions network was drawn employing the R package network[92]. See supplemental materials and methods for full description.

Additional materials and methods. For the materials and methods of the culture supernatant analysis, analysis of cell proliferation, meta-analysis for comparison of urine-derived renal progenitors to public nephron progenitor data sets and cell lines used in this study and culture conditions, please see supplemental materials and methods.

Statistics. All data are presented as arithmetic means ± standard error of mean. At least 3 independent experiments were used for the calculation of mean values. P values of < 0.05 were considered significant.

Data availability

All raw and processed data used in this study have been archived in NCBI gene expression omnibus under GEO accession number GSE128281. (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128281).

Received: 24 June 2019; Accepted: 31 December 2019;
Published online: 20 January 2020

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Acknowledgements

James Adjaye acknowledges support from the medical faculty of the Heinrich Heine University- Düsseldorf, Germany. In addition, James Adjaye and Md Shaifur Rahman acknowledges support from the German Academic Exchange Service (DAAD-91607303). We acknowledge Dipl. Ing Katharina Raba, Institute of Transplantation Diagnostics and Cell Therapeutics, Heinrich Heine University, Germany for her kind assistance with the FACS analyses.

Author contributions

:Conception: J.A.; Cell culture experiments: M.S.R., L.S.S., F.A., M.B. and J.A.; Real Time PCR: F.A., L.E.; Bisulphite sequencing: L.E.; Bioinformatics: W.W. and J.A.; Proliferation assays and Western blot analyses: S.M.; Derivation and characterisation of iPSCs: M.B., N.G. and A.N.; Immunostaining and FACS analysis: M.B., L.S.S., A.N., M.S.R., L.N.; Writing of manuscript: M.S.R., W.W., L.S.S., L.N. and J.A.; Final edits: J.A. All authors approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-57723-2.

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II. Book Chapters

ii. Stem Cell Therapy In: Hock F., Gralinski M. (eds) Drug Discovery and Evaluation: Methods in Clinical Pharmacology.

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Springer, Cham (2020)

Stem cell therapy and regenerative medicine have a tremendous potential for the treatment of a wide variety of currently fatal diseases. So far, routine stem cell based therapies are limited to the treatment of hematologic and dermatologic malignancies, but the field is continuously evolving. One research focus lies on mesenchymal stem cells (MSCs) which have an immunomodulatory potential and support regenerative processes throughout the body. Pluripotent stem cells, on the other hand, can be differentiated into every cell type and might be able to replace damaged tissue in the future. Currently, stem cell based clinical trials are ongoing for a broad range of diseases affecting every organ, and the number of therapies that are obtaining approval, at least in certain countries, is continuously increasing. In this chapter we summarize the current state of research regarding stem cell based therapies. We introduce stem cell sources along with selected indications for their use and relevant clinical trials. We discuss options as well as limitations and risks of these treatments.

Authors Contribution: 10 %

L.N. contributed to the manuscript writing.

Status: Published in *Drug Discovery and Evaluation: Methods in Clinical Pharmacology* (doi.org/10.1007/978-3-319-56637-5_72-1).

Stem Cell Therapy

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Contents

Abstract

Stem cell therapy and regenerative medicine have a tremendous potential for the treatment of a wide variety of currently fatal diseases. So far, routine stem cell based therapies are limited to the treatment of hematologic and dermatologic malignancies, but the field is continuously evolving. One research focus lies on mesenchymal stem cells (MSCs) which have an immunomodulatory potential and support regenerative processes throughout the body. Pluripotent stem cells, on the other hand, can be differentiated into every cell type and might be able to replace damaged tissue in the future. Currently, stem cell based clinical trials are ongoing for a broad range of diseases affecting every organ, and the number of therapies that are obtaining approval, at least in

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F. J. Hock, M. R. Gralinski (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*,
https://doi.org/10.1007/978-3-319-56637-5_72-1

certain countries, is continuously increasing. In this chapter we summarize the current state of research regarding stem cell based therapies. We introduce stem cell sources along with selected indications for their use and relevant clinical trials. We discuss options as well as limitations and risks of these treatments.

Introduction

It is a long-standing goal of regenerative medicine to cure diseases by replacing defective cells/tissues with healthy ones. Nowadays, with increasing improvements in stem cell technology, this dream comes closer to reality, although there are still many obstacles to overcome.

Stem cell therapy was initiated as a treatment for blood malignancies over 50 years ago and is now an integral part of handling these conditions. Preparations containing skin stem cell have been used to treat severe burns since 1981. However, for all other diseases, stem cell therapy is still at a stage of intense research with many ongoing clinical trials. New techniques for genome editing as, for example, CRISPR/cas9 (clustered regularly interspaced short palindromic repeat) or TALEN (transcription activator-like effector nucleases) have also found their way into regenerative medicine and augment hopes that genetic defects can be efficiently and routinely corrected in the future.

Stem Cell Sources

Adult and Pluripotent Stem Cells

Stem cells are undifferentiated cells capable of dividing symmetrically to maintain a stem cell pool or asymmetrically to produce terminally differentiated cells. They can be separated into pluripotent and multipotent cells according to their differentiation potential(Table 1). Pluripotent stem cells can generate every cell type of the human body, while multipotent stem cells are restricted to a few, closely related cell types. Because of their ability to replace damaged cells and restore tissues, stem cells are of major interest for the field of regenerative medicine (Chagastelles and Nardi 2011).

Stem cells can be obtained from many different sources, each associated with specific advantages and disadvantages. Usually, the disease that should be treated defines which stem cell sources are suitable and if autologous or allogeneic transplantation is needed. In the case of autologous transplantation, stem cells are obtained directly from the patient which obviates the risk of immune rejection. Cells for allogeneic transplantation are derived from an independent donor who is not necessarily related to the patient. Therefore, it is always associated with varying intensities of immune reactions and usually necessitates, at least temporarily, an immunosuppressive treatment. Although autologous stem cells are safe from an immunological point of view, they often cannot be applied due to other shortcomings. If the disease is caused by a genetic defect, all cells of the patient carry this mutation and hence are not suitable for restoring the function of the affected organ. In addition, in some conditions, there is simply not enough time for the sometimes lengthy process of isolation, purification, and potential biotechnological modification of autologous stem cells (Champlin 2003).

Pluripotent Stem Cells (PSCs)

Embryonic stem cells (ESCs), which are pluripotent, are isolated from the inner cell mass of the blastocyst during preimplantation development (Thomson et al. 1998). High expectations are evoked by these cells as they renew themselves indefinitely, do not age in culture, and can be directed in vitro to differentiate into every cell type of the human body (Chagastelles and Nardi 2011). However, their use is limited by major ethical considerations, as they can only be obtained by destroying the embryo. Regulations on the use of ESCs vary from country to country and influence research as well as cell therapy (Chagastelles and Nardi 2011; Elstner et al. 2009).

In 2006/2007 these ethical limitations were overcome, when the group of Shinya Yamanaka in Kyoto, Japan, managed to reprogram terminally

Table 1 Definitions of stem cell-related terms

Term	Definition
Regenerative medicine	An area of medicine which uses cells or biological materials to treat diseases or improve organ/tissue functions
Advanced therapy medicinal product (ATMP)	Novel class of medicinal products comprising somatic cell therapeutics, gene therapeutics, and bioengineered tissue
Stem cells	Non-committed cells which have the ability to self-renew or generate higher differentiated daughter cells
Multipotent	Adult stem cells which differentiate only into a limited number of cell types, e.g., HSCs, MSCs, and NSCs
Pluripotent	Stem cells which can give rise to all cell types of the body. Naturally, only embryonic stem cells (ESCs), which reside in the inner cell mass of the blastocyst, are pluripotent. In culture, all cells can be coerced into pluripotency by overexpression of necessary factors (induced pluripotent stem cells, iPSCs)
Hematopoietic stem cells (HSCs)	Multipotent stem cells which reside in the bone marrow and form all cells of the blood
Mesenchymal stem cells (MSCs)	Multipotent stem cells which reside in almost all tissues and can differentiate into mesodermal cell types such as osteocytes, chondrocytes, and adipocytes. They secrete a variety of factors which reduce inflammation and promote regeneration
Neural stem cells (NSCs)	Multipotent stem cells which reside in the subventricular and the subgranular zone of the adult brain can differentiate into neurons, astrocytes, and oligodendrocytes. They secrete neurotrophic factors, cytokines, and growth factors
Holoclones	Subset of skin stem cells, with high proliferative and self-renewal potential
Gene editing	Molecular technique (e.g., zinc-finger nucleases, TALENs, and CRISPR/Cas9) for changing the DNA sequence in order to alter a gene's function or expression level
Autologous transplantation	Transplantation of cells that are taken from the patient
Allogeneic transplantation	Transplantation of cells which are taken from an independent donor
Good manufacturing practice (GMP)	(Inter)national rules to ensure high and consistent quality of medicinal products
Graft-versus-host disease (GvHD)	Potentially life-threatening conditions after HSC transplantation, where donor-derived immune cells attack cells from the patient

differentiated murine and later also human cells into pluripotent stem cells. These so-called induced pluripotent stem cells (iPSCs) can be generated by overexpressing a cocktail of pluripotency-associated transcription factors in somatic cells which eventually resets these cells into an undifferentiated state (Takahashi et al. 2007). This was a major breakthrough in the field of stem cell research and was honored in 2012 with the Nobel Prize in Physiology or Medicine for Yamanaka (shared with John B. Gurdon) (Fig. 1).

Today, almost every cell type has been successfully reprogrammed, and the technique itself has been optimized in order to increase efficiency as well as safety. In the earliest experiments, retroviruses were employed to overexpress the pluripotency factors. However, as retroviruses randomly integrate into the genome, they can potentially induce cancer formation if, for example, their integration destroys a tumor-suppressor gene or activates an oncogene. Nowadays, reprogramming is usually performed by using non-integrating episomal plasmids or Sendai viruses (Abu-Dawud et al. 2018). Nonetheless, undifferentiated PSCs still might elicit tumors in the recipient, simply because of their proliferative

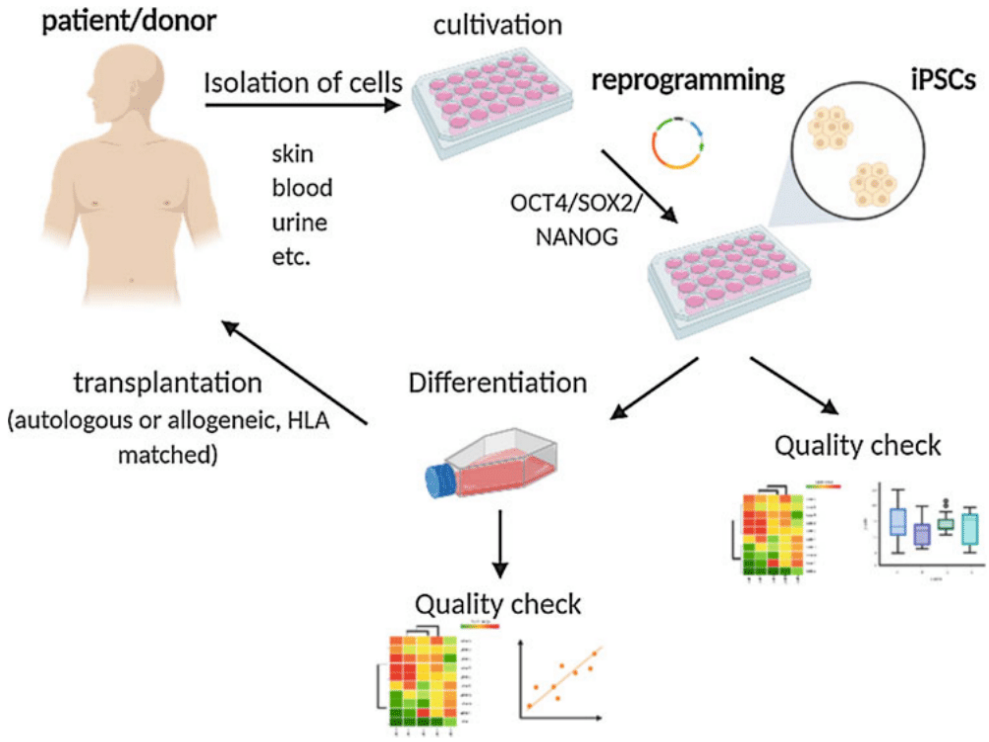

Fig. 1 Generation of iPSCs and their use as a potential cell therapeutics. Somatic cells are isolated from a donor, cultured, and reprogrammed into iPSCs. IPSCs are expanded and thoroughly checked, to ensure pluripotency and genomic integrity. Cells that pass the quality control are differentiated into the required cell type. After a quality assessment of the differentiated cells, ensuring successful differentiation and loss of pluripotent cells, the cells can be transplanted into the donor. Figure made in ©BioRender – biorender.com

potential. This is especially dangerous in the autologous setting where the transplanted cells are not recognized by the recipient's immune system. Therefore, sophisticated protocols have been established that allow efficient differentiation into all major cell types and include stringent quality controls to avoid transplantation of residual undifferentiated cells (Inoue et al. 2014).

iPSCs are associated with great anticipations for therapeutic employment as they can be generated from every patient; hence, their transplantation does not elicit immunological rejection. In addition, every given cell type can be reprogrammed which is especially interesting as it abolishes the need for invasive procedures, if, for instance, urine cells are taken as a source (Table 2). However, current data raise doubts regarding feasibility as reprogramming and all associated quality controls for excluding tumorigenic potential are not only very costly but, more importantly, time-intensive. Therefore, currently efforts are made to establish banks of iPSCs with homozygous HLA types in order to provide tolerable cells for a major part of the population. Japan with its very homogenous population is at the forefront of this and aims at having covered most of its population by 2030 (Kim et al. 2017).

Adult Multipotent Stem Cells

Hematopoietic Stem Cells (HSCs)

HSCs reside in specific niches in the bone marrow (BM) and can differentiate into all cells of the hematopoietic system. For transplantation, either they are isolated directly from BM aspirates, or they are collected by apheresis from peripheral blood after mobilization with granulocyte colony-stimulating factor (G-CSF). In both cases, the surface marker cluster of differentiation (CD)

Table 2 Ongoing clinical trials using PSCs listed at https://clinicaltrials.gov/ in August 2019

Disease	Therapy	Study ID	Sponsor	Country	Start
Spinal cord injury	ESC-derived OPCs	NCT01217008	Asterias Biotherapeutics	USA	2010
Stargardt macular dystrophy	hESC-derived RPE	NCT01345006	Astellas Institute for Regenerative Medicine	USA	2011
Dry AMD	hESC-derived RPE	NCT01344993	Astellas Institute for Regenerative Medicine	USA	2011
Stargardt macular dystrophy	hESC-derived RPE	NCT01469832	Astellas Institute for Regenerative Medicine	UK	2011
Dry AMD	hESC-derived RPE	NCT01674829	CHABiotech CO., Ltd	Korea	2012
Stargardt macular dystrophy	hESC-derived RPE	NCT01625559	CHABiotech CO., Ltd	Korea	2012
Exudative AMD	Autologous iPSC-derived RPE	UMIN000011929	RIKEN	Japan	2013
Spinal cord injury	ESC-derived OPCs	NCT02302157	Asterias Biotherapeutics	USA	2014
Ischemic heart disease	ESC derived $CD15^{+}$ $Isl\text{-}1^{+}$ progenitors	NCT02057900	Assistance Publique – Hôpitaux de Paris	France	2014
Type 1 diabetes mellitus	VC-01 combination product (device loaded with ESC-derived β-like cells)	NCT02239354	ViaCyte, California Institute for Regenerative Medicine (CIRM)	Canada, USA	2014
Dry AMD	hESC-derived RPE	NCT02286089	BioTime Inc.	Israel, USA	2015
Retinitis pigmentosa	hESC-derived RPE	ChiCTR-OCB-15007055	Institute of Zoology, Chinese Academy of Sciences	China	2015
Stargardt macular dystrophy, AMD, exudative AMD	hESC-derived RPE	NCT02903576	Federal University of São Paulo	Brazil	2015
AMD	hESC-derived RPE	NCT01691261	Pfizer	UK	2015
Dry AMD	hESC-derived RPE	NCT02590692	Regenerative Patch Technologies	USA	2015
AMD, Stargardt macular dystrophy	hESC-derived RPE	NCT02749734	Southwest Hospital, China	China	2015
PD	Human parthenogenetic neural stem cells	NCT02452723	Cyto Therapeutics Pty Limited	Australia	2016
Dry AMD	hESC-derived RPE	ChiCTR-OCB-15007054	Institute of Zoology, Chinese Academy of Sciences	China	2016
GvHD	CYP-001: iMSCs	NCT02923375	Cynata Therapeutics Ltd.	Australia, UK	2016
PD	ESC-derived NPC	NCT03119636	Chinese Academy of Sciences	China	2017
Dry AMD	hESC-derived RPE	NCT03046407	Chinese Academy of Sciences	China	2017
Neovascular AMD	Allogenic iPSC-derived RPE	UMIN000026003	Kobe City Medical Center General Hospital	Japan	2017

(*continued*)

Table 2 (continued)

Disease	Therapy	Study ID	Sponsor	Country	Start
Type 1 diabetes mellitus	VC-02 combination product (device loaded with ESC-derived β-like cells)	NCT03162926	ViaCyte	Canada	2017
Type 1 diabetes mellitus	VC-02 combination product (device loaded with ESC-derived β-like cells)	NCT03163511	ViaCyte, California Institute for Regenerative Medicine (CIRM)	Canada, USA	2017
PD	iPSC-derived dopaminergic progenitors	UMIN000033564	Kyoto University Hospital	Japan	2018
Dry AMD	hESC-derived RPE	NCT02755428	Chinese Academy of Sciences	China	2018
ALS	hESC-derived astrocytes	NCT03482050	Kadimastem	Israel	2018
Spinal cord injury	iPSC-derived NSCs	Approved	Keio University School of Medicine	Japan	2019
Retinitis pigmentosa	hESC-derived RPE	NCT03944239	Qi Zhou	China	2019
Retinitis pigmentosa due to monogenic mutation	hESC-derived RPE	NCT03963154	Centre d'Etude des Cellules Souches	France	2019
Heart failure	Allogenic iPSC-derived cardiomyocytes	NCT03763136	Help Therapeutics	China	2019
meniscus injury	hESC-derived MSC like cell	NCT03839238	Tongji Hospital	China	2019

34 can be used to separate HSCs from other cell types (Panch et al. 2017).

A third source of HSCs is the umbilical cord which contains stem cells in addition to very naïve immune cells. These cells can be collected directly after birth and are in contrast to HSCs from BM or apheresis frequently stored in liquid nitrogen for usage in the far future (Panch et al. 2017). Parents can choose to store the umbilical cord blood (UCB) either in public banks making it available for everyone or in private banks where it is kept exclusively for the donating family who also has to cover the costs. At the moment about six times more UCB units are stored in private banks than in public ones although the vast majority of UCB units used for transplantation are released by public banks (Ballen et al. 2015).

Routine allogeneic transplantation of HSCs only became possible after the human leukocyte antigen (HLA) system, which is fundamental for the function of the human immune system, was understood (Klein and Sato 2000; van Rood 1966). Nowadays, great efforts are undertaken to ensure that donor and recipient HLA type match in order to reduce the risk of graft-versus-host disease (GvHD) (Klein and Sato 2000). In this potentially life-threatening condition, donor-derived immune cells attack not only residual tumor cells in the recipient but also healthy tissue. Symptoms are ranging from rather mild skin reddening and itching toward massive damage of the gastrointestinal tract or the liver and always need special immunosuppressive treatments. If GvHD occurs in the first 100 days after transplantation, it is classified as acute and later as chronic. Over time, the new immune system is capable to adapt to the recipient's tissue surface antigens, and symptoms recede.

Mesenchymal Stem Cells (MSCs)

In 1924, Alexander A. Maximow identified cells residing within the bone marrow (BM) which had some similarities to fibroblasts. These cells were described to support hematopoiesis, the

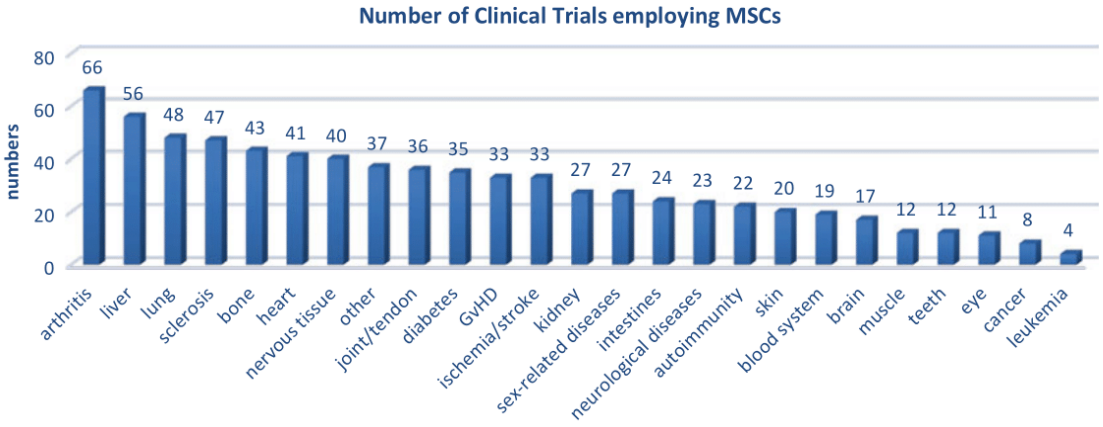

Fig. 2 Use of MSCs in clinical trials. Information on over 740 clinical trials using MSCs have been deposited at https://clinicaltrials.gov/ in March 2019

generation of blood cells (Maximow 1924). By exploiting their ability of plastic adherence, Friedenstein and colleagues isolated these cells from BM (Friedenstein et al. 1970). The term "mesenchymal stem cells" (MSCs) was established by Arnold I. Caplan in the 1990s (Caplan 1991). Although in the beginning MSCs were exclusively isolated from the perivascular region of the BM, several other sources for MSCs have been established in recent years: placenta, umbilical cord blood (UCB), heart, skin, pancreas, lung, brain, kidney, adipose tissue, cartilage, tendon, as well as amniotic fluid and urine (Wu et al. 2018; Fitzsimmons et al. 2018; Spitzhorn et al. 2017; Rahman et al. 2018).

In addition to their extensive in vitro proliferation potential, MSCs exhibit a multipotential differentiation capacity which manifests in their ability to form various cell types from the mesodermal lineage including the cartilage, bone, and fat (Fitzsimmons et al. 2018). In vitro, they can also differentiate in cell types from other lineages such as pancreatic β cells or cardiomyocytes, although it is controversially discussed if this happens in vivo, too (Segers and Lee 2008; Cho et al. 2018). The fate-determining decision is triggered by various factors such as cytokines and growth factors present in the stem cell niche – a distinct microenvironment in which the stem cells are located (Fitzsimmons et al. 2018).

MSCs exhibit a fibroblast-like, spindle-shaped morphology. They are characterized by the expression of specific cell surface markers (also referred to as immunophenotype) such as CD73, CD90, and CD105 and the of lack hematopoietic markers. Together with their differentiation capacity and their plastic adherence, these features were set as the minimal criteria to identify MSCs by the international society for cellular therapy (ISCT) (Dominici et al. 2006). MSCs also have the ability to secrete various cytokines and growth factors. Thus, they can support hematopoiesis in the BM and are able to suppress reactions of the immune system. They support regeneration processes by the release of distinct molecules, for example, vascular endothelial growth factor A (VEGF-A), hepatic growth factor (HGF), platelet-derived growth factor (PDGF), and various interleukins (Fitzsimmons et al. 2018). Furthermore, MSCs can react to factors released by the environment. They migrate to sites of inflammation or injury to support the regeneration process. As MSCs express only very low levels of HLA, they can be transplanted even without matching their HLA type to that of the patient (Fitzsimmons et al. 2018).

To investigate their therapeutic potential, MSCs have been employed in over 740 clinical trials (ongoing or completed) for a broad range of diseases and organs affected which underlines their versatility (Fig. 2).

In most clinical trials, MSCs from adult BM are used followed by the adipose tissue, umbilical cord tissue, and placenta (Galipeau and Sensebe

Table 3 Approved MSC-based therapies (alliancerm.org)

Product	Company	Indication	Treatment	Approval	Country
Queencell	Anterogen Co., Ltd.	Connective tissue disorders	Autologous adipose tissue MSCs	2010	South Korea
Cellgram-AMI	Pharmicell	AMI	Autologous BM-MSCs	2011	South Korea
Cupistem	Anterogen Co., Ltd.	Perianal fistula in Crohn's disease	Autologous adipose tissue MSCs	2012	South Korea
Cartistem	Medipost	Knee cartilage defects	Allogeneic UCB-MSC	2012	South Korea
Prochymal	Mesoblast Inc.	GvHD	Allogeneic BM-MSCs	2013	Canada, New Zealand
Neuronata-R	Corestem Inc.	ALS	Autologous BM-MSCs	2014	South Korea
Temcell HS	JCR Pharmaceuticals Co., Ltd.	GvHD	Allogeneic BM-MSCs	2015	Japan
Stempeucel	Stempeutics Research Pvt	Critical limb ischemia	Allogeneic BM-MSC	2017	India
Alofisel	TiGenix NV, Takeda Ltd.	Perianal fistulas in Crohn's disease	Allogeneic adipose tissue MSCs	2018	EU
Stemirac	Nipro Corp	Spinal cord injury	Autologous BM-MSC	2018	Japan
Neuronata-R	Corestem Inc.	ALS	Autologous BM-MSCs	2018	USA, orphan drug
Neuronata-R	Corestem Inc.	ALS	Autologous BM-MSCs	2019	EU, orphan drug

2018). The most prevalent way of administration is intravenously. This is very critical, because cell aggregation after infusion is one of the most prevalent adverse effects of MSC treatments (Caplan et al. 2019). MSCs are thawed immediately prior to transfusion. This is also a key step since it is well-known that the fitness of MSCs declines within the first 24 h after thawing which could impair their function (Caplan et al. 2019).

Despite the plethora of MSC-based clinical trials, so far worldwide only a few products based on MSCs have obtained clinical approval (Table 3).

MSC-Based Clinical Trials

In 1995 MSCs were the first cellular pharmaceutical tested in human. They were isolated from the BM of patients with hematologic malignancies in complete remission and reinfused intravenously (Lazarus et al. 1995). This study confirmed the safety of autologous MSC transplantation.

Later, MSCs were used in clinical trials to support hematopoietic recovery following high-dose myeloablative chemotherapy and accelerating hematopoiesis after BM transplantation (Fitzsimmons et al. 2018). The immunosuppressive capacity of MSCs is frequently used as a support in other transplantation settings, to reduce the risk of organ rejection or GvHD, especially if the HLA types of perfectly donor and recipient are not perfectly matching (Fitzsimmons et al. 2018).

In 2009, the first major industry-sponsored (Osiris Therapeutics (USA)) phase III trial of allogeneic MSCs for treatment of steroid-refractory GvHD (NCT00366145) was completed. Although it did not show an overall significant positive effect of the MSCs, it demonstrated that children with GvHD were responsive to this cellular treatment (Kurtzberg et al. 2014). In 2013, the therapeutic called Prochymal/remestemcel-L was conditionally approved in Canada and New Zealand for application in children with an obligation to prove its efficacy. Mesoblast Inc.

(Australia) took over the MSC therapeutic agents and focused on the prospective phase III trial (NCT00759018) (Kurtzberg et al. 2014). The overall response in the children 28 days after treatment was significantly improved in comparison to control patients. In Canada reimbursement issues prevented establishment as a common clinical treatment. In Japan on the other hand, the MSC therapy for GvHD and a proper reimbursement were approved and installed as Temcell in 2015 (JCR Pharmaceuticals Co. 2017).

For treating patients with complex perianal fistulas without inflammatory bowel disease, Cellerix S.A. sponsored a study of autologous adipose MSCs (NCT00475410). Forty-eight hours after thawing, the cells were injected in the area of the fistula. The outcome of the MSC group was not superior to the control group. After Cellerix S.A. was acquired by TiGenix NV in 2011, a new phase III clinical trial was initiated (NCT01541579), treating only Crohn's disease patients with an increased dose of allogeneic cells. The group receiving MSCs significantly improved their condition in comparison to the control group. The results disclosed in 2015 were reported to be the first completely successful outcome of MSCs in a clinical trial of an advanced state (Panés et al. 2016). The product, named Alofisel, was EMA approved first in 2009 as an orphan drug until it obtained full approval in 2018. A similar product, called Cupistem, got approval in South Korea.

The highest number of clinical trials with MSCs targets bone and cartilage diseases, for example, osteogenesis imperfecta, a genetic disorder which is characterized by mutations in the genes *collagen type I alpha 1 chain* (*COL1A)1* and *2*. This leads to osteopenia, multiple fractures, severe bone deformities, and considerably shortened stature. The first clinical trial to address this used allogeneic BM (containing MSCs) in children. It was shown that MSCs could migrate to the bone and improve bone structure (Horwitz et al. 1999). In a special case of such a treatment in 2005, allogeneic MSCs were transplanted in utero into a fetus with severe osteogenesis imperfecta. Within the first 2 years, only three fractures occurred, and correct growth tendencies as well as normal psychomotoric behavior were reported. At the age of 8 years, the same patient was treated again with MSCs of the same donor resulting in low-level beneficial outcome. This study has shown long-term safety, but still more work has to be done to unravel the effectiveness (Gotherstrom et al. 2014).

In patients suffering from osteoarthritis (OA), the articular cartilage is degenerated, and subchondral bone sclerosis and synovial inflammation lead to pain in the joints, local inflammations, and restricted movement. Intense research and many clinical trials are performed in this field, with limited positive outcome. In general, patients reported less pain and improved mobility after treatment, and improved cartilage quality could be observed, but due to study design, the evidence is not very reliable (Iijima et al. 2018). Nevertheless, South Korea approved Medipost's Cartistem in 2012, a product based on allogeneic UCB-MSCs which are plied to the joint during orthopedic surgery.

MSCs from Pluripotent Stem Cells (iMSCs)

The derivation of MSCs from BM or other sources is done routinely, but these invasive procedures are associated with certain risks (Sheyn et al. 2016). In the everyday clinical scenario with a high percentage of elderly people, the quality of MSCs is compromised due to cellular aging. This is manifested in higher levels of cellular senescence, DNA damage, oxidative stress, genomic instability, and immunogenicity which reduces their therapeutic potential (Yang 2018).

These hurdles can be bypassed by the generation of induced MSCs (iMSCs) from PSCs which have a rejuvenated phenotype (Frobel et al. 2014; Spitzhorn et al. 2019). Human iMSCs have been used in many preclinical studies for, e.g., liver and bone regeneration (Spitzhorn et al. 2018; Jungbluth et al. 2019, in press). A very important aspect for transplantation of iMSCs is the fact that although they are derived from PSCs, they are not tumorigenic and thus safe for transplantation. To date iMSCs have been successfully used in two clinical trials. Cynata Therapeutics Ltd. (Australia) is funding a phase I clinical study in which iMSCs coming from iPSCs are used for the treatment of steroid-resistant acute GvHD with preliminary

promising results (NCT02923375). The Tongji Hospital (China) in cooperation with the Chinese Academy of Sciences is running a phase I clinical trial for iMSCs generated from ESCs with the focus to evaluate the safety in treating meniscus injury patients (NCT03839238).

Neural Stem Cells (NSCs)

Neural stem cells (NSCs) are self-renewing multipotent cells with the potential to differentiate into neurons, astrocytes, and oligodendrocytes, the three main cell types in the central nervous system (CNS) (Seaberg and van der Kooy 2003). Additionally, NSCs are capable of secreting soluble molecules such as neurotrophic factors, cytokines, and growth factors. Due to these special characteristics, transplantation of NSCs is a promising treatment option for diseases associated with the CNS, for both regeneration of neural cells and restoration of the microenvironment at the injury site by providing trophic support.

First evidences of adult neurogenesis (the endogenous generation of new neurons) were described by Altman and Das in 1965 (Altman and Das 1965). In the adult brain, NSCs are confined primarily to two regions, the subventricular zone (SVZ) and the subgranular zone (SGZ). Adult neurogenesis is a dynamic but extremely coordinated process, where the proliferation, migration, and differentiation of the NSCs are controlled by microenvironmental stimuli. Although adult neurogenesis in the CNS is limited under normal physiological conditions, it can be induced after injury to recruit NSCs and reconstruct neural tissues. Unfortunately, in the case of severe injury, the response of activated NSCs is ineffective in keeping the balance between self-renewal and differentiation (Ming and Song 2011).

Cell therapy based on NSCs is now possible thanks to the comprehensive development of protocols based on growth factors to expand and differentiate these cells in vitro. NSCs can be derived from three major sources: directly extracted from primary CNS tissue (fetal and adult brain and spinal cord tissue), differentiated from pluripotent stem cells (PSCs), and through transdifferentiation from somatic cells (Tang et al. 2017).

NSCs derived from primary CNS: Human NSCs can be isolated directly from the ventricular zone of fetal brain tissue or from the SVZ of adult brain. After isolation, these cells can be propagated as single-cell suspensions that ultimately will form three-dimensional neurospheres in a medium supplemented with appropriate growth factors that allow proliferation, self-renewal, and expansion of NSCs in vitro (Gonzalez et al. 2016; Tang et al. 2017).

NSCs derived from PSCs: NSCs can be in vitro differentiated from ESCs and iPSCs via a process called neuroinduction. After this stage, NSCs can be maintained in culture in the same conditions as the NSCs directly isolated from the CNS. The use of iPSC-derived NSCs has advantages over ESC-derived NSCs since iPSCs can be generated from adult tissue, bypassing ethical issues. In addition, iPSC-derived NSCs can be obtained in a patient-specific manner, allowing autologous transplantation and thus overcoming the immune rejection (Tang et al. 2017; Gonzalez et al. 2016).

Transdifferentiation of somatic cells into NSCs: The generation of NSCs by transdifferentiation of somatic cells, a process that comprises the transformation of a somatic cell into another somatic cell type without undergoing the pluripotent stage, is a promising tool for therapeutic purposes. Although it was already shown that NPCs can be transdifferentiated from fibroblasts, urine cells, and MSCs, thus bypassing the tumorigenic potential of PSC-derived NSCs, it is a very recent field that needs further investigation (Tang et al. 2017).

Approval of Stem Cell Treatments

Stem cell therapy and regenerative medicine elicit high hopes – in patients who expect cures from a disease, as well as in industry that sees the great economic potential. This has led to the emergence of many clinics which perform unapproved stem cell treatments (Sipp et al. 2017).

The ethical and legal view on stem cells is highly divergent between countries. In the USA as well as in Europe, human cells and tissues are

mostly defined as advanced therapy technical medicinal products (ATMP). That means that they have to be approved by the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA), respectively (Sakai et al. 2017). Usually this approval is only granted after thoroughly proving safety and efficacy of the treatment in controlled clinical trials. However, patients as well as industry express the need for a streamlined process which would reduce time and costs for approval. In 2014, Japan revised its law and introduced the Pharmaceuticals, Medical Devices, and Other Therapeutic Products (PMD) Act which provides conditional approval as long as the procedure has been proven to be safe. However, the new product has to prove its efficacy within 7 years. In the USA "the right to try" laws provide terminally ill patients the opportunity to test stem cell therapies before their final approval (Sakai et al. 2017). This provoked controversies as proper clinical trials are a highly valuable tool for determining safety and efficacy of novel treatments. On the other hand, many stem cell-based clinical trials (even in phase I/II) are only performed on terminally sick patients due to the high risks they are associated with, which decreases the likelihood of a positive outcome.

In parallel, a lot of unapproved stem cell therapies are on the market, many of them consisting in gaining MSCs, e.g., from liposuction and using these cells for a variety of treatments (Sipp et al. 2017; Goff et al. 2017). However, for these treatments neither safety nor efficacy has been studied in an accredited way, and in several cases there were dramatic outcomes such as severe infections or blinding (Marks et al. 2017). As clinics performing these unapproved treatments frequently advert them directly to patients with no independent physician explaining risks and benefits, they are very attractive for patients who are inclined to spend a lot of money and take incalculable risks (Sipp et al. 2017). Therefore, it is important to provide unbiased information to patients and educate the general population on risks and benefits of stem cell treatments.

Stem Cell-Based Regenerative Therapies

Hematopoietic System

From all areas of stem cell therapy, applications in the hematopoietic system have the longest history. The first allogeneic BM transplantation was performed in 1957 by E. Donnall Thomas, who paved the way for this kind of treatment and received the Nobel Prize in 1990 (Thomas et al. 1957). About 10 years after this first transplantation, techniques had developed that allowed for HLA typing, and the usage of stem cells from HLA-matched donors dramatically improved the outcomes of transplantations (Henig and Zuckerman 2014). As the HLA genotype limits the number of putative donors, international collaboration is mandatory. To this end the International Bone Marrow Transplant Registry (IBMTR) was established in 1972. The indications for HSC transplantation shifted with increasing knowledge about the diseases that are to be treated as well as with improved technologies that enable a broader spectrum of patients to be included (Little and Storb 2002).

In the beginning, predominantly hematopoietic cancers were treated with allogeneic stem cells. Later, also other blood malignancies were included, e.g., thalassemia or sickle cell anemia, and today also unrelated conditions as, e.g., solid tumors and autoimmune diseases are treated by HSC transplantation (Passweg et al. 2019).

Although therapy schemes have improved dramatically over time and the number of transplantations has increased immensely, HSC transplantation still is a very risky and stressful procedure for the patient. In a first step, the host's immune system and its stem cells have to be destroyed by irradiation and/or chemotherapy in order to remove the malignant cells. Nowadays there exist sophisticated protocols depending on the actual disease, which enable physicians to limit the destruction, especially of the stem cells, and thus allow also elder and fragile patients to undergo HSC transplantation (Gyurkocza et al. 2010). During the phase of engraftment, patients lack a functioning immune system and are very much at risk of developing

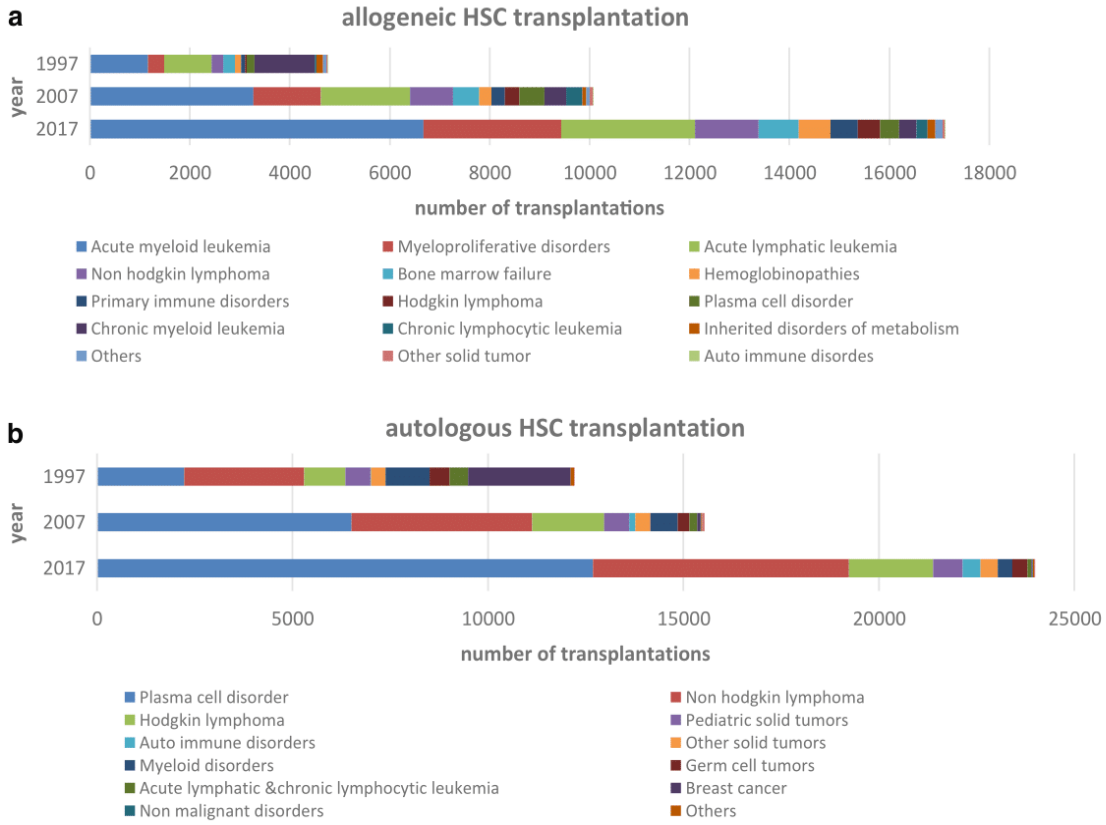

Fig. 3 Development of allogeneic (**a**) and autologous (**b**) HSC transplantation in Europe from 1997 to 2017. The number of HSC transplantations increased massively, with the frequency of distinct indications changing over time (Passweg et al. 2019)

infectious diseases. Only after the stem cells have homed into the BM niche, they efficiently produce new immune cells. However, in this phase the risk of GvHD is also augmented (Dowse and McLornan 2017) (Fig. 3).

Today, most HSCs for transplantation are collected via apheresis because this allows for a higher enrichment of stem cells, and the samples contain more T cells which are valuable for fighting the disease, called graft-versus-tumor/leukemia reaction. In addition, an increasing number of UCB transplants are performed. Although they contain less HSCs, which increases the risk of engraftment failure, their T cells are immature which reduces the risk of GvHD and allows for more HLA mismatches being present. Transplanting a combination of two independent UCB units seems to be the optimal solution in this case (Dowse and McLornan 2017; Panch et al. 2017).

Although HSC transplantation has been a routine treatment for many years, the mortality rates are still quite high. In acute leukemia it ranges between 7% and 27%, at 100 days post-allogeneic transplantation depending on disease severity. The leading causes are GvHD and infections. If patients survive for more than 2 years without relapse, their long-time survival rates are 80–92% (Henig and Zuckerman 2014).

Besides being a risky procedure, there are cases where allogeneic stem cell transplantation has additional benefits for the patients. Two patients who were infected with human immunodeficiency virus (HIV) reached remission for at least 20 and 18 months, respectively, after receiving allogeneic HSC transplants for treatment of leukemia or Hodgkin's lymphoma, respectively. Both patients were transplanted with HSCs from donors homozygous for a 32 base-pair deletion

within the *C-C chemokine receptor type 5 receptor* (*CCR5*) gene, which prevents expression of the receptor and thus precludes infection with HIV strains that need CCR5 as a co-receptor (Gupta et al. 2019).

Initial observations in the 1990s that HSC transplantation in cancer patients also improved coincidental autoimmune diseases (AD) developed this field as a new target for HSC transplantation. To date, several thousand patients have received HSC transplants (primarily autologous) to treat ADs, especially multiple sclerosis, Crohn's disease, and systemic sclerosis (Jessop et al. 2019). All ADs have in common that the patient's immune system attacks cells of its own body, leading to permanent inflammation followed by scar formation and impairment of the respective organ's function. HSC transplantation is capable of resetting the immune system, and it has been shown that after transplantation the amount of autoreactive immune cells can be reduced. However, HSC transplantation is still associated with many risks, and treatment outcome varies between patients and depends on disease severity. Nonetheless, many patients have long-term improvements, and for some ADs randomized controlled clinical trials have demonstrated the benefit of HSC transplantation compared to the best available conventional therapy (Jessop et al. 2019).

As HSC transplantation has been established a long time ago, it is also one of the fields where the most advanced techniques in terms of gene therapy are currently used. The first successful gene therapy trial was performed as early as 1990, although not targeting HSCs but T cells (Al-Saif 2019). Two patients who suffered from severe combined immune deficiency (SCID) caused by a mutation in the enzyme adenosine deaminase were included in this trial. The defective gene was exchanged for a functional copy in patients' T cells. Their blood values improved and they needed less medication after this treatment.

Current studies involve gene therapy directly in HSCs which can be performed in vivo as well as ex vivo. For in vivo gene therapy, genes are delivered to the HSCs by vectors or liposomes which can be injected into the bone or – after HSC mobilization – even intravenously. However, it has to be taken into account that these particles potentially can transfect also other cell types and frequently elicit strong immune reactions. On the other hand, they overcome transplantation-related issues as the need for chemotherapy in advance and the risk of poor engraftment. In ex vivo gene therapy, HSCs are collected from the patient and genes are modified in vitro. Besides simply overexpressing a healthy variant of the target genes, other possibilities include gene modification by applying, for example, TALENs or CRISPR/cas9. After the procedure, it is possible to selectively only transfer successfully modified cells back into the patient (Al-Saif 2019).

Currently, several clinical trials employing genetically engineered HSCs for transplantation are in progress. In sickle cell disease (SCD), a transversion of A to T within the coding region of the β-globin gene causes hemoglobin polymerization, which is associated with morphological changes of erythrocytes and impaired oxygen transport capacity. Standard treatment for SCD consists in applying hydroxyurea which increases the expression of the fetal y-globin, a molecule that prevents hemoglobin polymerization. Two gene therapies in clinical trials aim at increasing y-globin expression, either by overexpressing it directly (NCT02186418) or by inactivating a repressive factor (NCT03282656). In a different approach, a β-globin variant, which also prevents hemoglobin polymerization, is introduced into HSCs by a self-inactivating lentivirus (NCT02151526). Data from a first patient treated with this system indicate safety as well as efficacy (Al-Saif 2019).

In the future many therapy improvements are likely to be implied. One interesting novel approach concerns the field of chimeric antigen receptor (CAR) T-cell therapy. For this kind of therapy, until now, patient-derived T cells are genetically modified to express a chimeric antigen receptor specific for a tumor-related antigen. The engineered cells are then reinfused into the patient and supposed to eliminate the cancerous cells. As the whole procedure of engineering is very time-consuming, several groups are working on

lymphoepithelial kazal-type inhibitor (LEKTI) that is responsible for the function of the skin barrier. The skin of *SPINK5*-mutated patients becomes red and scaly (ichthyosiform erythroderma). Functionally, the skin leaks fluid and loses the capacity to bind water which causes skin dryness and hypernatremic dehydration. This and other complications, e.g., bronchopneumonia or sepsis, lead to a high mortality rate in early life (Di et al. 2019).

Currently, there are no approved treatments to cure this lethal condition. Based on the principle of ex vivo gene modification in stem cells, a clinical phase I trial of grafting autologous epithelial sheets harboring intact *SPINK5* for NS has been conducted (Di et al. 2019) (NCT01545323). It proved successful in terms of gene modification and engraftment, but only transient functional correction was observed, possibly due to a limited number of stem cell holoclones within the graft.

A groundbreaking example of skin stem cell therapy is that of a child having recovered from a fatal form of JEB in Germany in 2017 after engraftment with epidermal sheets consisting of autologous skin stem cells which were genetically modified to contain healthy copies of the *Laminin Subunit Beta 3* (*LAMB3)* gene. Impressively, this *LAMB3* harboring epidermal autograft could restore dermal-epidermal adherence, and the long-lived skin stem cell holoclones could regenerate and renew the epidermis. Upon 1-month post-grafting, the new epidermis had formed without blisters, and the regenerated epidermis was strong and elastic alike healthy skin (Hirsch et al. 2017). Previously, in 2006, genetically engineered autologous keratinocyte grafts have been transplanted in a patient with laminin 332 deficiency (non-Herlitz junctional EB) (Mavilio et al. 2006). In a clinical study from Austria in 2014, genetically modified autologous epidermal sheets were grafted in an adult woman to cure from laminin 332-β3-dependent JEB with a large non-healing epidermal ulceration (Bauer et al. 2017). The transgenic epidermis expressed a normal amount of properly functional laminin 332 which is precisely located at the dermal-epidermal junction. These experimental results could benefit JEB patients with disrupted laminin 332.

The above-stated cases of JEB have demonstrated that it is possible to cure genetically damaged non-healing skin by using gene therapy in epidermal stem cells. However, for the EB simplex, which is not caused by the lack of the LAMB3 protein but by an active dysfunctional version of the protein, other approaches are necessary. Corrections with a gene-editing innovative tool like CRISPR/Cas9 might be possible. The feasibility of genome editing in skin stem cells using a self-inactivating (SIN) retroviral vector and CRISPR/Cas9 approach in a preclinical study in mice has already been reported (Hainzl et al. 2017; Izmiryan et al. 2018). Currently, this technique is also used in clinical trials in RDEB patients. The aim is to completely cure the disease using autologous skin grafts made of primary keratinocytes and fibroblasts with genetically edited type 7 collagen (NCT01263379, NCT02493816).

A special tissue composed of keratinocytes is the cornea of the eye. The integrity of this epithelial layer and the absence of blood vessels are crucial for proper vision. In a healthy setting, corneal cells are regenerated by limbal stem cells, located between the cornea and the bulbar conjunctiva. Thermal and chemical burns of the eye often destroy the limbal stem cells which results in vascularization of the cornea and ultimately vision loss (Pellegrini et al. 2009). Beginning in the late 1990s, the first patients were treated with transplants generated from limbal stem cells, and permanent restoration of the cornea was achieved in 76.6% of the cases. Importantly, a 10-year follow-up proved long-term stability of the regenerated tissue (Rama et al. 2010). The treatment relies on autologous cells which can be obtained if at least 1–2 mm intact limbal tissue is available. The cells are expanded and prepared for transplantation on a fibrin matrix. This product, marketed as Holoclar, was the first stem cell-based ATMP authorized by the European Medicines Agency in 2015.

Tremendous advancement of ESCs, iPSCs, and genome editing technology has been made during the last 10 years; now the major goal is to harness these technologies to generate unlimited amounts of genetically corrected keratinocytes not only from autologous but also from allogeneic donors.

Human PSCs can be differentiated into keratinocytes which are the basis for in vitro-generated functional epidermal sheets. These sheets could provide temporary skin substitutes for patients awaiting autografts (Guenou et al. 2009). Tolar and colleagues demonstrated that gene-corrected iPSCs could be generated from the skin of patients with a mosaic form of RDEB (Tolar et al. 2014). Compared to adult stem cells, iPSCs have a higher proliferation potential, allowing genetic manipulations and to overcome the autograft shortages. By generating iPSCs and, subsequently, iPSC-derived keratinocytes, they were able to provide proof of principle that iPSC technology can be used to generate essentially unlimited amounts of clinically normal epidermis from patients (Sebastiano et al. 2014; Wenzel et al. 2014).

Cardiac Diseases

The heart is the most important muscle in the human body. It pumps blood through the body and provides the essential oxygen and nutrient supply. Heart diseases can severely restrict the life of patients. Ischemic or coronary heart disease (CHD) is the most common form of heart diseases. It manifests in a reduced blood flow to the heart due to plaques which partially block its arteries. Complete blockage of the blood flow causes damage to the heart cells leading to myocardial infarction (MI) and even heart failure. Heart failure is defined as a syndrome that restricts the cardiac function of filling or ejecting blood in the ventricle, irrespective of cause. This condition has far-reaching consequences for the whole body and is not restricted to the heart only but can effect almost every other organ (Segers and Lee 2008).

Similar to other cases of organ failure, treatment of patients with end-stage heart failure includes organ transplantation, which greatly enhances the patients' quality of life as they regain physical endurance and are less hospitalized (Michler 2018). However, the demand of donor organs is much higher than the pool of available donors can manage. Many patients are also not eligible for transplantations depending on their conditions. They are particularly in the focus for the future use of stem cell therapy (Michler 2018).

The goal of stem cell treatment for heart diseases is the generation of cardiomyocytes and blood vessels, which support the regeneration of cardiac function (Michler 2018).

Some clinical trials already applied BM-MSCs. In an in vivo rodent model, differentiation of BM-MSCs to cardiomyocytes was reported when injected into the murine myocardium (Toma et al. 2002). However, it is debatable if these cells are able to generate cardiomyocytes in human patients, too. It is not proven that injected stem cells regenerate the heart tissue by differentiating into cardiomyocytes, but it is believed that secreted factors comprising growth factors, cytokines, and chemokines may improve the regeneration by activating reparative mechanisms and inhibiting apoptosis, fibrosis, and hypertrophy. Moreover, it seems that infused stem cells support the proliferation of cardiomyocytes and also recruit cardiac stem cells which regenerate the cardiac tissue (Segers and Lee 2008).

With regard to ischemic heart diseases, some clinical trials also showed that the injection of stem cells may lead to the formation of blood vessels which improved cardiac performance (Segers and Lee 2008).

In March 2019, more than 300 stem cell-based clinical studies for heart diseases were listed at https://clinicaltrials.gov/. Many of these are performed with BM-MSCs in various cell doses and varying infusion time points. Even though these cell therapies did not show significant improvements in the healing process of the heart diseases, there are some points which could be learned from these trials: (a) stem cell therapy is safe for patients, (b) therapy with cells is minimally effective, and (c) transdifferentiation of BM-MSCs does not occur in a frequency high enough to have an effect (Michler 2018).

In 2001/2002, the PERFECT phase II clinical trial, sponsored by Miltenyi Biotec GmbH, was initiated in order to study safety as well as efficiency of autologous $CD133^{+}$ BM-derived stem cells when injected into the myocardium during a coronary artery bypass graft (CABG). As primary

outcome, left ventricular contractility was measured and compared to CABG alone. Meanwhile, this study has developed into the first phase III clinical trial. Eighty-two patients with myocardial infarct were either injected with autologous $CD133^+$ BM-MSCs or placebo product while undergoing bypass surgery in a double-blinded and randomized fashion (Steinhoff et al. 2017). Analyses 180 days after the treatment showed that cardiac tissue repair and the improvement of left ventricular function were induced, possibly due to the presence of CD133 endothelial progenitor cells in the stem cell preparation (Steinhoff et al. 2017).

Between 2004 and 2005, 204 patients suffering from acute myocardial infarction (AMI) were treated in a phase III clinical trial called REPAIR-AMI (NCT00279175). The patients were randomly injected with autologous BM-MSCs or placebo medium into the infarct artery 3–7 days after infarct reperfusion therapy. The 12-month follow-up results showed a reduced number of patients who met the endpoint death, needed a revascularization, or had another MI in the BM-MSC-treated group than in the placebo group (Schachinger et al. 2006).

Another clinical trial combined the use of left ventricular assist devices (LVAD) with the injection of allogeneic MSCs (Ascheim et al. 2014) (NCT01442129). Thirty randomized patients suffering from end-stage heart failure were injected 25 million MSCs each during LVAD implantation. After 90 days, the functionality of the ventricle was checked while temporarily weaned from the LVAD. Even though an effect of MSCs was not clearly observed, the treated patients were able to wean of the LVAD more often and for longer periods.

Alternative to the injection of single cells, sphere-derived cells can be transplanted into the diseased tissue. Spheres are three-dimensional cell aggregates composed of cell types specific for one tissue or organ. In the clinical trial CADUCEUS (NCT00893360), patients with ischemic LV dysfunction were randomly injected with autologous cardiosphere-derived cells (CDC) generated from myocardial biopsies into the infarct-related artery 90 days after the myocardial infarct. The control group received standard treatment (Malliaras et al. 2014). Follow-up examination of the patients revealed no improvement of end-diastolic volume, end-systolic volume, and left ventricular ejection fraction. However, CDC treatment reduced scar mass and improved viable heart mass as well as regional contractility and systolic wall thickening (Malliaras et al. 2014).

Between 2013 and 2018, hESC-derived cardiovascular progenitors were transplanted into ten patients who suffered from ischemic heart failure in a clinical trial performed in France (NCT02057900). The generated cells were embedded into fibrin gel and were injected while the patients surgically received a coronary artery bypass grafting and/or a mitral valve procedure. A follow-up after 1 year revealed no adverse events during the recoveries and no tumor formation. None of the patients suffered from arrhythmias, and the patients' symptoms improved with increased systolic motion. Silent alloimmunization (immune response to antigens of an allogeneic donor) occurred in three patients. The conclusion was that the derivation of cardiovascular progenitor cells from hESCs was feasible, and transplantation of these cells was safe for short and medium term (Menasche et al. 2018).

All in all, many clinical trials with stem cell-based therapies for heart diseases were performed in the last 20 years. Especially, BM-MSCs were used extensively in these trials but with no or only minimal success. Nevertheless, there are, alone in the USA, more than 60 direct-to-costumer businesses cardiomyocytes promising to cure patients suffering from heart diseases with stem cells. These treatments are not authorized by the US FDA and expose the patients to incalculable risks (Goff et al. 2017). Until the mechanisms related to heart development and metabolism are better understood and risk factors are eliminated, stem cell therapy for heart diseases is still only at the very beginning.

Diabetes

Diabetes is one of the major health problems of our time. In 2017 about 425 million adults worldwide were affected with increasing tendencies. Ninety percent of the patients suffer from type 2 diabetes (T2D) which usually develops later in

life. Type 1 diabetes (T1D) can already start at a young age and currently affects more than 1.1 million children (Diabetes Facts & Figures 2019). T1D is an autoimmune disease where immune cells attack and destroy pancreatic β cells. These cells are responsible for the production of insulin in response to dietary glucose. Insulin enables cells, especially those of the muscles, to take up glucose for energy generation. If insulin is missing, blood glucose levels increase dangerously high which acutely can cause a diabetic coma. In the long term, elevated glucose levels can harm almost every organ, leading to cardiovascular problems, retinopathy, nephropathy, and neuropathy. In T2D the pancreas still produces insulin, but the peripheral tissues become resistant toward it, which means that insulin loses its ability to channel glucose into the cells. Other insulin-associated functions, especially its promoting effect on fat storage, remain intact. Over time, the pancreas becomes exhausted in T2D and stops producing insulin similar to T1D. While T1D always needs treatment with insulin injections, only later stages of T2D depend on injections (Kharroubi and Darwish 2015).

From the 145 stem cell-based clinical studies for diabetes listed in August 2019 at https://clinicaltrials.gov/, about one third deal with T2D, while the majority has been designed for T1D. Several approaches for stem cell therapy of diabetes have been tested. They are based either on the concept of replacing nonfunctional β cells by stem cell-derived ones or on exploiting the capacities of MSCs or HSCs to act as immunomodulators and improve the regeneration of lost cells. In view of this, MSCs are able to suppress T-cell responses and inhibit differentiation of dendritic cells as well as B-cell proliferation. They can stimulate the production of anti-inflammatory cytokines and suppress that of pro-inflammatory cytokines, as well as of reactive oxygen species. In addition, they can improve insulin signal transduction, while HSCs stimulate angiogenesis in the damaged islets as well as regeneration of endothelial progenitor cells (Sneddon et al. 2018).

Clinical trials for T1D and T2D usually employ MSCs from different sources or BM-derived mononuclear cells/HSCs either alone or in combination by intravenous injection or injection into the pancreatic artery. All clinical trials so far proved safety of the procedure and also showed limited therapeutic effects of the treatment. However, follow-up periods usually were no longer than 12 months, so we lack information on long-term success, and also no systematic study has been performed so far to determine which stem cell source and which mode of application are the best (Sneddon et al. 2018).

MSCs also have a capacity for in vitro differentiation into islet-like insulin-producing cells. These differentiated cells have been successfully used in clinical trials, although it is not yet clear whether they really replaced damaged β cells or if the positive effects were again due to immunomodulatory effects of MSCs (Cho et al. 2018).

In order to replace non-functioning β cells, novel techniques based on ESCs have been developed. To this end, ESCs are in vitro differentiated into insulin-producing β-like cells. Unfortunately, these cells are less efficient in insulin synthesis than primary β cells, which is probably due to the missing 3D structure of the organ, which provides a dedicated niche for β cells. Therefore, differentiation protocols are currently being developed which take also the 3D structure into account (Sneddon et al. 2018).

Besides cell maturation/function, cell therapy of the pancreas faces two main challenges which even hamper treatment with adult pancreatic cells. First, the transplanted cells home-in inefficiently into the pancreas, and second, they are frequently attacked by the host's immune system. To overcome both issues, several companies have developed encapsulation devices made of, e.g., alginate, polytetrafluoroethylene, silicone, or polycaprolactone. These devices can be transplanted either intraperitoneally or subcutaneously. Ideally, they provide sufficient blood and oxygen supply which ensures proper function of the encapsulated cells and allow the secreted insulin to exit the device. In addition, they protect the transplanted cells from the host's immune system and in the worst case prevent the escape of tumorigenic cells (Sneddon et al. 2018). One clinical phase I/II trial has been conducted by Sernova

Corp. employing isolated islet cells in a pouch (NCT01652911). The method proved to be safe. Six weeks after transplantation, the pouches were explanted, and histological analysis demonstrated that islets retained their macrostructure and were connected to new blood vessels. However, the end goal of long-term insulin independence of the patients could not be achieved. Another phase I/II clinical trial which is running until 2021 is currently conducted by ViaCyte to test safety and efficacy for a device loaded with ESC-derived insulin-producing β-like cells (NCT02239354). Preliminary results indicate that the devices are well-tolerated and that insulin-producing cells persist up to 2 years after transplantation (Pullen 2018).

Another stem cell-based technique tested for the treatment of diabetes is the so-called stem cell educator therapy. In this setting, mononuclear cells are separated from the patient's blood in a closed-loop cell separator. They are then educated to gain immune tolerance by briefly co-culturing them with adherent UCB stem cells. The educated mononuclear cells are reinfused into the patient (Zhao et al. 2013). A clinical phase I/II trial, performed by Tianhe Stem Cell Biotechnologies, demonstrated safety as well as efficacy of this treatment in T2D patients over up to 56 weeks (NCT01415726). Patients not only tolerated the treatment well but also had improved values for glycosylated hemoglobin (HbA1C), c-peptide, and homeostasis model assessment (HOMA) of insulin resistance. The company is now performing a phase II trial including T1D patients as well (NCT03390231) which is supposed to be completed by the end of 2020.

Liver Diseases

The liver is the central metabolic organ of the human body. It is involved in nutrient as well as in drug metabolism, and it produces a plethora of proteins which are secreted and have essential functions elsewhere in the body. The liver is a very complex organ. Eighty percent of its cells are hepatocytes which are responsible for the metabolic homeostasis. They store, release, and synthesize glucose and lipids according to the body's energy needs. They are involved in protein breakdown and detoxify ammonium ions in the urea cycle. The most important function of hepatocytes from a pharmacological point of view is the so-called first- and second-phase metabolism where drugs either get activated or detoxified by cytochrome P450 enzymes and afterward by conjugation with distinct cofactors (Stanger 2015).

There are mainly two indications for stem cell therapy in the liver. First, stem cells can be used to mitigate congenital metabolic defects. Second they are employed to restore liver tissue in the fibrotic/cirrhotic liver.

Several metabolic diseases are known where genetic defects of key enzymes either reduce the output of a specific pathway or impair this pathway completely. Usually, these are rare diseases, affecting less than 1 in 1500–2500 people, and they manifest early in childhood. Well-known examples are Crigler-Najjar syndrome or the various urea cycle defects. In Crigler-Najjar syndrome, patients lack the enzyme uridine diphosphate glucuronosyltransferase which is responsible for conjugating bilirubin – a degradation product of hemoglobin – and thus enabling its excretion. If unconjugated bilirubin accumulates in the body, it causes severe icterus which even affects the brain (kernicterus) leading to encephalopathy and thus triggering neurological damage and long-term cognitive defects. The current standard of care is a lifelong phototherapy for 10–12 h a day in order to convert unconjugated bilirubin with blue-light irradiation into a water-soluble form that can be excreted via the bile. Urea cycle defects result from mutations in any of the cycle's enzymes. This cycle is needed to detoxify ammonium ions which are generated during protein degradation and can, like unconjugated bilirubin, cause encephalopathy (Sokal 2014).

Two characteristics make metabolic diseases especially amenable for stem cell therapy. First, they are caused by an isolated mutation of one specific gene, and second, significant metabolic improvement can be obtained by replacing only 5% of total hepatocytes with healthy ones, while as few as 10% of healthy cells are expected to normalize liver function (Sokal 2014). From 2012

to 2014, a clinical phase I/II trial took place to evaluate the safety of a stem cell treatment in 6 pediatric Crigler-Najjar syndrome and 14 urea cycle disorder patients (NCT01765283) (Smets et al. 2019). Patients obtained transhepatic infusions with Heterologous Human Adult Liver-Derived Progenitor Cells (HepaStem), a product by the Belgian company Promethera. These stem cells are obtained by isolation and short-term cultivation of parenchymal liver cells which display a mesenchymal-like phenotype and preferentially differentiate into hepatocytes. They were administered in an immunosuppressive setting, and the safety could be proven in a 12-month follow-up period. In addition, a certain level of efficiency could also be shown. A proof-of-concept study in a 3-year-old girl suffering from a urea cycle disorder not only showed preliminary efficiency of the transplanted cells but also indicated that these cells were proliferating in the liver which means that maybe less cells are needed than initially expected (Sokal et al. 2014).

Currently, there are 20 clinical trials listed which employ stem cells to treat liver cirrhosis, while an additional 40 trials cover both cirrhosis and fibrosis (https://clinicaltrials.gov/, 5.8.2019). Most of these trials are conducted with autologous or allogeneic MSCs from various sources. All chronic liver diseases associated with inflammation (viral hepatitis, primary biliary cirrhosis, alcoholic and nonalcoholic fatty liver disease) can potentially progress via a fibrotic stage to the end stage of liver cirrhosis. In this condition, functional liver tissue is replaced by scars made of fibrotic material. The liver gets rigid and cannot perform its functional tasks properly anymore. In this phase, orthotopic liver transplantation is the only option to save the patient's life. However, there is a shortage of suitable donor organs, and researchers are working on cell therapies in order to temporarily improve the liver's function, enabling the patient to survive longer time periods before transplantation with the final goal to replace liver transplantation altogether. The first completed trials which all employed some kind of MSC obtained, e.g., from autologous fat tissue or BM indicate an overall safety of the procedure with limited success (Hu et al. 2019).

Kidney Diseases

The kidneys are a bean-shaped pair of organs, which are essential for removal of toxins and waste products from the blood, maintenance of fluid homoeostasis, secretion of hormones, and water reabsorption from urine. An adult kidney is composed of around one million nephrons, the functional units of the kidney.

Conditions such as diabetes and high blood pressure as well as genetic disorders are the main causes of chronic kidney disease (CKD). CKD is characterized by a gradual loss of renal functions which peaks in kidney failure at the worst outcome. In addition, acute kidney injury (AKI), which is defined as abrupt occurring kidney damage or failure, can cause renal diseases. Until now, kidney failure is only treatable with dialysis and finally organ transplantation. Transplantation is the only treatment which can recover kidney function, but it is accompanied with lifelong immunosuppression. Moreover, the organ demand is much higher than the available donor organs.

An alternative to organ transplantation and dialysis could be stem cell-based therapy. Researchers work on growing stem cell-derived organs in vitro, which may be used for transplantations and might cope with the high demand for donor organs. Another option may be injection of isolated stem cells into the affected region. It is proposed that these cells then differentiate into the required cell type within the organ.

One hereditary kidney disease is polycystic kidney disease (PKD). Characteristically, fluid-filled cysts form in the tubules of the kidney which increase the organ size and can spread to other organs. Damage in the kidney may result in end-stage renal disease and kidney failure. The autosomal dominant form of this disease (ADPKD) is caused by a mutation of the genes *polycystin* (*PKD*) *1* or *2*. The respective proteins are located in primary cilia and involved in calcium-dependent signalling pathways. Their dysfunction influences cell proliferation and structure

as well as fluid secretion (Igarashi and Somlo 2007). A clinical trial, performed in 2014, with a focus on ADPKD patients depended on the use of MSCs. It was hypothesized that an introduction of BM-MSCs can improve kidney function while acting anti-apoptotic, anti-fibrotic, and anti-inflammatory (NCT02166489). Six patients were chosen for this trial. 2×10^6 cells/kg autologous BM-MSCs were infused intravenously. The patients were observed afterward during a time span of 12 months. Overall, the procedure was safe for the patients, but the effects of the MSCs could not be evaluated due to the trial design (Makhlough et al. 2017).

Since the kidney contains a great variety of different cell types, this organ is especially challenging to model in vitro. Successful treatment of kidney diseases can only be obtained, when the affected or damaged cell types are recognized. Upon better understanding about the affected cell types in the different kinds of kidney diseases, more treatments and therapies will be established in the future.

Diseases of the Central Nervous System (CNS)

The central nervous system (CNS), composed of the brain, spinal cord, and retina, is one of the most complex and less understood systems in the human body. CNS disorders and injuries, among them neurodegenerative diseases, retinal degeneration, and spinal cord injury, not only cause devastating consequences for the patients and family members but are also a major economic burden.

Neurological disorders are complex and usually irreversible, partially due to the limited potential of endogenous regeneration of the CNS. With the lack of effective therapeutic approaches, stem cell-based therapy holds promising potential to treat CNS disorders.

Retinal Degradation

Retinal disorders were the first CNS conditions to be targeted with cell therapy. The retina comprises several neuronal layers, among them the photoreceptors (PRs) which convert the light inputs into signals that are then transmitted to the brain via the optic nerve. The PRs are in contact with a monolayer of retinal pigment epithelium (RPE), essential to maintain PR homeostasis. Degeneration of the PR due to their malfunction or due to degeneration of the RPE can lead to visual decline that ultimately ends up in blindness.

The leading causes of retina degeneration and blindness in Western countries are age-related macular degeneration (AMD) and retinitis pigmentosa. AMD can be subclassified into non-vascular AMD (dry form) and neovascular AMD (wet form). Another variant of this disease, which is not age-associated, is Stargardt macular degeneration. It is the most prevalent inherited macular dystrophy affecting both children and adults. While AMD is caused by the degeneration of the RPE, retinitis pigmentosa is caused by the degeneration of the PR and/or RPE. Retinitis pigmentosa is a group of heterogeneous inherited conditions with a prevalence of 1:4000. Since the PR and the RPE cannot be endogenously regenerated, stem cell therapy is a promising therapeutic alternative for treating these retinal degeneration conditions (Ben M'Barek and Monville 2019).

RPE and PR cells can be obtained from fetal and adult tissue, specifically from cadavers (allogeneic RPE) or from patients (autologous RPE) through a nasal surgery. The use of fetal tissue implies ethical concerns and showed variability related to the developmental state of the fetus, leading to the abandonment of this source. Adult RPE cells showed a huge limitation due to the low amount of cells that can be obtained, as well as the complications inherent to the surgery in case of autologous RPE isolation. With the development of efficient protocols to differentiate ESCs and iPSCs into specific retinal subtypes like RPE cells, retinal cell replacement is a promising target of CNS cell therapy, with multiple clinical trials ongoing (Goldman 2016).

The first clinical trials using ESC-derived RPEs to treat AMD and Stargardt disease were performed in 2012 (NCT01344993 and NCT013450060). The results of these studies provided evidence that ESC-derived RPEs were safe to treat these diseases. As a consequence, many other clinical trials emerged

placing retinal degeneration at the forefront of stem cell-based therapies. In 2014, the first clinical trial using autologous iPSC-derived RPEs was conducted at the RIKEN institute in Japan (UMIN000011929). However, it was aborted 1 year later due to safety concerns. There are currently three ongoing clinical trials using iPSC-derived RPEs, with more expected to be approved. The results from these trials will help to optimize the best formulation strategy to combat retinal degeneration (Ben M'Barek and Monville 2019; Schwartz et al. 2015).

Neurodegenerative Diseases

Neurodegenerative diseases are a heterogeneous group that can have a hereditary or a spontaneous origin, which all culminate in the loss of neurons or glia cells in the CNS. Stem cell-based therapy is emerging as a promising candidate to treat some of these diseases including Parkinson's disease (PD), Alzheimer's disease, Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), among others. Cell replacement therapy was already performed as a proof of concept in some of these conditions.

PD is the second most common degenerative disease of the CNS, affecting 1% of the population above 60 years (Tysnes and Storstein 2017). PD is characterized by the degeneration of the dopaminergic neurons (DAn) in the *substantia nigra pars compacta* (SNpc), causing the characteristic motor factors of PD like rigidity, bradykinesia, tremors, and postural instability, along with other non-motor symptoms. Another hallmark of PD is the presence of aggregated α-synuclein in the Lewy bodies and Lewy neurites in the substantia nigra. The loss of DAn is initially restricted to the SNpc, but it will progressively spread to other regions of the brain during the development of the disease. The exact etiology of PD remains unknown, and nowadays it is considered to be a multifactorial disease resulting from a complex interplay between genetic factors and environmental cues. Currently, the available pharmacological regiments for PD only treat the symptoms of the disease and not the underlying neurodegeneration process (Antony et al. 2013; Kalia and Lang 2015). Therefore, new therapeutic strategies are needed, with stem cell-based therapy being the most promising approach to slow or even stop the progression of this devastating disease. The first cell therapy trials for PD were performed more than 30 years ago, using fetal ventral mesencephalic cells (fVM), a source of DAn. These first trials showed long-term efficacy and survival. However, it has been reported that in some cases the transplanted cells exhibited disease-related pathology and there was a huge variability in the clinical response probably due to the variability of the cells transplanted (Barker et al. 2015). In order to standardize the approach of cell therapy for PD, TRANSEURO (NCT01898390), a European consortium conducting phase I clinical trials to investigate the benefits of transplantation of allogeneic dopaminergic neuroblasts derived from fVM into PD patients, was created.

High expectations for future PD treatments lie on emerging therapies based on hPSC-derived DAns. The preferential use of iPSCs over ESCs has been fostered by the systematic banking of HLA-matched iPSCs, avoiding the need for immunosuppression regime in the recipient. The development of good manufacturing practice (GMP)-grade protocols to obtain authentic and functional midbrain DAn cells from hPSCs lead to the creation of the global network initiative GForce-PD. This network encompasses different consortia like the European NeuroStemCellRepair and the NYSTEM-PD based on ESC-derived DAn and the Japanese Center for iPS Cell Research and Application (CiRA) based on allogeneic transplantation from HLA-matched iPSC-derived DAn cells. As part of the GForce-PD network, the first clinical trial using iPSC-derived DAns to treat PD started in August 2018 at Kyoto University by a team headed by Takahashi as part of the Japanese Haplobank CiRA (UMIN000033564). Furthermore, some clinical trials outside the GForce-PD have already started. The Chinese Academy of Sciences is using HLA-matched ESCs (NCT03119636), while an Australian Clinical trial (NCT02452723) sponsored by Cyto Therapeutics Pty Limited is based on human parthenogenetic stem cell-derived neural cells (Barker et al. 2019; Wenker and Pitossi 2019).

With more iPSC-derived cell therapies to treat PD already planned, the follow-up of these trials will likely be beneficial, not only for PD but also to develop new approaches for other neurodegenerative diseases.

ALS is a neurodegenerative disorder characterized by progressive degeneration of the upper and lower motor neurons. ALS has a devastating impact on the patient, and although the pathogenic basis is still unclear, stem cell-based therapy seems to have potential. The motor neuronal degeneration observed in ALS may arise from defective trophic support from astrocytes and microglia, whose replacement appears technically more feasible. Furthermore, the delivery of growth factors or extracellular vesicles might exert a neuroprotective effect.

Injection of human NSCs (NSI-566) originating from the spinal cord of an 8-week-old aborted fetus was performed in a phase I (NCT01348451) and phase II (NCT01730716) clinical trial. The results from these trials showed improved functional status scores of these patients, although survival did not differ from control groups. A phase 3 clinical trial is already planned (Goutman et al. 2018; Abati et al. 2019). Another trial using fetal tissue-derived NSCs in combination with gene therapy was approved in 2016. The human NSCs are engineered to produce Glial cell-derived neurotrophic factor (GDNF) (CNS10-NPC-GDNF) and injected in the spinal cord (NCT02943850). It is expected that these cells differentiate into neuroprotective astrocytes, increasing the survival of motor neuros in ALS patients. With more preclinical data suggesting that the malfunctioning of astrocytes is involved in ALS pathogenesis, a new clinical trial using ESC-derived astrocytes (NCT03482050) was approved in 2018. The results from these trials will give us insights about the safety and potential of PSCs in the treatment of ALS.

Beside these treatments, which are in the trial state, the Korean company Corestem has obtained approval for their product Neuronata-R in South Korea in 2014 after successful phase I and II clinical trials (NCT01363401). The treatment is based on repeated intrathecal injections of autologous BM-MSCs (Oh et al. 2015). In 2018 it also got permission as an orphan drug from the US FDA and EMA followed this classification in 2019.

Spinal Cord Injury (SCI)

SCI is one of the most devastating neurological injuries, including motor, sensory, and autonomic paralysis, with a prevalence (2016) of 27.04 million cases (GBD 2016 et al. 2019). SCI results from dislocation or fracture of the spine in the cervical of thoracic region and can be divided into two different phases, the acute and the chronic phases. In the acute phase, neurons and glia cells can undergo fast necrosis seconds to minutes after the injury (primary injury). The secondary injuries, depending on the extent of the primary injury, comprise the most critical phase. A cascade of destructive events occurs, including aberrant molecular signalling, inflammation, vascular changes, and secondary cellular dysfunctions. Weeks to months after the secondary injuries, the chronic phase takes place with scar formation as a consequence of the reactive gliolysis, formation of cysts, and gray matter demyelination (Cofano et al. 2019). Treatments for acute SCI are limited to the stabilization of the injured spine followed by rehabilitation in the chronic phase. Although some early medical treatments accounted for some significance recovery, there is no effective treatment.

The development of cell transplantation therapies to replace the damaged cells and to promote neural protection and regeneration of the injured spinal cord has shown great potential. A variety of different cell types have already been transplanted into injured spinal cord, in rodents and human, like olfactory ensheathing cells, oligodendrocyte precursor cells (OPCs), Schwann cells, NSCs derived from fetal tissue, and MSCs. In particular, from the 18 clinical trials conducted, 16 were based on MSCs. The big disadvantage of using MSCs for transplantation is their restricted differentiation potential. To overcome this, the first clinical trial using ESC-derived OPCs was approved in 2010 (NCT01217008). This study was terminated based on financial issues, and a second clinical trial was approved in 2015 (NCT02302157). This is the only PSCs-based clinical trial currently active, and the results are

expected to be published soon (Csobonyeiova et al. 2019; Pereira et al. 2019). Recently the beneficial effects of iPSC-derived NSCs transplantation for tissue recovery were shown. After transplantation these cells can differentiate into neurons and oligodendrocytes, myelinate the host axons, and secrete neurotrophic factors to prevent the secondary damage. In February 2019, the first clinical trial using allogenic iPSC-derived NSCs was approved. The study will be performed on four patients at the Keio University School of Medicine by a research team headed by Prof. Hideyuki Okano (Nagoshi et al. 2019; Tsuji et al. 2019)

Future Perspectives in Stem Cell-Based Therapy of the CNS

Although some neurodegenerative diseases share pathological mechanisms such as protein aggregation, misfolded proteins, and loss of local neurons, the underlying molecular mechanisms that lead to the CNS's progressive degeneration are unknown. There are still considerable problems in treating these diseases, and so far they do not make good targets for stem cell therapy, especially because the neurodegeneration is too widespread, diffuse, and currently irreversible. Alzheimer's disease, the most common and devastating form of dementia, is so far not a target for stem cell-based replacement therapy due to its high complexity, heterogeneity, and unclear pathogenesis. Other diseases are still not a target of stem cell-based clinical trials, but preclinical studies have shown the potential of such therapy. Huntington's disease(HD) is a fatal autosomal-dominant disease characterized by motor dysfunction, cognitive impairment, and psychiatric disturbances due to neurodegeneration. HD is caused by a mutation of the huntingtin (HTT) gene, and stem-cell based therapy is a potential strategy to restore neuronal function, replacing the lost neurons, and provide neurotrophic support. Several clinical trials using human fetal tissue to treat HD have been performed since 1990. The results from these very heterogeneous trials indicated that fetal transplantation in HD is safe but the clinical improvement did not last more than 4–6 years after transplantation. Finding an alternative to the fetal striatal graft transplantation is being a focus of research, with a set of preclinical studies based on PSCs ongoing based on PSCs. The results from these studies using PSC-based therapy are largely inconsistent. Thus, more preclinical work is necessary to confirm if PSCs have a therapeutic potential to treat HD.

Another disease where stem cell-based therapy is a potential target is ischemic stroke, an acute cerebrovascular disease caused by a decreased or interrupted blood supply in the brain. Ischemic stroke destroys a heterogeneous population of brain cells, making cell replacement therapy a difficult approach since neurons and glia cells that should be replaced are affected. Therefore, most clinical trials shifted to use MSCs in order to suppress the post-ischemic inflammatory response. The results from these clinical trials showed an improvement of the neurological symptoms in the patients. Although MSCs are an attractive and promising therapy, it is not a cell replacement therapy. The UK started a clinical trial already in phase II (NCT03629275) where an immortalized human NSC line (CTX0E03) is being used. Although (HLA-matched) iPSCs are still considered as a potential source for stem cell therapy to treat stroke, where the results from preclinical studies have been encouraging, additional studies and improvement in the strategies for cell replacement therapy are required.

Summary

A tremendous number of stem cell-based clinical trials (also for organs which we could not mention in this book chapter) are currently underway. Many of these focus on MSCs which can be applied in a wide variety of settings, to support regeneration, modulate the immune response, and maybe even restore damaged tissue (Fig. 4). As they do not have to be applied in an HLA-matched manner, allogeneic transplantation is facilitated. Cells differentiated from PSCs are currently emerging as treatment options for various diseases. Here, the hurdles for transplantation are higher because the

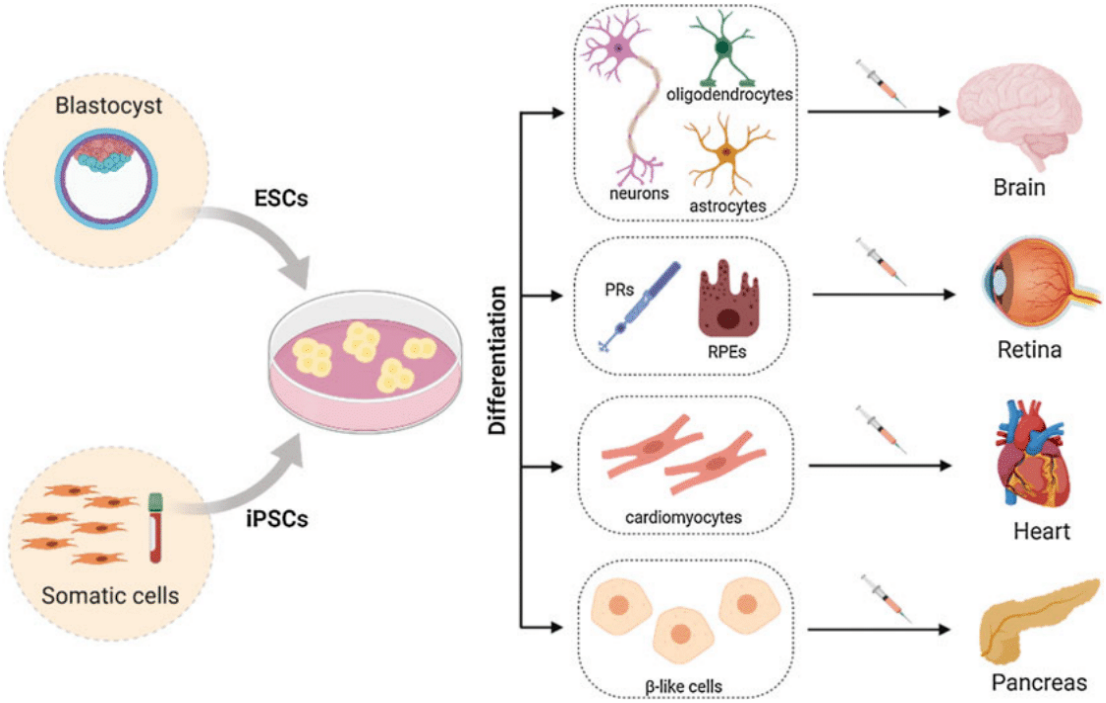

Fig. 4 Overview of the ongoing PSC-based clinical trials. PSCs isolated from the blastocyst (ESCs) or reprogrammed from somatic cells (iPSCs) are differentiated in vitro to generate neurons, oligodendrocytes, and astrocytes to treat central nervous system disorders, PRs and RPEs to treat retinal degenerative diseases, cardiomyocytes to treat heart diseases, and β-like cells to treat type 1 diabetes. After differentiation the cells are transplanted into the brain, retina, heart, or pancreas from the donor. Figure made in ©BioRender – biorender.com

possibility of immune rejection requires HLA matching and the tumorigenic potential of PSCs needs to be controlled.

While HSC and keratinocyte transplantation have been in the clinic for a long time, only selected MSC-based treatments have been approved in a limited number of countries. It will probably take much longer until PSC-based therapies will be available to a broad spectrum of patients. These novel treatments will need a close surveillance until we have fully understood their risks and benefits.

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III. Oral Presentations

Lisa Nguyen, Wasco Wruck, Lars Erichsen, Nina Graffmann and James Adjaye. Puromycin Aminonucleoside induces kidney injury in iPSC-derived kidney organoids. EUSAAT Conference in Linz, Austria, 2022.

IV. Poster Presentations

i. **Lisa Nguyen**, Wasco Wruck, Lars Erichsen, Nina Graffmann and James Adjaye**.** Puromycin-Aminonucleoside induces kidney injury in iPSC-derived kidney organoids. 13th internal Meeting Stem Cell Network NRW in Herne, Germany, 2022.

ii. **Lisa Nguyen**, Martina Bohndorf, Lucas-Sebastian Spitzhorn and James Adjaye. Modelling nephrogenesis in a 3D-culture system using human urine derived renal progenitor cells. ACTC Conference in Cardiff, Wales, 2019.

Eidesstaatliche Versicherung

Ich, Thien Dieu Lisa Nguyen, versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Ort, Datum: ..... Unterschrift: .....

(Thien Dieu Lisa Nguyen)