Generation of vascularized 3-dimensional (3D) kidney organoids from human pluripotent stem cells

Low, Jian Hui

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**GENERATION OF VASCULARIZED 3-DIMENSIONAL (3D)
KIDNEY ORGANOIDS FROM HUMAN PLURIPOTENT
STEM CELLS**

LOW JIAN HUI

LEE KONG CHIAN SCHOOL OF MEDICINE

2020

**GENERATION OF VASCULARIZED 3-DIMENSIONAL (3D)
KIDNEY ORGANOIDS FROM HUMAN PLURIPOTENT
STEM CELLS**

LOW JIAN HUI

Lee Kong Chian School of Medicine

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution

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Low Jian Hui

Supervisor Declaration Statement

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26th July 2020

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Xia Yun

Authorship Attribution Statement

This thesis contains materials from 1 paper published in the following peer-reviewed journal where I was first and/or corresponding author.

Chapter 3 is published as Low JH, Li P, Chew EGY, Zhou BR, Suzuki K, Zhang T, Lian MM, Liu M, Aizawa E, Esteban CR, Yong KSM, Chen Q, Campistol JM, Fang M, Khor CC, Foo JN, Izpisua Belmonte JC, Xia Y (2019). Generation of Human PSC-Derived Kidney Organoids with Patterned Nephron Segments and a De Novo Vascular Network. Cell Stem Cell. 25:1-15. DOI: 10.1016/j.stem.2019.06.009.

The contributions of the co-authors are as follows:

- I conceptualized the flow of the project, assisted in manuscript preparation, performed the experiments, collected, and analysed the data.
- Dr Li Pin, Dr Zhou Bingrui, Miss Zhang Tian, Mr Liu Meng provided valuable scientific and technical inputs, performed the experiments, collected, and analysed the data.
- Dr Chew Guo Yan Elaine, Miss Michelle Lian Mulan and Asst Prof Foo Jia Nee performed and analysed the single cell transcriptomics experiments.
- Dr Keiichiro Suzuki and Dr Emi Aizawa performed gene correction on the ARPKD patient-derived induced pluripotent stem cells.
- Dr Kylie Yong Su Mei and Dr Chen Qingfeng provided the human fetal kidney samples and performed flow cytometry experiments.
- Dr Chiea Chuen Khor, Asst Prof Fang Mingliang, Dr Concepcion Rodriguez Esteban and Dr Josep M Campistol, Prof Juan Carlos Izpisua Belmonte provided the broad project directions and assisted in data interpretation.
- Asst Prof Xia Yun wrote the manuscript, provided the broad project direction, supervised the planning and execution of the experiments and data analysis.

26th July 2020

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Date

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Low Jian Hui

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Abbreviations

3D	Three-dimensional
ABC	Adenosine triphosphate-binding cassette transporter (gene)
ACE	Anterior chamber of the eye
ADH	Anti-diuretic hormone
APS	Anterior primitive streak
AQP	Aquaporin (gene)
BEAM	Branched expression analysis modelling
BMP	Bone morphogenetic protein (gene)
BSA	Bovine serum albumin
CAKUT	Congenital anomalies of the kidney and urinary tract
CAM	Chorioallantoic membrane
CD	Cluster of differentiation (gene)
CDH	Cadherin (gene)
CK8	Cytokeratin 8 (gene), also known as KRT8
CKD	Chronic kidney disease
CRISPR	Clustered regularly interspaced short palindromic repeats
CSB	Comma shaped body
DALYs	Disability adjusted life years
DBA	Dolichos biflorus agglutinin
DEG	Differentially expressed genes
DMEM/F12	Dulbecco's modified eagle media and Ham's F-12 media
DMSO	Dimethyl sulfoxide
E(number)	Embryonic age of mice

EB	Embryoid body
ELISA	Enzyme-linked immunosorbent assay
ESRD	End stage renal disease
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDR	False discovery rate
FGF	Fibroblast growth factor (gene)
FITC	Fluorescein isothiocyanate
FOX	Forkhead box (gene)
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
GBM	Glomerular basement membrane
GDNF	Glial-derived neurotropic factor (gene)
GO	Gene ontology
GWAS	Genome-wide association studies
hESC	Human embryonic stem cell
HGF	Hepatocyte growth factor
HTS	High-throughput screening
hPSC	Human pluripotent stem cell
IACUC	Institutional Animal Care and Use Committee
IM	Intermediate mesoderm
iPSC	Induced pluripotent stem cell
JAG1	Jagged 1 (gene)
kDa	Kilo-dalton
KDR	Kinase domain receptor (gene), also known as VEGFR2
KOSR	Knock out serum replacement

LMN1	Laminin (gene)
LHX1	LIM/ homeobox protein (gene)
LTL	Lotus tetranogolobus lectin
LPM	Lateral plate mesoderm
MEIS1	Meis homeobox 1 (gene)
MET	Mesenchyme-to-epithelial transition
MIXL1	Mix paired-like homeobox 1 (gene)
MM	Metanephric mesenchyme
NEAA	Non-essential amino acids
NICD	Notch intracellular domain
NPC	Nephron progenitor cells
NPHS1	Nephrin (gene)
NPHS2	Podocin (gene)
NSG	Nod scid gamma (mouse)
OAT	Organic anion transporter (gene)
OSR1	Odd-skipped-related 1 (gene)
PAX	Paired box (gene)
PBS	Phosphate buffered saline
PDGFR	Platelet-derived growth factor receptor (gene)
PFA	Paraformaldehyde
PC	Principal component
PD	Peritoneal dialysis
PKD	Polycystic kidney disease
PKHD1	Polycystic kidney and hepatic disease 1 (gene)
PODXL	Podocalyxin (gene)

PS	Primitive streak
PTA	Pre-tubular aggregate
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RA	Retinoic acid
RAAS	Renin angiotensin aldosterone system
RNA	Ribonucleic acid
RRT	Renal replacement therapy
RV	Renal vesicles
SALL1	Sal-like protein 1 (gene)
scRNA-seq	Single-cell RNA-sequencing
SEM	Scanning electron microscopy
SIX	Sine oculis homeobox (gene)
SLC	Solute carrier family (gene)
SSB	S-shaped body
TEM	Transmission electron microscopy
TeSR	Tenneille's secret recipe
TGF β	Transforming growth factor beta
tSNE	t-distributed stochastic neighbour embedding
UB	Ureteric bud
UMOD	Uromodulin (gene)
VEGF	Vascular endothelial growth factors (gene)
WT1	Wilms Tumour (gene)
XEN	Extra-embryonic endoderm

Summary

Our kidneys work continuously by regulating fluid homeostasis in our body. However, when they fail to function, due to age, disease or injury, we develop chronic kidney disease (CKD). The heavy health and economic implications of CKD were reflected in The Global Burden of Disease study, which reported that CKD claimed 1.2 million lives and 38.8 million disability-adjusted life-years (DALYs) in 2017 alone. Attributing to an aging population worldwide, these numbers are projected to increase, and will impose a heavy burden on economies and healthcare systems. Despite these consequences, renal replacement options for CKD, including both forms of dialysis, remains largely unimproved since their inception 70 years ago, and do not fully replace the function of a kidney. Kidney transplantations offers the most favourable treatment outcome, but there is a growing imbalance between donors and patients on waiting lists. Two emerging technologies – cellular reprogramming and directed differentiation, has rekindled the field of organ engineering. Kidney organoids are three-dimensional cellular aggregates that harbour multiple kidney cell types, recapitulate rudimentary features of the kidney, and can be generated from human pluripotent stem cells (hPSCs). In the past 5 years, many studies have established novel differentiation methodologies to generate these kidney organoids and utilize them to investigate kidney development and disease *in vitro*. However, as this technology is at its infancy, several challenges, which include a lack of control over differentiating cell types, and glomerular vascularization, have hampered the translation of these technology for medical applications.

I begin this thesis with a literature review of kidney function and its physiopathology. In **Chapter 1**, I give an introduction to the kidneys, discuss the current situation of chronic kidney disease, treatment methods and why it is difficult to replace kidney function. Then, I discuss the current progresses in kidney development and the signalling pathways governing the

specification of each intermediate stages. I discuss the key progenitor populations of the kidney, and how reciprocal interaction initiates kidney development. Next, I describe the establishment of hPSCs and organoid-based directed differentiation approaches to derive kidney-specific cells and tissues by harnessing decades of information uncovered from studying kidney development in model organisms. **Chapter 2** details the materials and methods in order to allow reproducibility in other laboratories. Using these knowledge and techniques, I developed an improved protocol to generate vascularized 3-dimensional kidney organoids from hPSCs in **Chapter 3**. I focused my efforts on trying to achieve a high efficiency of nephron progenitor cells induction, in order to obtain kidney organoids that contain a large number of nephrons. Both the differentiation intermediates and the vascularized kidney organoids were extensively characterized and shown to be reflective of the events going on during *in vivo* development. The functionality of these kidney organoids was also assessed by their ability for size selective dextran and albumin uptake. I took advantage of this highly efficient and robust protocol, to investigate the mechanisms underpinning vascular formation in kidney organoids. I confirmed that VEGF-A derived from the nascent podocytes induced vascularization in a paracrine manner. The origin of these vascular progenitors was found to be from a subpopulation of nephron progenitor cells at around day 11 of differentiation. These findings were confirmed using single cell RNA-seq and bioinformatics analysis. These hPSC-derived organoids were utilized to study early nephron patterning events. I developed an approach and elucidated the critical window to modulate the different cell types within kidney organoids using a single molecule. This approach, which recapitulates nephron patterning, enables us to adjust the amount of proximal-to-distal nephron segments and vasculature within kidney organoids. I explored the *in vivo* potential of these hPSC-derived kidney by implanting them underneath the renal capsule of immunodeficient hosts. There, kidney organoids undergo rapid engraftment, with the formation of new blood vessels. Histological, immunofluorescence and

ultrastructural analysis identified structural and functional maturation features that would otherwise be unachieved with *in vitro* approaches. Implanted kidney organoids demonstrated rudimentary functionality with size-specific dextran handling and the formation of urinary filtrate. The discussion of our results was summarized in **Chapter 4**, where I discuss the potential implications and limitations of our discoveries. I conclude the thesis and discuss potential future works in **Chapter 5**. Altogether, I believe these findings will help advance the field of renal regenerative medicine, as we inch closer to growing functional tissues in-a-dish.

1. Introduction

1.1. Kidney structure and function

The kidneys are structurally complex organs that play an important role in regulating bodily fluid homeostasis and excretion of waste metabolites. In an adult human, each kidney is about 11 centimetres in length, or about the size of a clenched fist. Despite their relatively small size, our kidneys receive about 20% of the overall cardiac output (Boron and Boulpaep, 2012). They filter the blood plasma, retaining large proteins while allowing the passage of small molecules *via* ultrafiltration. About 180 litres of ultrafiltrate is generated a day, but more than 99% is recovered after passing through the tubules and collecting duct (Kaufman et al., 2020). Ultrafiltration and toxin clearance is the main function of the kidneys and can be measured as the glomerular filtration rate (GFR). The GFR is hence clinically utilized as a measure of kidney function. The kidneys also have a myriad of other functions. For example, it regulates blood pressure by the renin-angiotensin aldosterone system (RAAS). For hormonal functions, it secretes erythropoietin and activates vitamin D into calcitriol, regulating erythropoiesis and calcium homeostasis respectively. The kidneys also play an indispensable role in regulating acid-base imbalances by a multitude of mechanisms, such as phosphate excretion and bicarbonate reabsorption (Katz and Lindheimer, 1977; McDonald et al., 1976; Robson, 2014).

1.2. Nephrons – the workhorse of the kidney

The functional unit of the kidney is the nephron, which contains more than a dozen cell types organized into a filtration apparatus called the glomerulus and a series of contiguous tubules (Figure 1.1 a). In a healthy adult human, each kidney is composed of approximately $0.2 - 1.8 \times 10^6$ nephrons (Brenner and Mackenzie, 1997; Hughson et al., 2003).

At the proximal region, a glomerulus comprised highly specialized cells called podocytes surround a bundle of blood vessels called the capillary tufts. Podocytes are unique epithelial cells characterized by numerous cellular projections called “foot processes”. These branching foot processes interdigitate extensively with neighbouring podocytes, forming an array of narrow gaps called the slit diaphragm on the basolateral side of the glomerular basement membrane (GBM). On the adjacent surface side of the GBM are endothelial cells, which are highly fenestrated and glycosylated. Fenestrae are large pores within the endothelial cell body and allows the blood plasma to come in contact the GBM (Figure 1.1c, d). Extensive glycosylation on the surface of the blood vessels confers a negative charge that repels essential proteins such as albumin (Singh et al., 2007). Together, the podocyte slit diaphragm, GBM and endothelial fenestrations forms the filtration barrier, which selectively allows the bypass of specific waste products while retaining essential molecules (Quaggin and Kreidberg, 2008).

Rudimentary filtrate generated by the filtration barrier is collected by the Bowman’s capsule. This “cup”-shaped epithelial structure channels the filtrate into the proximal tubules (Figure 1.1 b, c). Armed with a battery of both basolateral and apical transporters, these cells reabsorb majority of the filtrate including water, glucose, amino acids, and ions. This process continues in a lesser but significant extent in the medial and distal segments, which ultimately adjusts ions and water reabsorption based on the current water and salt composition in the plasma. Importantly, each tubular segment has a distinct collection of solute transporters which mediates its activity and solute selectivity (Carone et al., 1979). These specific transporters have also served as important biomarkers for identification of tubular identity and segmentation (Fassett et al., 2011; Takasato et al., 2015; Tramonti and Kanwar, 2013). Finally, the filtrate is led to the collecting duct, a branching network which consolidates the filtrate towards the bladder for disposal.

1.3. Collecting ducts – final touches to the filtrate

The collect duct is a converging epithelial network that connects the entire milieu of nephrons to the renal pelvis (Figure 1.1 a, b). Apart from being a plumbing network that drains the kidneys, the collecting duct is also an important site for urine concentration and osmolarity adjustments (Kokko, 1987).

Two main types of cells make up the entirety of the collecting duct system – principal cells (PCs) and intercalated cells (ICs). PCs are also called “light cells” as they are paler than intercalated cells when stained, allowing them to be readily distinguished from the darker ICs. More than two thirds of the epithelial population in the collecting ducts are PCs, and their main role is to modulate water uptake via sodium and potassium transport. These cells execute the function of RAAS and sympathetic systems by responding to aldosterone and vasopressin, regulating the expression of various transporter proteins such as Na^+/K^+ -ATPase pumps and aquaporins respectively (PhD, 2016). In turn, these transporters concentrate or dilute the urine according to the volume status of the body. The remaining one third of cells comprises of ICs, which regulate pH and acid-base balance by H^+ secretion and HCO_3^- reabsorption (Pearce et al., 2015; Stokes, 1990). Three main types of ICs play complementary roles to achieve this balance. Type A ICs secrete H^+ and reabsorbs HCO_3^- , while type B ICs secrete HCO_3^- and reabsorbs H^+ (Roy et al., 2015). The third type of IC, termed as non-A, non-B IC secrete both H^+ and HCO_3^- (Kim et al., 2002; Wall et al., 2003). Together, these mechanisms concentrate urine and modulates acid-base balances in the blood plasma to ensure fluid homeostasis in the body.

Figure 1.1 Microanatomy of the kidney

(a) Illustration of a human kidney. (b) Illustration of a single nephron. (c) Illustration of a glomerulus. (d) Illustration of the glomerular basement membrane. Images were adapted from (“12. Glomerular Filtration Barrier - Talbot Flashcards,” n.d.; “Glomerular capillaries,” n.d.; “Kidneys, Bladder and Prostate Scans,” n.d.; Biga et al., 2019).

1.3. Kidney disease – epidemiology and therapeutic interventions

1.3.1. Chronic kidney disease

Chronic kidney disease is characterized by the progressive loss of GFR and loss of renal function. The prevalence of CKD is increasing worldwide, and is currently estimated to affect about 13% of the entire population (Hill et al., 2016; Saran et al., 2017). Clinically, CKD is diagnosed when patients exhibit a GFR of < 60 mL/min for more than 3 months (Kyte et al.,

2018; Weckmann et al., 2018). Additional parameters such as albuminuria, renal ultrasound, blood chemistry analysis or kidney biopsy may be evaluated to confirm the diagnosis. End-stage renal disease (ESRD), which is the terminal form of CKD is diagnosed when the patient has a GFR of < 15 mL/min. ESRD is estimated to affect up to 1% of the global health population, and up to a half a million adults in the United States alone (Acosta-Ochoa et al., 2019; Benjamin and Lappin, 2020).

Kidney failure is a consequence of genetic and metabolic diseases, including but not limited to diabetes, high blood pressure and obesity (Gansevoort et al., 2013). These comorbidities cause vascular damage in the kidney which ultimately impairs kidney function and CKD. High blood pressure puts chronic strain on the renal capillaries, causing reduced glomerular mass, endothelial dysfunction and complications arising from a maladaptive RAAS system (Ku et al., 2019). In diabetic CKD patients, hyperglycemia causes peripheral vascular damage which is accompanied by mesangial expansion, basement membrane thickening and podocyte effacement (Reidy et al., 2014). Hypertriglyceridemia caused by a chronic high fat diet and sedentary lifestyle leads to the formation of atherogenic particles, altered vascular tone and inflammation (Bulbul et al., 2018; Florens et al., 2016). Together, these changes inflict oxidative stress and vascular damage in the renal arteries and microcapillaries, driving CKD progression. Conversely, the most common cause of death in CKD patients arises from cardiovascular complications. Fluctuations in blood pressure and persistent hypertension, confounded by the effects of dialysis, inflicts up to a 30-fold increase in cardiovascular mortality risk compared to healthy individuals (Fujii et al., 2019; Go et al., 2004). Hence, apart from administering RRT, an important aspect of treating CKD and ESRD patients is to mitigate the underlying cause of disease and slow the rate of kidney disease progression.

1.3.2. Congenital kidney disease

Many genetic mutations may influence the pre-natal development of the urogenital systems, causing congenital abnormalities of the kidney and urinary tract (CAKUT). About 1 out of 500 infants are born with CAKUT and may represent up to 30% of birth anomalies (Schedl, 2007). CAKUT present as a diverse range of clinical presentation – including renal agenesis, hyplasia, ureteric branching defects, undifferentiated blastema or polycystic kidney diseases. Depending on the target gene and penetrance of the mutation, CAKUT presents with varying severity. A perturbation occurring early in gestation may result in a more severe phenotype, such as kidney agenesis, whereas a perturbation later in gestation may be less severe. Genes implicated in key kidney development pathways, such as those along the glial-derived neurotropic factor (GDNF)-RET axis, may have the most severe phenotype (Davis et al., 2014; Jain, 2009). Wilm's tumour, caused by neoplasm of poorly differentiated mesenchyme and usually presented as sporadic tumours, may arise due to mutations in the Wilms tumour (WT1) gene. Depending on the severity and size of the tumour, surgical removal of the primary tumour may enable the patient to carry on with a normal life. Genes implicated in CAKUT are not restricted to kidney development, as mutations in cilia-associated genes such as *PKD1*, *PKD2* and *PKHD1*. Kidneys with these mutations harbour numerous cysts that originate from the nephrons or collecting ducts (Colbert et al., 2020; Igarashi and Somlo, 2007). Newborns that have polycystic kidney disease (PKD) also suffer from cysts formation in other organs, such as the lung and liver (Kaimori and Germino, 2008).

Many infants with CAKUT cannot survive postnatally without RRT (Hiraoka, 2003; Murugapoopathy and Gupta, 2020). In less severe cases, the infant can survive on limited kidney function but later develops CKD in adulthood due to a low starting nephron number. Hence, early identification of CAKUT, together with pre-emptive measures to halt to progression to CKD are essential in maintaining the patient's quality of life.

1.3.3. Renal replacement therapies

Current treatment methods for CKD are largely focusing on renal functional replacement, blood pressure management and mitigation of associated syndromes. The overarching aim of these therapeutic interventions aim to maintain overall fluid homeostasis, remove waste metabolites and delay progression to ESRD. Significant lifestyle modifications and dietary restrictions will be imposed in order to slow down the rate of renal decline. These may include adhering to a strict low salt, phosphorus and protein diet (Benjamin and Lappin, 2020).

Three key treatment methods, including peritoneal dialysis, hemodialysis and kidney transplantation were invented over 70 years ago and remains as the only few options in the mitigation of CKD today. According to the 2016 US Renal Data System Annual Data Report, 9.6% of all cases began treatment with peritoneal dialysis (PD) and 87.9% with hemodialysis. Up to 2.6% of patients managed to receive a kidney transplant as a prophylactic measure before the CKD progressed further (Saran et al., 2017).

1.3.3.1. Peritoneal dialysis

For patients with less severe forms of CKD, the first line of treatment is PD. During PD, an isotonic dialysate, which is comprised of deionized water containing sodium bicarbonate, sodium chloride and acid concentrate, is injected into the peritoneal cavity. The peritoneum capillaries and membrane is exploited as a natural semipermeable membrane, where metabolic waste such as urea and creatinine are removed *via* diffusion into the dialysate (Nolph, 2013; Ronco et al., 2009).

The PD process begins with the surgical implantation of a catheter with one end in the abdomen and another end protruding the skin. Up to three liters of dialysate is introduced into the abdomen cavity at once. This fluid, also referred to as a “dwell”, remains in the body for 4 to

6 hours before it is discarded. This process may be repeated once daily, or continually, depending on the severity of CKD.

While this approach is not considered to be the most invasive, it requires discipline and technicality from patients. Infections are often encountered, due to the fluid transfer process and the introduction of a foreign substance into the body. While the patient may resume with their daily activities, the large pressure within the abdomen may cause discomfort, lower back pain, or hernia.

1.3.3.2. Hemodialysis

Hemodialysis, is the process of purifying the blood plasma of an affected patient by extracorporeal removal of metabolic waste products. The process starts by gaining access to the blood through the use of an intravenous catheter. Long-term constructs such as an arteriovenous fistula or a synthetic graft could be engineered to increase blood supply and enable repeated access.

Blood is drawn out through a tube at a rate of around 200 to 400 mL per minute into a hemodialysis machine. In the machine, blood flow from one direction meets the dialysate flowing in the opposite direction, creating a counter current flow. This mechanism establishes a concentration gradient across the membrane and enable the diffusion of solute across a semi-permeable membrane. By tuning the hydrostatic pressure of the dialysate, it is possible to regulate the amount of fluid removal from the body. Minerals of bicarbonates may also be directly supplemented to alter the composition of the plasma.

A standard hemodialysis regime may take as long as three to four hours at a specialized facility with up to three sessions a week (Susantitaphong et al., 2012). There are also options to perform daily dialysis or nocturnal dialysis at home, which are considered to be gentler forms of dialysis but take longer periods of time. Quality of life is heavily compromised, due to time and energy

commitment to therapy and the stress due to the high cost of treatment. Surprisingly, hemodialysis was not found to be associated with marked improvements in survival rates, despite significant loss of quality of life (Ranganathan and John, 2012). Despite its drawbacks, hemodialysis is currently regarded as the most optimal option for patients on a waiting list for a kidney.

1.3.3.3. Transplantation

A kidney transplantation from a living or cadaveric donor offers the most favourable transplantation outcome, by taking on the responsibility of plasma filtration from the diseased kidneys. Sources of these kidneys may include a living donor who is a genetically compatible family member, or from cadaveric kidneys. Nonetheless, there are multiple limitations with this approach. Firstly, this is considered as a highly invasive surgery, with the risk of complications increasing with age, obesity and other underlying symptoms (Pham et al., 2010; Segev et al., 2008). Secondly, the recipient must be on lifelong immunosuppressants to prevent organ rejections and maintain the survival of the graft. Low rejections rates have since been reported but this is accompanied with higher incidence of cancer and infections (Kalluri and Hardinger, 2012; Lam et al., 2015). Lastly, the growing imbalance between the number of donors and patients on a waiting list delays the availability and accessibility of treatment. Alarmingly, only about 0.7% of cadaveric kidneys were accepted for transplantation due to age, obesity, diabetic or underlying disease of the donors (Davis and Cooper, 2019). Hence, there is an urgent and unmet need for alternative therapeutic options, or an alternative source of kidneys.

1.4. Mammalian kidney development

Kidney morphogenesis is a subject of intensive investigation for more than half a century, with the overarching goal of recreating this process *in vitro*. It is now understood that kidney

development entails a reciprocal interaction between the metanephric mesenchyme (MM) and the ureteric bud (UB). Both populations are derived from the intermediate mesoderm (IM). Together, these two multipotent populations give rise to the main bulk of cells that make up the definitive kidney. The UB generates the collecting duct tree and ureter, eventually leading to the bladder from a single duct, and the MM forms the main body of the nephrons, ranging from glomeruli, proximal tubule, loop of Henle, and distal tubules (Auerbach and Grobstein, 1958; Grobstein, 1955; Kobayashi et al., 2008; Osafune et al., 2006).

1.4.1. Primitive streak specification

At the onset of gastrulation, pluripotent epiblast cells ingress into the embryo *via* the primitive streak (PS), a structure located at the posterior of the embryo. The PS is the origin of both the endoderm and mesoderm. In the mouse, this happens at E6.25 and E7.5, and the establishment of PS signals the initiation of germ layer formation.

Gastrulation and its molecular underpinnings have been a subject of intensive investigation by early developmental biologists. Several concepts and pathways have been described and will be briefly discussed here. The mechanics of streak formation and forces driving cellular ingression is believed to be driven by two counter-rotating flows of cells towards the posterior embryo (Cui et al., 2005). A complex signalling network orchestrates this dynamic migratory phenotype, including BMP, Nodal and Wnt. Where did the initial signals originate from? Funa and colleagues identified that these signals might originate from the extra-embryonic endoderm (XEN)(Funa et al., 2015). The XEN is a region of close proximity to the PS and secretes both BMP and Wnt. These signals collectively activate canonical Wnt Signalling and the PS programme by transcription of *MIXL1*, *EOMES*, *T*, *TBX6* and *Nodal* (Funa et al., 2015). As the streak begins to ingress, Wnt3a turns on the expression of *T* and *Tbx6* to sustain the expression

of *Wnt3a* in an autocrine manner. *Tbx6* in turn represses the expression of *Sox2*, inhibiting ectoderm formation (Takemoto et al., 2011; Wardle and Papaioannou, 2008).

Nodal, which is activated by the initial XEN signals, amplifies the signalling cascade of BMP4 and reinforces the PS cellular identity. Binding regions for SMAD2 and SMAD3 within the *Mixl1* promoter region indicates that the gene could be regulated by both BMP and Nodal signalling (Mizutani et al., 2011). Nodal conditional knockout in mouse epiblasts resulted in a dramatic reduction of BMP4 in the XEN, implying that Nodal functions upstream of, and regulates BMP4 (Lu and Robertson, 2004). Importantly, the inhibition of Nodal by hypoblast-derived Cerberus ensures proper activation of Nodal-related genes and ensures that only a single streak is formed at the posterior end (Bertocchini and Stern, 2002; Perea-Gomez et al., 2002)

1.4.2. Intermediate mesoderm specification

The PS is subdivided into the anterior and posterior regions by Nodal and BMP signalling. The anterior PS gives rise to the definitive endoderm while the posterior PS generates the mesoderm (Tam and Loebel, 2007). The posterior PS is induced due to its proximity to the XEN-derived BMP4, which induces the activation of BMP-related genes and also autocrine BMP autocrine (Ben-Haim et al., 2006). Inversely, chordin and noggin, derived from the node, antagonizes BMP4 and establishes the anterior PS. (Beppu et al., 2000; Winnier et al., 1995).

The posterior primitive streak-derived mesoderm sits in the trunk of both sides of the developing embryo. The nascent mesoderm proliferates and elongates rostrally, with the spatial location exposing these cells to different morphogen gradients along the mediolateral axis of the embryo (James and Schultheiss, 2003; Sweetman et al., 2008). FGF signalling in a push and pull mechanism regulates the migration of these cells (Yang et al., 2002). The expression of FGF receptors indicates their responsiveness and migration rostrally to somite derived FGF4

(Shamim and Mason, 1999). Conversely, FGF8 production from daughter cells work as chemorepellents to drive these rapidly proliferating progenitors out of the region (Yang et al., 2002).

Three sub-classes of mesoderm can be derived from this population of cells. These are the paraxial mesoderm (PM), lateral plate mesoderm (LPM) and the IM. Closest to the notochord, the PM gives rise to the somite, followed by the IM, which forms to the kidney and gonads. The LPM, which is furthest away from the spinal cord, develops into the heart and circulatory network. Differentiation of the mesodermal derivatives are influenced by the positioning of PS cells along the anterior-posterior and medio-lateral axes. Medio-lateral mesoderm patterning is established by BMP4 and Nodal morphogen gradients. The LPM is specified by autocrine BMP4 signalling, where it expresses BMP4 for its own specification (James and Schultheiss, 2005). In addition, the neighbouring extra XEN also contributes a substantial amount of BMP4 to drive LPM specification (James and Schultheiss, 2005). The notochord, which is present at the medial region of the embryo, secretes Noggin, an antagonist of BMP4. Inactivation of BMP4 is required for the differentiation of the PM (Wijgerde et al., 2005). The IM, which is sandwiched between these two layers, is specified with little BMP4 activity (James and Schultheiss, 2005; Obara-Ishihara et al., 1999). BMPs are essential for turning on *Osr1* and *Pax2*, both early markers essential for IM (James and Schultheiss, 2005). In addition to BMP, Nodal is also implicated in the medio-lateral patterning of the mesoderm. The induced PM expresses *Cerberus*, an antagonist of Nodal signalling (Biben et al., 1998). It appears that Nodal, in combination with BMP signalling is required to establish the IM. This was shown by several experiments performed using chicken embryos, where an expansion of IM compartment was observed with forced activation of Nodal signalling. Expectedly, Nodal inhibition by either *Cerberus* or *Lefty2* inhibited IM formation accompanied by a significant reduction in *Pax2* (Chen and Schier, 2002; Fleming et al., 2013).

1.4.3. Specification of the UB

In the mouse, nephric duct development from the IM precedes the specification of the UB. The nephric duct is formed at E8.0 by the MET of IM cells into a simple epithelial tubule. This nascent nephric duct must migrate along the trunk of the developing embryo into the urogenital sinus at E9.5. There, an outward branching forms the UB, which interacts with the surrounding MM cells to initiate kidney formation.

Induction of the UB is dependent on a network of an interplay of signalling molecules that drive nephric duct specification, migration and differentiation. Aberrant signalling in UB formation has profound consequences, due to its central importance in metanephros development. Several key genes regulate the development of the nephric duct, including Pax2, Pax8, Gata3, Ret and Lhx1.

Pax2 and Pax8 play essential roles in the development of many renal lineages and are first expressed at E8.5 in the nephric duct (Bouchard et al., 2002; Torres et al., 1995).

In Pax8 knockouts, the nephric duct forms normally, but only in the presence of a functioning Pax2 gene. In Pax2 knockouts, the nephric duct fails to migrate caudally (Dressler et al., 1990; Narlis et al., 2007; Torres et al., 1995). In chicks, Pax2 alone was able to rescue and induce nephric duct formation (Bouchard et al., 2002). However, in Pax2 and Pax8 double knockouts, the IM undergoes apoptosis at E9.5 resulting in no kidney formation. Furthermore, nephric duct markers including Ret, Lhx1 and Gata3 were not detected, indicating a complete failure in nephric duct induction (Bouchard, 2004; Bouchard et al., 2002; Grote et al., 2006). Taken together, it appears that there may be a redundancy mechanism in place during nephric duct specification as either Pax2 or Pax8 alone is sufficient. However, for its proper development and migration, Pax2 is imperative.

In the kidney, Gata3 is expressed in the nephric ducts and is predicted to be a direct binding target for Pax2 and Pax8 (Grote et al., 2006). Gata3 knockout results in embryonic lethality at E11.0, due to its essentiality in other developmental aspects of the embryo (Grote et al., 2008). However, conditional inactivation in the nephric duct causes the formation of ectopic UB budding along its entire segment and formation of multiple collecting systems (Grote et al., 2006). Gata3 is also necessary for the nephric duct to express the Ret receptor, enabling its response to MM-derived GDNF. Mice directly lacking Ret do not display nephric duct formation defects, but fails to form proper functioning kidneys and do not survive post-natal (Grote et al., 2008; Schuchardt et al., 1994).

Lhx1, or the LIM1 homeobox 1 gene, is essential for appropriate migration of the nephric duct. Lhx1 is broadly expressed in the IM and LPM, but is gradually restricted to the nephric duct and subsequently UB at around E9.5 to E10.5 (Barnes et al., 1994; Fujii et al., 1994; A. Kobayashi et al., 2005; Tsang et al., 2000). Lhx1 mutants exhibits a migratory defect, with the nephric duct failing to migrate caudally and undergo premature necrosis. Additionally, loss of *Wnt9b* and *Cdh1* expression suggests that Lhx1 may be essential in maintaining nephric duct identity (Pedersen et al., 2005). Hence it appears that Lhx1 is absolutely essential for nephric duct survival as mutants fail to form the kidneys (Kobayashi et al., 2005; Pedersen et al., 2005; Shawlot and Behringer, 1995).

Together, nephric duct specification, migration and differentiation is dependent on a milieu of spatially and temporally expressed signalling factors. The elucidation of these signalling networks is essential in enabling regeneration and generation of UB cell types. As previously described, the nephric duct is an indispensable precursor in kidney development, and its inappropriate specification has drastic implications, such as kidney agenesis.

1.4.4. Specification of the MM

The MM is the primordium of the adult kidney, generating almost all the cells that contribute to the nephron epithelium and stroma. Like the UB, the MM is also induced from the IM, and presents as a loosely condensed mesenchyme at the dorsal flank of the embryo.

Specification of the UB and MM employs different transcriptional regulation programmes, evident by the successful formation of the MM even in the absence of the nephric duct. Several key transcriptional regulators, such as *Wt1*, *Six1*, *Osr1* and *Hoxd11* were identified to establish an MM identity and will be briefly discussed here.

Wt1 plays a central role in organ development, which includes the heart, lungs and kidneys. In the developing urogenital system, *Wt1* deletion results in impaired UB invasion into the MM, and also MM apoptosis (Kreidberg et al., 1993). Hence, *Wt1* likely acts as a pro-survival or anti-apoptotic factor, as well as a regulator of UB chemoattractant. *Six1* and *Osr1* has a more direct implication in MM specification. The loss of *Six1* did not appear to affect nephric duct formation, but *Six1* mutants failed to undergo branching morphogenesis due to perturbed *Grem1* expression (Nie et al., 2011). The loss of *Six1* also results in the loss of *Pax2*, *Six2* and *Sall1*, suggesting that *Six1* functions upstream of, and regulates the expression of these MM-specific genes (Xu et al., 2003). *Osr1* was shown to inhibit the endodermal-inducing signals. In the absence of *Osr1*, no IM and MM were formed but an expansion of endodermal population was observed (Mudumana et al., 2008). In early kidney development, both the UB and MM expresses *Osr1*, but its expression is gradually restricted to just the nephron progenitor cells at E11.5 (Mugford et al., 2008b). *Hoxd11* is specifically expressed in the posterior IM but not the anterior IM. The posterior IM is the precursor of the MM, and the activity of *Hoxd11* was believed to activate the expression of *Six2*. When *Hoxd11* is ectopically expressed in the anterior IM, the MM marker *Six2* is also expressed, suggesting that *Hoxd11* may be a central regulator of *Six2* and hence MM specification (Mugford et al., 2008a).

Lastly, interaction of these transcription factors likely was shown to orchestrate the transcriptional regulation of different targets. Two such protein complexes have been reported. Pax2-Eya1-Hoxd11 complex was shown to bind to the enhancer regions of Gdnf and Six2 (Gong et al., 2007). The Hox-Eya-Pax complex, first discovered in eye development, was postulated to regulate the expression of Gdnf (Brodbeck and Englert, 2004). These results indicate the existence of a complex transcriptional network governing MM specification by the coordination and coexpression of these transcriptional factors.

1.4.5. Interaction of the UB and MM generates the metanephric kidney

Human kidney development originates from the generation of three excretory organs namely, the pronephros, mesonephros and metanephros. The metanephros gives rise to the definitive kidney in mammals and persists throughout the organism's life while the pronephros and mesonephros are subsequently degenerated during gestation. The development of the metanephros, also known as the metanephric kidney, is a subject of extensive research and its development will be discussed in detail here.

Using lineage tracing, $Osr1^{+}$ cells were identified to mark the IM, which undergoes differentiation into cells of the definitive kidney, including the nephrons, vasculature, interstitial cells and nephric duct (which give rise to the ureteric bud) (Mugford et al., 2008b). Reciprocal interactions between the MM and UB initiates kidney development and are essential for nephrogenesis and branching morphogenesis. The survival and differentiation of these two populations are interdependent, as they will not survive when separated and cultured in isolation (Saxén and Lehtonen, 1987; Saxén and Sariola, 1987).

At E9.5, the nephric duct starts migrating in a rostral to caudal direction. Upon reaching the hind limb region at E10.5, an outward UB branching invades into the loosely condensed MM cells. Upon contact with inductive signals from the MM, the ureteric epithelium undergoes

budding and branching morphogenesis (Figure 1.2). Similarly, inductive signals from the UB initiate mesenchyme-to-epithelial (MET) transition to initiate nephrogenesis. Around each UB tip, the condensation of approximately 30 MM cells constitutes all of the progenitor cells that make up the entirety of the nephron (Bard et al., 2001; Boyle et al., 2008; Herzlinger et al., 1992; Kobayashi et al., 2008; Osafune et al., 2006).

Nephrogenesis describes the morphogenesis of MM into nephrons and is well-characterized morphologically. Firstly, condensed MM cells forms pre-tubular aggregates (PTA) underneath the UB tips and undergo MET to form the renal vesicles (RV)(Carroll et al., 2005). β -catenin-dependent activation of Fgf8 and Wnt4 are believed to be the target genes driving PTA transition into RV. These RVs are epithelial aggregates which acquire basal-apical cell polarity to form lumen. Within 24 to 36 hours, the RV elongates, patterns into a transient comma-shaped body (CSB) and subsequent forms the S-shaped body (SSB)(Little and McMahon, 2012).

Figure 1.2. Morphogenesis of the mammalian kidney.

Schematic of nephron development, from the onset of reciprocal interaction to developing the full length of the nephron (Lopez and Gomez, 2011).

Within the SSB, cells are actively undergoing morphogenesis and differentiation, and exhibit a highly diverse transcriptomics landscape (Brunskill et al., 2008; Combes et al., 2019; Kohda et al., 2000; McMahon et al., 2008; Menon et al., 2018; Wang et al., 2018). Proximal cells at

the S-shaped body express WT1, and further differentiate into podocytes. These podocytes then produce large amounts of VEGF-A to attract vascular progenitors, or angioblasts that migrate into the glomerulus to form the glomerular capillary tufts (Eremina et al., 2006). In mice, Flt1⁺ vascular progenitors exist within the MM and mainly contribute to glomerular microcapillaries through vasculogenesis (Gao et al., 2005; Mugford et al., 2008b). These microvessels are believed to form specialized endothelial cells lining the glomerular capillary tufts before connecting to larger vessels migrating into the developing kidney through angiogenesis (Abrahamson and Robert, 2003).

The nascent nephron proliferates excessively, driving extension and lengthening of tubular segments. The Loop of Henle, also known as the medial segment, is established at the intersection of proximal and distal tubule development. The proximal-distal patterning of these tubular segments is mainly driven by Notch and Wnt signalling. In Notch2 mutants, podocytes and proximal tubules fail to form in the kidney (Cheng et al., 2007). These Notch mutants do not display any UB-developmental defects and have significantly more distal tubules overlapping into the missing proximal space. Conversely, overexpression of Notch intracellular domain (NICD) generates more proximal tubules and less distal tubules (Cheng et al., 2007; Lindström et al., 2015). The UB produces high amount of Wnts in the form of Wnt9b, which establishes a morphogen gradient across the condensed MM cells. Hence, Wnt signalling is believed to be strongest in regions in closest proximity to the UB and was found to repress a proximal fate while promoting a distal tubular fate (Lindström et al., 2018a, 2015). The amount of Wnt exposure was found to be time-dependent, with early migrating cap mesenchyme cells exposed to longest duration of Wnt and generating the distal segments. Cells that are recruited late into the cap are exposed to shortest durations of Wnt signalling, and acquire a proximal fate (Lindström et al., 2018a). Finally, a luminal connection between nephrons and collecting ducts is established when the distal SSB connects to the tip of the UB (Georgas et al., 2009).

In addition to the UB, MM and vascular endothelial cells, the renal stromal population is a critical component to the developing kidney. These stromal cells express *Foxd1* and secrete important regulatory signals, such as RAs, that govern the differentiation and self-renewal abilities of MM cells. In mouse models with ablated *Foxd1*⁺ population, the capping mesenchyme expansion is aberrant, leading to dysregulated growth rates and organ sizes (Fetting et al., 2014; Kobayashi et al., 2014). This might imply that apart from being a supportive population, the renal stromal population may regulate CM differentiation and self-renewal.

1.5. Development of the renal vasculature

The circulatory system within our kidneys are paramount for its function. The kidneys receive around 25% of the final cardiac output, which equates to about 1,800 liters per day in a healthy adult. The extensive network of branching vessels, and a highly orchestrated microcapillary network enables the efficient distribution of this large volume. The efficiency of its design is highlighted by the fact that an estimated 94% of the blood that enters the kidneys reaches the kidney cortex, where it contacts the glomerulus (Garcia-Sanz et al., 1998; Liu et al., 2017).

However, the establishment of this vascular network is still a subject of intensive research. Adding on to this challenge, there are several types of vascular cells within the kidney, of which some are unique to this organ system. For example, endothelial cells in the glomerular capillary tufts are fenestrated, with pores in the cell body allowing for molecular passage.

1.5.1. Mechanisms of renal vasculature development

The renal vasculature is believed to originate from two distinct processes – angiogenesis and vasculogenesis, which work synergistically to compose the entire vascular network (Abrahamson et al., 1998; Hyink et al., 1996; Lopez and Gomez, 2011; Robert et al., 1996). Angiogenesis, which is defined as the sprouting and extension of existing blood vessels, is

believed to be the primary process for construction of large vessels, including the renal artery, veins, and secondary branches. The concept of angiogenesis in kidney vasculature development is established and well accepted by the field, as it was relatively simpler to document an extra-renal source of large vessels invading into the kidney mesenchyme. Vasculogenesis is defined as the creation of new blood vessels from a non-vascular progenitor into the vascular endothelial cells. This process is difficult to investigate, and often necessitates the use of transgenic organisms and reporter systems to trace the origin of these progenitors. It is believed that the process of vasculogenesis generates the vascular progenitors that form the glomerular and peritubular microcapillaries, which eventually links up with the vessels derived from the sprouting dorsal aorta to elaborate the entire arborized and functional vasculature. However, despite the growing body of literature supporting kidney vasculogenesis, emerging studies have provided evidences that refute this theory (Munro et al., 2017).

In the study of kidney vasculature development, the mouse model is a powerful toolkit due to its amenability for genetic alterations, lineage tracing and timed pregnancy. It would not be feasible to conduct these approaches in humans due to ethical considerations and the scarcity of resources.

Early cross-transplantation experiments have attempted to prove if the renal vasculature originate from an intra-renal source, that is within the kidney mesenchyme per se. Some of the earliest xenotransplantation studies provide robust evidences of an extra-renal origin of kidney vascularization (Figure 1.3). When embryonic mice or quail kidneys were transplanted into quail chorioallantoic membrane (CAM), the origin of some endothelial and mesangial cells could be traced back to the host, suggesting that the rudiments were able to draw in exogenous vasculature through angiogenesis (Hyink et al., 1996; Robert et al., 1996; Sariola et al., 1983)

Emerging evidences supporting an intra-renal origin have stemmed from findings that the E12 MM are avascular but may harbour endothelial precursors (Robert et al., 1996). *Ex vivo* cultures of *Flk-lacZ* kidneys did not result in any vasculature formation. However, when these avascular cultures were grafted onto the anterior chamber of the eye (ACE) of wildtype mice, graft-derived *lacZ*⁺ endothelial cells were found in the glomerular microcapillaries (Robert et al., 1998). Hence, these experiments showed that under favourable conditions achieved *via* grafting and not by culture, angioblasts within the metanephros may contribute to vasculature formation. However, follow up allograft experiments in mouse models yielded conflicting results. When wildtype E12 mouse embryonic kidney rudiments were grafted into the ACE or kidney capsule of *ROSA26-lacZ* mice, the resultant vascular network were *lacZ*⁻, an indication of vascularization by graft-derived cells (Hyink et al., 1996; Robert et al., 1996). In contrast, when embryonic kidneys were grafted into newborn *ROSA26-lacZ* mice, host vasculature was found in large numbers in the graft (Robert et al., 1996). This difference might be attributed to differential angioblast activity in different recipient sites, highlighting the critical importance of the niche in vascular development (Lopez and Gomez, 2011).

Figure 1.3. Elucidating the origin of the renal vasculature

A summary of early cross-transplantation studies to elucidate the extra-renal and intra-renal origin of the kidney microvasculature.

1.5.2. The cellular origin for kidney vasculogenesis

Efforts were then placed on trying to identify the angioblast progenitors within the MM, the discovery of which would provide compelling evidence for kidney vasculogenesis. Using inducible genetic models, it was found that *Osr1*⁺ IM cells could give rise to renal microcapillaries and glomerular ECs between E7.5 to 9.5 (Mugford et al., 2008b). However, a recent study performed using time-lapse imaging of culture embryonic kidneys have shown that the vasculature always develop from pre-existing endothelial cells (Munro et al., 2017). The study also provided robust evidence that it may be impossible to dissect kidney rudiments that are completely avascular, challenging results obtained earlier. Hence, the concept of kidney vasculogenesis is still a widely debated concept.

1.6. Human pluripotent stem cells

1.6.1. Human embryonic stem cells

In the early embryo, cells of the inner cell mass and epiblast contribute to almost every cell that makes up the adult body. These cells, endowed with near-limitless self-renewal and the ability for trilineage differentiation, are known as pluripotent stem cells. In development, pluripotency is a transient state that is on the precipice of imminent differentiation. Unsurprisingly, without substantial knowledge on the signals that maintain pluripotency and self-renewal, it was difficult to maintain this status in culture. It was only in 1998 that conditions for maintaining the pluripotency status of human embryonic stem cells (hESCs) were established (Thomson et al., 1998). When left to undergo spontaneous differentiation *in vitro* or implantation into immunodeficient hosts, these cells form embryoid bodies and teratomas, which are both indicative of pluripotency. In addition, as they represent the earliest stage of human development, they have enabled an opportunity to study development in-a-dish. However, as the generation of hESCs requires the use of live human embryos, there are substantial difficulties in obtaining these cells.

1.6.2. Induced pluripotent stem cells

In 2006, it was discovered that the forced expression of four transgenes, namely *OCT4*, *SOX2*, *KLF4* and *c-MYC* was able to reprogram somatic cells into an ESC-like state (Takahashi and Yamanaka, 2006). These induced pluripotent stem cells (iPSCs) rekindled significant interest in the field as they harbour similar abilities for self-renewal and pluripotency. Unlike ESCs, iPSCs can be generated from a skin biopsy or blood sample, bypassing the need for human embryos (Loh et al., 2010; Nishikawa et al., 2008). Additionally, iPSCs contain the same genetic makeup as the donor, offering a patient-specific resource for studying mechanisms of genetic diseases. Furthermore, as an expandable and allogenic source of cells, considerable interest

was placed on directing the differentiation of these cells into clinically relevant cell types with the hope of cell transplantations.

1.6.3. Directed differentiation of hPSCs

Both human ESCs and iPSCs spontaneously differentiate into embryoid bodies, or teratoma when provided with a nutrient-abundant niche *in vitro*, or *in vivo* respectively. This demonstrates the intrinsic capacity of these cells to generate cells of the three germ layers. To harness its full potential, there is a need derive functional cell types of interest from these cells.

Restricting cell fate into one of the three germ layers is the first step in deriving more specialized cell types (Murry and Keller, 2008). Earlier studies have shown that by regulating BMP, Wnt and Nodal pathways in hPSC cultures, it was possible to induce germ layer formation *in vitro*, which is similar to gastrulation *in vivo*. For ectoderm induction, the inhibition of BMP, Wnt and activin/Nodal signalling is required (Aubert et al., 2002; Ying et al., 2003). BMPs alone were able to generate a primitive streak-like state, and this was accelerated by the addition of Wnt (Nostro et al., 2011; Wiles and Johansson, 1999). Nodal activation by Activin supplementation was required for the differentiation into endoderm (Kubo et al., 2004).

Following induction of the desired germ layer, re-creation of the developmental niche is instrumental in obtaining the cell type of interest. Lessons from embryonic development usually function as a guide, by painting a very rudimentary roadmap highlighting the genes that are required to be activated, and how to switch them on. These usually involve large-scale screening approaches to identify the timing, dosage, and combination of supplements for intermediate of each stage to be derived.

1.6.4. Organoids as the new paradigm of directed differentiation

Organoids have emerged as highly relevant and physiological *in vitro* biological models over the past decade. As defined by Lancaster and Knoblich, organoids are three dimensional cellular aggregates that contain multiple cell types and recapitulate basic features of the organ that they represent (Lancaster and Knoblich, 2014). The building blocks of organoids are highly diverse. This includes embryonic progenitor, cancer stem cells, adult stem cells as well as pluripotent stem cells (Fatehullah et al., 2016).

The generation of organoids from hPSCs represents a new paradigm of directed differentiation. It is possible to derive target cells from monolayer cultures. However, they lack the complex tissue architecture found in the organs that they represent (D'Amour et al., 2006; Osakada et al., 2009). The advent of 3D, or organotypic systems have enabled the culture of cellular aggregates. These cellular aggregates are then able to form tissue-like compartmentalization using their intrinsic ability to self-organize (Itskovitz-Eldor et al., 2000; Lancaster and Knoblich, 2014). In the past decade, hPSC-derived organoids representing almost every organ and functional structures have been developed, and are emerging as cutting edge tools in biological research (Sharma et al., 2020). Using organoids, we can investigate several aspects of organogenesis that may not be recapitulated in monolayer cultures. This includes cell-cell, cell-matrix, and other spatial interactions with the surroundings, enabling the study of innate cell behaviour. Integration of organoids with engineering approaches are also enabling the design and construction of organ constructs, which may one day be able to achieve higher-order structures and functions (Park et al., 2019; Takebe and Wells, 2019; Xia and Izpisua Belmonte, 2019).

1.7. Generation of kidney organoids and tissues from directed differentiation

1.7.1. Translating insights from mouse kidney development into mPSCs and murine-derived stem cells.

We owe much of our current understanding of kidney formation to developmental biologists who have been studying kidney development in various model organisms, such as zebrafishes, chicks, and mice. These insights have painted a roadmap for the development of culture conditions to create kidney tissue from mouse pluripotent stem cells (mPSCs) or its progenitors.

Kidney-specific cell types and organoids were first generated using murine-derived materials, as kidney development was better understood in mice as compared to humans. By starting with the mouse, confounding issues such as species-specific knowledge gaps and molecular differences may be avoided, enabling proof-of-concepts to be tested. To validate the renal identity of these differentiation products, the embryonic kidney niche and reaggregation assay are commonly used. This niche was widely regarded as a specialized niche permission for kidney cell types, but not non-kidney cell types (Unbekandt and Davies, 2010). Hence, the ability to integrate into developing glomerulus or tubular structures will provide evidence of a renal identity.

In the early 2000s, directed differentiation of mPSCs were largely based on embryonic body (EB) cultures, which closely recapitulates multicellular organotypic processes occurring during development (Doetschman et al., 1985; Hirst et al., 2006; Ng et al., 2005). The next challenge was to identify the signalling pathways required to direct mPSCs into the early intermediate stages of kidney development. Several signals, such as BMPs were selected due to their potent ability to induce mesoderm in ESCs (Johansson and Wiles, 1995; Wiles and Johansson, 1999). Combinations with RA and activin A were also evaluated as they were able to induce pronephric development in *Xenopus* (Osafune et al., 2002).

Using this concept, mPSCs were first coaxed into the IM lineage using a combination of Activin A, RA and BMP7 (Kim and Dressler, 2005; Kramer et al., 2006). Upon injection into mouse embryonic kidney rudiments, these cells integrated into proximal tubular epithelial structures within the rudiments. In another study, Kobayashi and colleagues found that Wnt signalling was also required for kidney differentiation. A Wnt4 transgene was stably engineered to constitutively activate Wnt signalling in mouse ES cells (Kobayashi et al., 2005). In three weeks, these cells expressed AQP2 when cultured in the presence of Activin A and hepatocyte growth factor (HGF).

With limited knowledge on the precise and timing of signalling pathways required, several studies have also employed the use of conditioned medium from embryonic kidneys with the rationale that the necessary factors were present to induce kidney differentiation. In one such study, Activin and RA were employed to differentiate mPSCs into mesoderm cells before conditioned medium from UB cells were used (Ren et al., 2010). These mesoderm cells acquired renal markers such as *WT1*, *PAX2*, *NPHS2* and *CDH1*, indicating that the conditioned medium contains nephrogenic promoting factors. Conditioned medium from cell lines, which were relatively easier to acquire, has also been tested. Nishikawa and colleagues explored the effects of cell line-derived conditioned medium on induced mesoderm. MM-conditioned medium was used to induce UB, and UB-conditioned medium was used to induce the MM (Nishikawa et al., 2012). This concept aimed to recapitulate reciprocal signals during kidney development and was shown to be effective in inducing MM populations expressing WT1 and CDH11.

With promising successes in the generation of renal cell types from mPSCs, as well as a clearer roadmap of which signalling pathways to activate, these knowledges were translated towards human-derived materials.

1.7.2. The genesis of human renal tissue from hPSCs

One of the first reports of achieving a renal-cell identity from human PSCs was from Xia and colleagues, where they described a protocol to generate ureteric bud-like progenitor cells using a two-stage, 4 days protocol (Xia et al., 2013). These cells expressed *OSR1*, *PAX2*, *GATA3*, *PAX8* and *LHX1*, which were markers of the Wolffian duct and ureteric bud. Upon introduction into mouse embryonic kidney reaggregates, these cells expressed CK8 and contributed exclusively to the ureteric bud tips, but not the nephrons. Hence, the ability to not only integrate into kidney organoids but also exclusively into the ureteric bud validates that these cells can be identified as UB-like progenitors (Xia et al., 2013).

Taguchi and colleagues later employed a reverse engineering approach to dissect the lineage origin of MM cells and defined the conditions for stepwise differentiation from mouse PSCs (Taguchi et al., 2014). They found that Wnt and BMP4 activation from epiblasts were required to induce the expression of T^+CDX2^+ nascent mesoderm. Using a cocktail of Activin A, BMP4, retinoic acid and CHIR99021, these cells formed $OSR1^+PAX2^+$ IM which generated MM-like cells upon later FGF9 exposure. They were able to induce MET and nephrogenesis from these cells when cultured alongside mouse embryonic spinal cord, which supposedly mimic UB-derived signals. Nephrons generated using this protocol formed glomeruli and renal tubules, and was widely recognized as one of the first instances of MM induction from hPSCs (Taguchi et al., 2014).

Takasato and colleagues reported two protocols in a span of two years. The first protocol employed a human ESC line with a knock in MIXL1 reporter (Takasato et al., 2014). Using this line, they identified that canonical Wnt activation alone was sufficient for primitive streak induction, without the need for BMP or Activin signalling (Davis et al., 2008; Takasato et al., 2014). Following a high dose of FGF9 exposure, they simultaneously derived both MM and UB progenitors, which self-organized into complex kidney structures upon reaggregation. The

later protocol was optimized to recapitulate early IM patterning, showing that the duration of Wnt exposure prior to FGF9 treatment was able to modulate the population of UB or MM progenitors (Takasato et al., 2015). The study also performed extensive characterization, demonstrating all four main populations of cells – UB, MM, endothelial and stroma within their kidney organoids.

Morizane and colleagues reported a highly efficient protocol to generate nephron progenitor cells (NPCs)(Morizane et al., 2015). Originating as a subpopulation of the MM, NPCs can give rise to almost all cells that make up the nephron. The study reported that it was possible to achieve an 80-90% of NPCs which were SIX2⁺SALL1⁺WT1⁺PAX2⁺. The high efficiency of NPC induction enables the formation nephron organoids without the need for cell sorting (Morizane et al., 2015).

In the same laboratory, Freedman generated “epiblast spheroids”, which were then differentiated into kidney organoids (Freedman et al., 2015). Epiblast spheroids are 3D cultures of hPSCs grown on a soft Matrigel substrate. They showed that with 1.5 days of CHIR99021 treatment followed by the basal media supplemented with just B27, they were able to generate kidney organoids containing glomerulus and tubules, which were later used for disease modelling. Results from this study suggests that it may be possible to prime hPSCs for renal differentiation by first providing them with an epiblast spheroid intermediate stage (Freedman et al., 2015).

1.7.3. Human kidney organoids go *in vivo*

Two studies in 2018 attempted to implant these hPSC-derived kidney organoids into immunocompromised mice to investigate *in vivo* functionality. Both studies used the Takasato protocol, but employed different implantation approaches (Takasato et al., 2015). Bantounas and colleagues subcutaneously implanted NPCs on day 12 of differentiation and allowed them

to continue nephrogenesis within the subcutaneous *in vivo* niche (Bantounas et al., 2018). They allowed cells to engraft and mature within the host for 3 months, of which they observed significant maturation. A caveat of their approach was low efficiency of nephrogenesis, with relatively abundant off-target cells such as cartilage and blastema. This could be a result of implanting progenitor cells, which were not terminally differentiated (Bantounas et al., 2018). Using an alternative approach, van den Berg and colleagues implanted matured kidney organoids at around 25 days of differentiation underneath the renal capsule (van den Berg et al., 2018). Although they allowed the graft to mature in the host for just a month, these kidney organoids underwent structural maturation as previously described (Bantounas et al., 2018). The implantation site was monitored in real time by an abdominal window using *in vivo* 2-photon microscopy, which they used to observe blood flow dynamics within the graft. Using kidney organoids expressing either MAFB⁺ and SOX17⁺ reporters, and fluorescently labelled dextran, they were able to detect blood flow within the graft and glomeruli. As kidney organoids contains more terminally differentiated cells, lesser off-target cells were observed as compared to the approach taken by Bantounas's group (Bantounas et al., 2018; van den Berg et al., 2018).

Implantation of kidney organoids was also attempted on chick embryos, which are nutrient and vasculature-rich niches amenable for grafting. Garreta and colleagues implanted kidney organoids onto 7 day old CAM (Garreta et al., 2019). Kidney organoids underwent rapid vascularization in 3 days, and structural maturation in 5 days. Next, they engineered a polyacrylamide hydrogel, and tuned its mechanical properties to recapitulate the CAM microenvironment. Using this substrate, they derived kidney organoids which were structurally more matured as compared to using classic cell culture conditions (Garreta et al., 2019).

1.7.4. Engineering microanatomical organization into kidney organoids

Kidney progenitor cells derived from embryonic kidneys have the potential to self-organize and reconstruct several rudimentary features of the kidney when single cells are reaggregated and cultured under the right condition (Unbekandt and Davies, 2010). These reconstructed kidney organoids are avascular and lack a unified collecting duct network, but these features may be introduced using other complementary means.

Xinaris and colleagues pioneered an approach to generate vascularized glomeruli by implanting embryonic progenitors underneath the renal capsule of a living host in the presence of VEGF. Vasculature from the recipient animal invades into the avascular graft, generating functional glomeruli containing well-developed foot processes, slit diaphragms and filtration barriers. (Xinaris et al., 2016a, 2012). The functionality and size-specific permeability were evaluated using a panel of dextran molecules, of which low molecular weight dextrans of 10 and 70 kDa were able to be filtered, but not high molecular weight dextrans exceeding 155 kDa. Importantly, human-derived amniotic fluid stem cells were able to integrate into developing mouse organoids, forming functional chimeric glomeruli (Xinaris et al., 2016b).

To generate higher order features such as a branching network, the MM may be dissociated and co-cultured with an intact UB. These MM cells harbour the ability to swarm towards the UB tips, where they organize into nephrons in a relatively similar manner to normal kidney development (Ganeva et al., 2011). This concept of “guided self-organization” has been harnessed by Taguchi and Nishinakamura, where modular assembly of both mouse and human-derived UB and MM, in the presence of mouse embryonic kidney stromal cells has shown to give rise to kidney organoids with an arborized collecting duct network (Taguchi and Nishinakamura, 2017). Importantly, the use of embryonic stromal cells was indispensable to recreate these higher order structures, providing an indication that they may be required for support and secretion.

1.7.5. Upscaling kidney organoid production

A prospect of hPSC-derived kidneys is the generation autologous kidney tissues for renal replacement therapies. Currently, the dimension of a single kidney organoid is about 1-2 mm in length, compared to an adult kidney which is 10-12 cm long. Hence, it would take numerous kidney organoids to replace the function of a normal kidney. A few recent studies have attempted to devise methods to scale up the production of kidney organoids

Przepiorski and colleagues performed differentiation in spinner flasks and devised a protocol that is significantly cheaper by omitting the use of FGFs (Przepiorski et al., 2018). Embryoid bodies were first formed using hPSCs and subjected to CHIR99021 treatment for 3 days. These organoids, presumptively of primitive streak stage were moved to bioreactors, where they were cultured in the presence of knock-out serum replacement (KOSR) supplement. Expectedly, with the use of EB induction method, there were marked differences in the organoid size, which was found to affect its structure and efficiency. They managed to circumvent this problem by removing kidney organoids that were either too small, or too large by using 200 μ M and 500 μ M strainers. Taken together, this presents a simple and affordable method to generate large quantities of kidney organoids (Przepiorski et al., 2018).

Using the modified Takasato protocol, Kumar and colleagues devise a culture bifurcation step to generate numerous micro-kidney organoids containing up to 10 nephrons each (Kumar et al., 2019; Takasato et al., 2015). Differentiating IM cells were replated under swirling, low attachment conditions in the presence of polyvinyl alcohol and methyl cellulose. The addition of a biomaterial provided the structural support that enabled numerous kidney organoids of 20 – 40 μ M to be generated, instead of aggregating into a large mass of tissue. In principle, this approach would be perfect for generating large quantities of evenly-sized kidney organoids (Kumar et al., 2019).

Although both approaches by Przepiorski and Kumar are able to scale up the amount of kidney organoids generated with minimal fuss, the approach is not adaptable for high-throughput screening approaches (Kumar et al., 2019; Przepiorski et al., 2018). To utilize organoids for high-throughput analysis, it is necessary to spatially separate them into different wells to expose them to different concentrations of various compounds. Czerniecki and colleagues initiated kidney organoid differentiation from hPSCs in either 96 or 384 well formats using the Freedman's protocol (Czerniecki et al., 2018; Freedman et al., 2015). Under adherent culture conditions, it was easy to manipulate these kidney organoids, perform media replenishments and compound testing. Each well was reported to contain up to hundreds of nephrons, which were amenable to automated imaging and phenotypic analyses (Czerniecki et al., 2018).

1.7.6. Limitations faced by the current generation of kidney organoids

The current generation of kidney organoids face several challenges, notably maturation, vascularization, and functionality. Nearly all the organoids generated by directed differentiation were reminiscent of their embryonic counterparts. Transcriptomics analysis revealed gene signatures representative of first, or second trimester organs (Garreta et al., 2019; Simunovic and Brivanlou, 2017; Takasato et al., 2015). It appears that that this could be an intrinsic limitation with current tissue engineering techniques

A caveat of the current generation of hPSC-derived organoids, including kidney organoids, revolve around the lack of vasculature. At present, most of the reported hPSC-derived kidney organoids places focus on achieving kidney epithelial populations. While various protocols have reported the generation of vascularized kidney organoids, several laboratories have reported that this is challenging to reproduce (Subramanian et al., 2019; Wu et al., 2018b). Even with the successful establishment of a vascular network, glomeruli within kidney organoids remained avascular under *in vitro* culture. This could be due to the lack of

hemodynamics, which might induce vascular remodelling and subsequently invasion (Homan et al., 2019; Serluca et al., 2002). The functionality of these constructs will be highly dependent on the appropriate assembly of vascular structures within, including the construction of a large vessel leading into the implant, down to the ultra-structural features of small microcapillaries in the glomeruli and the tubules.

Few organoids have been reported were able to recapitulate several features of the organ that they represent. For instance, cardiomyocytes were able to exhibit contractility and brain organoids were shown to fire brain waves reminiscent of neonatal brains (Ronaldson-Bouchard et al., 2018; Trujillo et al., 2019). Endocrinal organoids such as liver and pancreas were able to produce albumin and insulin, respectively (Millman et al., 2016; Pagliuca et al., 2014; Takebe et al., 2013). For kidney organoids, basic functions such as filtration and reabsorption has been proved to be difficult to achieve. To a large extent, this is due to the difficulty in recapitulating the structural organization within kidney functional units using *in vitro* cultures. However, several implantation experiments have shown plasticity of implanted kidney organoids to undergo remodelling and exhibit rudimentary filtration (Bantounas et al., 2018; Taguchi et al., 2014; van den Berg et al., 2018). While promising, extensive development is still required to translate them into therapeutics.

1.8. Rationale for the thesis

The establishment of reprogramming methodologies to derive individual-specific iPSCs and differentiate them into kidney organoids is a renaissance in the field of renal biology and regenerative medicine. Kidney organoids recapitulate the cellular diversity and microanatomical structures, offering a better *in vitro* model for dissecting kidney development and physiopathology. However, comparison between these hPSC-derived kidney organoids and the *in vivo* kidney still reveal numerous differences. Continual improvements are required, to improve the relevance of these models and to advance them towards medical applications.

Current protocols struggle with several drawbacks as previously described. hPSCs, endowed with the remarkable ability for self-renewal and trilineage differentiation served as an ideal cellular resource for this study. This project aimed to develop an improved version of kidney organoids with an additional emphasis on generating an endogenous vasculature network, more precise cell-fate control and rudimentary functionality. Our specific aim was to simply achieve *de novo* vascular co-development within hPSC-derived kidney organoids using a single approach. The development of such a protocol will enable the study of kidney vascular development in-a-dish, allowing us to perform mechanistic studies, drug screening and disease modelling. The advent of a technique that allows for more precise cell-fate control would improve reproducibility of different batches of kidney organoids and enable the enrichment of the desired cell populations. Finally, as the holy grail of kidney organoids is to ultimately use them for renal replacement therapies. To explore their *in vivo* potential, we will perform kidney organoid implantation underneath the renal capsule of immunodeficient mice. By implanting kidney organoids that are pre-vascularized, it would be likely that the grafts will have a higher chance of engrafting successfully and rapidly. This would enable us to study the functionality of kidney organoids within a living host.

1.9. Aims of this study

The aim of this thesis is to develop a method to generate an improved version of 3D kidney organoids from hPSCs. The successful establishment of such a protocol would serve as an invaluable model for interrogating kidney development and disease, with potential implications in regenerative medicine. To begin, **Chapter 1** provides a literature review on mammalian kidney development, hPSCs and harnessing the principles of directed differentiation and self-organization to generate kidney organoids. **Chapter 2** explain in detail the materials and methods that were used in this study. **Chapter 3** describes the results and findings that were uncovered in this study, including 1) the development of a novel protocol to generate vascularized 3D kidney organoids and characterization of both endpoint kidney organoids and intermediate stages using established markers. 2) the applicability of hPSC-derived kidney organoids in modelling human kidney vascular development and the discovery of a novel renal vascular progenitor population. 3) the ability to modulate the cellular composition within nephron-like segments in our kidney organoids by temporal modulation of canonical Wnt signalling. 4) the *in vivo* maturation and functionality of these kidney organoids, following implantation into an immunocompromised recipient host. 5) the extended applications of this technology to perform high throughput compound screening. **Chapter 4** discusses the potential implications and limitations of each finding. Finally, this thesis was concluded in **Chapter 5**, and the future trajectory of this technology was also discussed.

2. Materials and methods

2.1. Pluripotent Stem Cell Culture

H9 (WA09), H1 (WA01) human embryonic stem cells (WiCell) and BJ-Epi3 human induced pluripotent stem cells were cultured in TeSR on growth factor-reduced Matrigel-coated plates (Corning). For routine maintenance, cells above 70% confluency were treated with 2mg/mL Dispase (Invitrogen) for 5 mins, then dissociated into small clumps by scraping and passaged onto Matrigel-coated plates at a ratio of 1:5-1:10.

2.2. Induction of kidney organoids from human pluripotent stem cells

To prepare cells for differentiation, hPSCs were dissociated into single cells with Accutase (STEMCELL Technologies) and plated onto Matrigel-coated plates at a density of ~15,000 to ~24,000 cells/cm² in the TeSR supplemented with 10 μ M Y-27632 (Sigma) and cultured for 24 hours. Y-27632 was removed and cells were cultured in TeSR for another 48 hours to reach a confluency of 50-60%.

To induce the primitive streak, cells were cultured in basal differentiation medium (Advanced RPMI 1640, 1x L-GlutaMAX, 1x NEAA)(Life Technologies) supplemented with 10 μ M CHIR99021 (Sigma) for 4 days with daily media change. Next, to induce the nephrogenic IM, cells were cultured in basal differentiation medium, without the addition of any growth factors for 3 days with daily media change. Subsequently, to induce nephron progenitors, cells were cultured in basal differentiation medium supplemented with 50 ng/mL FGF9 (Peprotech) and 3 μ M CHIR99021 with daily media change for 2 days. From day 9 of differentiation onwards, cells were cultured in basal differentiation medium supplemented with 50 ng/mL FGF9 until day 20. Finally, kidney organoids can be maintained in basic differentiation medium from day 20 onwards without the addition of growth factors.

2.3. Self-organization of NPCs into 3D kidney organoids

To generate 3D kidney organoids from nephron progenitor cells (NPCs), day 10-12 differentiating cells were dissociated into single cells using Accutase. $2.5\text{-}5 \times 10^4$ cells per well were aggregated in round bottom ultra-low attachment 96-well plates (Corning) in basal differentiation medium containing 50 ng/mL FGF9 and 10 μM Y-27632. Y-27632 was removed after 24 hours and cells were cultured in the presence of 50 ng/mL FGF9 with media change every 3 days. By default, kidney organoids generated using this protocol is enriched with podocytes. Patterning may be performed using a low dose of CHIR99021 (1 μM) at day 14 of differentiation to increase the amount of distal nephron segments. To perform liquid-air interface culture, 3D kidney organoids at day 16-18 of differentiation (around 5-7 days after aggregation) are transferred onto the upper chamber of a Transwell insert (Corning) with daily media change at the bottom chamber.

For high content analysis, day 10-12 differentiating cells were dissociated into single cells using Accutase. Approximately $0.75\text{-}1.25 \times 10^5$ cells per well were seeded onto Matrigel-coated 96-well plates in basal differentiation medium containing 50 ng/mL FGF9 and 10 μM Y-27632. Y-27632 was removed after 24 hours and cells were cultured in the presence of 50 ng/mL FGF9 with media change every day.

2.4. Animals

NOD.Cg-Prkdcscid.II2rgtm1Wjl/SzJ (NSG) mice and CD-1 mice were purchased from Jackson Laboratory. Mice were housed in a specific pathogen-free animal facility in plastic cages on 12 h/12 h light/dark cycle and fed with standard chow diet. 8-10 weeks old NSG mice were used for implantation assay, followed by in vivo dextran assay (IACUC #A0346). 8-12 weeks old CD-1 mice were used for in vivo dextran assay (IACUC #A0346). 8-10 weeks old CD-1 mice were bred for E12.5 embryonic kidneys for generating mouse embryonic kidney

aggregates (IACUC #A0289). All animal experiments were performed in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University Singapore.

2.5. Generation of mouse kidney aggregates from embryonic mouse kidneys

Mouse kidney aggregates, or “organoids” were generated as described with minor modification (Xia et al., 2014). E12.5 mouse embryonic kidneys were obtained from BALB/cJInv mice and treated with 0.25% (w/v) trypsin for 15 mins at 37 °C. After neutralizing trypsin using kidney culture medium (KCM, MEM supplemented with 10% FBS, 10 μ M Y-27632 and 1:1,000 normocin) for 10 mins, mechanical force by gentle pipetting was applied to fully dissociate the kidney rudiments into single cells for reaggregation. 40,000 cells were aggregated in KCM overnight in liquid culture using low attachment 96-well plates. After 24 hours aggregation, Y-27632 was removed from the culture medium, and aggregates were transferred onto liquid-air interface culture.

2.6. Renal capsule transplantation

Mice were first anesthetized using a xylazine-ketamine cocktail and placed on a heating pad. Ophthalmic ointment was placed on the eyes to prevent the cornea from drying out. The fur was removed from the area surrounding the kidney area, from the flank area bounded caudally by the last rib and dorsoventrally by the spine and abdominal wall. The skin surface was sterilized using surgical iodine and 70% isopropyl alcohol. A skin incision is made above the anatomical region of the kidney with a similar incision on the abdominal wall. The kidney is externalized using a Moria spoon (Fine Science Tools, CA). From this step onwards, the kidney must be kept moist. A nick is made on the base of the kidney capsule before a subcapsular pocket is made using a blunt glass picket. Up to 8 – 12 kidney organoids are delivered into the subcapsular pocket using a Drummond pipette. A dry cotton bud is used to dry the opening of

the capsule, which seals on its own without the need for cautery. The kidney is gently returned back into the abdomen and the incision is closed by suture stitching. After which, wound clips were used to close the skin incision. Buprenorphine is administered subcutaneously as an analgesia (and administered daily) and the animal is returned to a clean cage. The wound clips are removed after a week, and the graft is harvested after 4 weeks.

2.7. *In vivo* luciferase assay

We injected 0.2 mg per gram body weight luciferin (Perkin Elmer) intraperitoneally into mice 5 mins prior to IVIS assay. Mice were anesthetized using isoflurane and placed in a prone position in the imaging chamber of the IVIS machine (Perkin Elmer Lumina XRMS). The injected mice were imaged over a 10 mins time period.

2.8. *In vitro* dextran uptake assay

Kidney organoids were incubated with 100 mg/mL of fluorescence-labeled dextran for 4 hours (pulse). 4 hours later, kidney organoids were switched to fresh medium and live cell culture images were captured using a wide-field fluorescence microscope (pulse). Kidney organoids were then cultured in the absence of dextran for another 24 hours (chase), before being fixed for immunofluorescence analysis.

2.9. Histology

Samples, which included kidney organoids, implanted kidney, or mouse tissue, were fixed overnight in 4% paraformaldehyde (PFA) at 4 °C. The next day, the PFA was removed, samples were kept at PBS for further processing.

For frozen sections, samples were subjected to an overnight sucrose gradient treatment prior to being embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek). Samples were gently

frozen and mounted on the cryostat chucks. Cryosections (8 μm) were generated using a Leica CM3050 S Research Cryostat cryostat (Leica).

For paraffin embedded sections, tissues were subjected to overnight processing followed by being embedded in paraffin (Leica). Tissue sections (2-4 μm) were generated using a microtome (Leica). Hematoxylin and Eosin (H&E) staining was performed according to the manufacturer's instruction. For immunostaining, sections were de-paraffinized and subjected to antigen retrieval procedure in citrate buffer (pH 6.0, 99 °C) for 45 mins before staining (see detailed staining protocol below).

2.10. Immunostaining and confocal microscopy

Fixed cell cultures or 3D kidney organoids were fixed with 4% paraformaldehyde (Sigma) at the stipulated time points for 15 mins at room temperature. Cells were washed once with phosphate buffered saline (PBS) before permeabilized using PBS supplemented with 0.5% Triton X-100 for 15 mins at room temperature. Blocking was performed for 1 hours at room temperature using blocking buffer (PBS supplemented with 2% FBS, 2% BSA and 0.1% Triton X-100). Cells were incubated with the indicated primary antibodies overnight at 4 °C with gentle agitation. The next day, cells were washed three times with PBST (PBS supplemented with 0.1% Tween-20) and followed by 2 hours incubation with secondary antibodies at room temperature. Cell were then washed three times with PBST before mounting with Fluoromount-G mounting solution (Southern Biotech). Confocal images were captured using a Zeiss LSM800 laser scanning microscope (Carl Zeiss) equipped with 10x, 20x, 40x or 63x oil-immersion objectives).

2.11. Image quantification and statistical analysis

Whole organoid images were acquired by me and image quantification was performed by Miss Nan Zheng at Lee Kong Chian School of Medicine.

Kidney organoids were treated with 0, 1, 3 or 10 days of patterning CHIR (1 μ M) before fixation and staining (LTL for proximal tubule, CDH1 for distal tubule and NPHS1 for glomerulus). Whole mount confocal images were acquired and used to visualize different segments using different colour channels. Images were processed and nephron segments were quantified using ImageJ (NIH). The scale of the images was first standardized and converted to 8-bits to adjust the threshold of pixel intensity. The percentage of specific segments were calculated against the sum of all three segments to obtain the relative area coverage.

2.12. VEGFR inhibition assay

Kidney organoids cultured on liquid-air interface were changed to basal differentiation medium supplemented with 10 nM each of FDA-approved VEGFR inhibitors (Axitinib/ Cediranib/ Tivozanib) for 1 to 3 days. After treatment, kidney organoids were fixed, and subjected to whole-mount immunofluorescence staining followed by confocal microscopy imaging.

2.13. Fluorescence-activated cell sorting (FACS)

Cell culture was dissociated into single cells using Accutase and blocked for 30 mins in blocking buffer (PBS supplemented with 2% FBS and 2% BSA). For cell surface protein, primary antibody incubation was performed on ice for 30 mins. Cells were washed once with washing buffer (PBS supplemented with 1% FBS and 1% BSA) and incubated with secondary antibody for 30 mins on ice. Cells were then washed with ice cold PBS before analysis or sorting. For intracellular staining, cells were fixed using 4% PFA for 15 min at room temperature, followed by permeabilization (PBS supplemented with 0.2% Triton X-100) for 15 mins at room temperature prior to blocking and staining. FACS analysis was performed on the LSRI Fortessa X-20. FACS sorting was performed on the FACS Aria Fusion with help from Miss Manisha Cooray at Lee Kong Chian School of Medicine.

2.14. Enzyme-linked immunosorbent assay (ELISA)

Cell culture medium was collected 24 hours after the previous medium change and centrifuged at 200 x g for 5 mins to remove cell debris. Human VEGFA levels were analyzed with human VEGF DuoSet ELISA (R&D systems) and DuoSet ELISA Ancillary Reagent Kit 2 (R&D systems) according to the manufacturer's instructions. All sample analyses were carried out in triplicates.

2.15. RNA isolation and qRT-PCR

Total RNA was isolated from cells using Trizol (Invitrogen). RNA was purified using Quick-RNA kit (Zymo Research) with DNase treatment. 500 ng total RNA of each sample was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using QuantStudio 6 (Applied Biosystems) and SYBR Green Supermix (Bio-Rad). Relative mRNA expression levels were quantified by the ddCt method and normalized to GAPDH or 18 s rRNA gene expression. All of the sample analyses were carried out in triplicates. In the event that the gene expression level is undetectable, a Ct value of 40 was assigned.

2.16. In vitro dextran and albumin uptake assay

Kidney organoids cultured on liquid-air interface were changed to basal differentiation medium supplemented with 100 µg/mL of fluorescently labelled dextrans (70 and 2,000 kDa) or 100 µg/mL of fluorescently labelled albumin for 6 hours. The medium is refreshed, and live cell culture images can be captured using a wide-field fluorescence microscope, if needed (pulse assess for uptake). Subsequently, kidney organoids are cultured for another 24-48 hours in fresh medium (chase assess for retention). The organoids are then fixed for immunofluorescence analysis.

2.17. Cryopreservation and revival of NPCs

At day 9 to 12 of differentiation, NPCs were dissociated into single cells with Accutase and cryopreserved in basal differentiation medium supplemented with 10 μ M of Y-27632, 50 ng/mL FGF9 and 10% DMSO. Cells were subjected to gentle freezing at a rate of 1-2 $^{\circ}$ C a minute using a specialized freezing container using a -80 $^{\circ}$ C freezer. After 24 to 48 hours, cells are transferred to a liquid nitrogen tank for long term storage.

For revival, cells were thawed in basal differentiation medium supplemented with 10 μ M of Y-27632 and 50 ng/mL FGF9 on Matrigel coated plates. After 24 hours, Y-27632 was removed and cells can be cultured according to the timing of the original protocol.

2.18. Electron Microscopy Sample Fixation

Implanted organoids were recovered two weeks after implantation and fixed in 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer for 15 mins. The implants were dissected into small cubes (~2 mm x 2 mm), fixed in fresh fixative solution overnight at 4 $^{\circ}$ C. After fixation, samples were washed 5 x 5 mins in 0.1 M phosphate buffer and stored at 4 $^{\circ}$ C.

2.19. Transmission Electron Microscopy

For TEM, samples were stained with 2% osmium tetroxide solution containing 2% potassium ferrocyanide for 2 h. Subsequently, samples were dehydrated in increasing concentrations of ethanol (25%, 50%, 75%, 95%, 100%) and acetone (100%) before infiltration with increasing concentrations of acetone: epon resin mixture (1:1 for 1 h, 1:6 for overnight; absolute resin for 1 h)(Polysciences Inc, PA, USA). Absolute resin was replaced every 1 h for 3 times before curing at 60 $^{\circ}$ C for 48 h. Blocks were sectioned using Leica EM UC7 ultramicrotome (Leica Microsystems, Wetzlar, DE) at 500 nm for semi-thin sections and 130 nm for ultra-thin sections. Sections were contrasted using 4% (w/v) uranyl acetate solution for 5 mins, 5 x

washes in distilled water, followed by Reynold's lead citrate solution for 5 mins and 5 x washes in distilled water. Images were collected using either JEOL JEM1010 or JEOL JEM2100 at the Advanced Bioimaging Facility at SingHealth Duke-NUS Academic Medical Centre or the Electron Microscopy Unit at Yong Loo Lin School of Medicine, National University of Singapore.

2.20. Scanning Electron Microscopy

For SEM, samples were processed by the Electron Microscopy Unit at Yong Loo Lin School of Medicine, National University of Singapore. Briefly, samples were fixed with 1% osmium tetroxide solution for 2 h. Samples were dehydrated in increasing concentrations of alcohol (25%, 50%, 75%, 95%, 100%) before drying in a critical point dryer. Finally, samples were mounted on the stub using carbon tape before sputter coated with gold. Images were collected using FEI Quanta 650 FEG.

2.21. Single-cell RNA-sequencing library preparation

In this particular experiment, cells were prepared by Dr Pin Li and Miss Tian Zhang at Lee Kong Chian School of Medicine and library preparation was performed by Dr Elaine Chew at Genome Institute of Singapore, Agency for Science, Technology and Research (A*STAR).

Day 10, Day 12 and Day 14 kidney organoids were dissociated into single cells with Accutase, followed by passing through a 40 µm filter thrice to remove cell clumps. Dissociated cells were subsequently stained with trypan blue and confirmed to have at least 80% viability. A minimum of 7000 dissociated cells were loaded per well for single-cell RNA-seq library preparation with the Chromium Single Cell 30 Gene Expression Solution according to the manufacturer's recommendations. Libraries generated were sequenced on Illumina HiSeq 4000 platform. We captured a total of 15,834, 6,554 and 41,534 single cells from Day10, Day12 and Day14 dissociated cells loading across 2, 1 and 6 capture wells, respectively. 73,000, 56,300 and

55,856 raw mean reads were obtained per cell from the captured Day10, Day12 and Day14 single cells.

2.22. Single-cell RNA sequencing data processing

The single-cell RNA sequencing dataset was analyzed by Dr Elaine Chew, Miss Michelle Lian and Assistant Professor Foo Jia Nee at Genome Institute of Singapore, Agency for Science, Technology and Research (A*STAR).

Cellranger analysis pipeline (version 2.2.0) was used to align sequenced reads to GRCh38 and aggregate output across all samples. Quality control and filtering was conducted with Seurat (version 2.3.0)(Butler et al., 2018) to retain cells with mitochondrial gene content $< 5\%$, total genes expressed between 200 and 7000, as well as number of detected unique molecules $< 50,000$. 15,560 Day10 single cells, 6,337 Day12 single cells and 40,609 Day14 single cells remained following quality control and filtering. Gene expression measurements for retained cells were log-transformed, normalized by total expression per cell, and scaled to 10,000 molecules per cell. We regressed out non-biological interesting variation in gene expression that is driven by processing batch, number of detected molecules, and mitochondrial gene content. Highly variable genes across the single cells were then identified and principal components (PCs) analysis was conducted. 8 PCs were selected as input for t-Distributed Stochastic Neighbour Embedding (t-SNE) for Day10, Day12, Day14 cells. We conducted unsupervised lineage trajectory analysis on a down-sampled set of Day10, Day12, Day14 cells (6,251 cells, 10% of all cells) with Monocle2 (version 2.10.0) (Qiu et al., 2017; Trapnell et al., 2014). In brief, genes with mean expression value $R \geq 0.05$ were used to order cells along the trajectory. Significant differentially expressed genes (DEGs, $q\text{-value} < 10^{-4}$) at branch points along the trajectory were identified using branched expression analysis modelling (BEAM) function of Monocle2. Genes with similar lineage-dependent expression patterns were

identified from the hierarchical clustering of branch specific DEGs and classified into up- or downregulated branch specific DEGs. GO analysis was conducted on the identified up- or downregulated branch specific DEGs to elucidate modulated biological processes at each branch point. t-SNE and unsupervised lineage trajectory analysis were also conducted on the endothelial subcluster identified in Day10, Day12, Day14 (cluster 10) using the above-described method with the variation of 7 PCs selection as input for t-SNE.

2.23. Bulk RNA sequencing analysis

The bulk RNA sequencing data in Figure 3.10 was processed by Dr Elaine Chew, Miss Michelle Lian and Assistant Professor Foo Jia Nee at Genome Institute of Singapore, Agency for Science, Technology and Research (A*STAR). The bulk RNA sequencing data in Figure 3.4 and 3.6 was processed by Mr Meng Liu and myself at Lee Kong Chian School of Medicine, Nanyang Technological University.

Sequenced reads were aligned to the Hg19 human genome (GRCh39) with STAR version 2.5.2a (Dobin et al., 2013). Cuffnorm version 2.2.1 (Trapnell et al., 2013, 2012) was used to obtain quartile normalize fragments per kilobase per million reads (FPKM) expression matrix of genes across all samples. Heatmap of relative expression of nephron stage markers and segment-specific markers was generated with Microsoft Excel. Cuffdiff version 2.2.1 (Trapnell et al., 2013, 2010) was used to identify differential genes between samples. Significant differentially expressed genes (DEGs) which have false discovery rate (FDR) ≤ 0.05 , fold change ≤ 1.5 , and FPKM ≥ 1 in one sample group were retained for subsequent analysis. Gene ontology (GO) clustering enrichment analysis was carried out on DEGs using the Functional Annotation tool in DAVID 6.7 (Huang et al., 2009, 2009) under medium classification stringency for all default annotation categories except protein domains.

2.24. Data and code availability

The accession number for the single-cell RNA sequencing data reported in this paper is NCBI GEO: GSE132026.

3. Results

3.1. Development of a novel protocol to generate 3D kidney organoids from human pluripotent stem cells

Genetic lineage tracing studies had demonstrated that the kidneys are derived from the IM, which originate from the PS. Logically, recreation of conditions to achieve specification into these intermediates are imperative for the derivation of kidney-specific lineages (Figure 3.1a). The prevailing view is that kidney development is divided into several progenitor stages over the course of a few weeks. These stages include the PS, followed by IM and subsequently MM, UB, endothelial and stromal cells (Takasato et al., 2015). Here, I have established a fully chemically defined medium system to direct the stepwise differentiation of hPSCs into NPCs and kidney organoids (Figure 3.1b,c). The use of a fully defined chemical media system enables reproducibility of differentiation across different cell lines, and the success of this protocol has been validated in multiple hPSC lines, including both human ESCs and iPSCs (Low et al., 2019). Additional emphasis was also placed on achieving simplicity and affordability, and the protocol was optimized to use the lowest concentration and combination of supplementation.

Figure 3.1. Development of a protocol to generate kidney organoids from hPSCs.

(a) A roadmap for the generation of NPCs and kidney organoids from hPSCs by recapitulating kidney development. (b) Schematic of the differentiation protocol. c) Brightfield image of a 3D kidney organoid at day 24 of differentiation.

3.1.1. Differentiate human pluripotent stem cells into primitive streak-like cells

The PS is an anatomical structure early in development which harbours multipotent progenitors belonging to both endoderm and mesoderm lineage. Cells that resemble the PS in terms of gene expression and multipotency can be generated from hPSCs via directed differentiation. For simplicity, we call these cells PS-like cells.

The specification of PS in the developing embryo is dependent on Wnt3a and BMP4 signalling (Beppu et al., 2000). Hence, the earliest reports in attempting to generate PS-like cells from human PSCs involved the use of a canonical Wnt agonist, CHIR99021 and BMP4 (Taguchi et al., 2014; Takasato et al., 2014). Later, it was discovered that the use of BMP4 may be omitted. Both the Morizane and Takasato protocol have shown that by using a high-dose of CHIR99021 treatment over 4 days, it was possible to induce a PS-like population expressing $T^+MIXL1^+TBX6^+$ at a very high efficiency (Morizane et al., 2015; Takasato et al., 2015). We have adopted the four days CHIR99021 method to derive PS-like cells from hPSCs.

Human ESCs or iPSCs were dissociated into single cells and replated onto Matrigel-coated plates at a concentration of around 2×10^4 cells per cm^2 three days prior to differentiation. In three days, these hPSCs formed small, evenly spaced colonies. Following 24 hours of CHIR99021 treatment, a significant number of cells undergo apoptosis, but surviving cells proliferate extensively and formed a confluent lawn in 4 days (Figure 3.2a). Immunofluorescence characterization revealed that almost all these cells expressed T and MIXL1, indicating the successful derivation of PS-like cells as previously reported (Figure 3.2b)(Morizane et al., 2015; Takasato et al., 2015).

Figure 3.2. Differentiating hPSCs into primitive streak-like cells.

(a) Brightfield images of differentiating culture from day 0 to 4. (b) Immunofluorescence image of differentiating cells at day 4 for markers of primitive streak. Representative markers used for each cell type – T and MIXL1 for PS-like cells and DAPI for nuclei.

3.1.2. Specify primitive streak-like cells into posterior intermediate mesoderm-like cells

Differential exposure to levels of BMP4 levels regulates mesoderm patterning. The IM, which is sandwiched between the LPM and PM, receives an intermediate amount of BMP4 signalling due to its anatomical position (James and Schultheiss, 2005). However, the exact amount of BMPs that is required for its specification *in vivo* remained obscure. Morizane and colleagues have shown that a 3-day treatment of late PS-like cells with 10 ng/mL of Activin A was required to induce posterior IM-like cells expressing WT1⁺HOXD11⁺ (Morizane et al., 2015).

The use of Activin A was usually involved in endodermal differentiation and may skew the trajectory of differentiating cells towards an endodermal fate (Sulzbacher et al., 2009; Teo et al., 2012). A better option for IM specification, which omits the use of Activin A, might have implications for higher efficiency. To examine this possibility, cells at day 4 of differentiation

was exposed to 10 ng/mL of Activin A (Morizane et al., 2015), or a gradient of Noggin for 3 days. Surprisingly, cells that were neither treated with Activin A nor Noggin expressed the highest levels of *WT1*, *LHX1*, *PAX2*, *OSR1* and *EYA1*, all of which were IM markers (Figure 3.3a). Markers of endoderm, paraxial and lateral plate mesoderm, including *TBX6*, *FOXF1*, *FOXH1*, *FOXA2*, *SOX17*, *GATA4* and *GATA6* were reduced using this growth factor-free approach. Interestingly, expression of *BMP2*, *BMP4* and *BMP7* was detected in the growth factor-free group, indicative of an autocrine BMP signalling mechanism within the differentiating culture (Figure 3.3a). At day 7 of differentiation, cells robustly expressed WT1 and HOXD11 protein, both markers of posterior IM as validated using immunofluorescence analysis (Figure 3.3b). These results provided support that the use of Activin A was dispensable for *in vitro* IM induction.

Figure 3.3. Specification of the primitive streak-like cells into intermediate mesoderm-like cells.

(a) Gene expression profiling of day 7 cells following 3 days of treatment (day 4 to 7). Colour swatch correlate to the relative fold change in gene expression levels as compared to day 0 hPSCs. (b) Immunofluorescence analysis of day 7 cells for markers of the intermediate mesoderm. Representative markers used for each cell type – *WT1* and *HOXD11* for IM-like cells and DAPI for nuclei.

To uncover transcriptomic alterations from the supplementation of Activin A, bulk RNA sequencing of our growth factor-free condition (“Activin A⁻”)(Low et al., 2019) was compared with Morizane’s condition (“Activin A⁺”)(Morizane et al., 2015). We detected 50 significant differentially expression genes (DEGS) between the two treatment groups. The DEGS were selected for gene ontology (GO) analysis and relevant pathways were presented. Activin A- group showed a higher expression of genes involved in SMAD and RA signalling pathway, which were critical pathways for kidney development, despite no addition of Activin, BMPs or RA. Signalling pathways involved in heart, lung and liver development were downregulated in the Activin A- group, indicative of lesser off-target cell types that originate from the endoderm and lateral plate mesoderm (Figure 3.4a,b,c).

Figure 3.4. Comparative bulk transcriptomics analysis of day 7 cells.

Bulk RNA sequencing analysis of day 7 cells with or without Activin A treatment (during day 4 to 7). (a) Comparative schematic of the differentiation protocol. (b) Selected gene ontology analysis based on differentially expressed genes (fold change more than 1.5 times and p value

< 0.05)(analysis performed with help from Mr Meng Liu). (c) Heatmap of upregulated and downregulated genes, values are mean FPKM of replicated samples (analysis performed with help from Mr Meng Liu).

Together, these results showed that 3 days of factor-free culture condition can efficiently generate cells belonging to the IM with high efficiency. This may present several advantages, including lesser off-target gene signatures, costs, and reducing the number of different growth factors required.

3.1.3. Generation of nephron progenitor cells

Fibroblast-growth factors (FGFs) play a key role in the establishment of NPC identity, and regulates its self-renewal and differentiation. Two key FGFs, including FGF9 and FGF20, were identified to play important roles in establishing the identity and multipotential properties of NPCs (Barak et al., 2012). Loss of these signals results in early progenitor differentiation and premature exhaustion of the NPC niche. While FGF20 is secreted by the NPCs and act in an autocrine manner, FGF9 is derived from the UB (Barak et al., 2012). Both the Takasato and Morizane protocol have employed FGF9, in different concentrations and timing, to derive NPCs from the IM. These NPCs then undergo nephrogenesis in response to CHIR99021 treatment (Morizane et al., 2015; Takasato et al., 2015).

At day 7 of differentiation, IM-like cells were cultured in the presence of 50 ng/mL FGF9 and 3 μ M CHIR99021 to drive their differentiation towards an NPC fate. After two days of culture, CHIR99021 treatment was lifted and cells were cultured in FGF9. Under brightfield microscopy, cells were a confluent lawn at day 9 but gradually acquire a patchy, undulating morphology at day 10 (Figure 3.5a). The acquisition of this morphology likely indicates early MET activity and provide early evidence that the differentiation is working as planned. Immunofluorescence analysis showed that these cells robustly expressed SIX2 and SALL1, indicating the acquisition of NPC identity (Figure 3.5b). Quantification using flow cytometry

at day 10 indicated that more than 90% of cells were SIX2⁺, indicating the successful generation of NPCs (Figure 3.5c).

Figure 3.5. Directed differentiation of intermediate mesoderm cells into nephron progenitor cells.

(a) Brightfield imaging of cells from day 7 to 10 of differentiation. (b) Immunofluorescence image of differentiating cells at day 9 for markers of nephron progenitor cells. (c) Flow cytometry analysis of cells at day 10 of differentiation (analysis performed with help from Miss Tian Zhang). Representative markers used for each cell type – SIX2 and SALL1 for NPCs and DAPI for nuclei.

I was interested in the transcriptomics differences between NPCs generated using two different protocols at day 10. Comparative bulk RNA sequencing and transcriptomics analysis was performed on day 10 NPC cells generated using either this protocol, or Morizane's protocol (Low et al., 2019; Morizane et al., 2015). A total of 36 significant DEGS were detected and selected for GO analysis, which revealed higher gene signatures related to vascularization, RA, BMP and organogenesis. Conversely, higher gene signatures related to cardiac development and Notch signalling pathways were detected in day 10 cells using the Morizane protocol (Figure 3.6a,b,c).

Figure 3.6. Comparative bulk transcriptomics analysis of day 10 cells.

Bulk RNA sequencing analysis of day 10 cells with or without Activin A treatment (during day 4 to 7). (a) Comparative schematic of the differentiation protocol. (b) Selected gene ontology analysis based on differentially expressed genes (fold change more than 1.5 times and p value < 0.05) (analysis performed with help from Mr Meng Liu). (c) Heatmap of upregulated and

downregulated genes, values are mean FPKM of replicated samples (analysis performed with help from Mr Meng Liu).

In conclusion, this protocol can generate around 90% of NPCs by day 10 of differentiation, and this was comparative to the Morizane protocol. However, transcriptomics analysis between cells from these two protocols informed us of differences in gene expression, which may either be caused by an off-target population, or differences in the quality of induced NPCs (Nishinakamura, 2019; Tanigawa et al., 2019).

3.1.4. Self-organization and morphogenesis of nephron progenitor cells

NPCs are able to undergo self-organization into complex structures reminiscent of the nascent nephrons when cultured under the appropriate confluency. In the developing kidney, NPCs rapidly undergo MET and nephrogenesis in response to UB-derived Wnt9b. Logically, hPSC-derived NPCs should harbour the same intrinsic potential as their *ex vivo* counterparts.

Indeed, these NPCs have the potential to undergo self-organization, forming numerous raised colonies resembling pre-tubular aggregates (PTA) at around day 11 to 12 of differentiation (Figure 3.7a). Immunofluorescence analysis revealed that these cells expressed the NPC markers SIX2, SALL1 and WT1. In addition, markers of developing PTA such as LHX1 and PAX8 were also detected. The absence of E-cadherin (CDH1) indicates that at this stage, NPCs are still mesenchymal in nature (Figure 3.7b). Over the course of a week, these PTAs undergo epithelialization with the formation of a basement membrane as marked by JAG1 and LMN1. These structures acquired polarization and formed structures reminiscent of comma-shaped bodies before elongation to form S-shaped body-like structures (Figure 3.7c). The morphogenesis of these NPCs into nephrons *in vitro* faithfully recapitulates nephrogenesis occurring during mammalian kidney development.

Figure 3.7. Self-organization and nephrogenesis of NPCs.

(a) Brightfield images of differentiating culture from day 10 to 14. (b) Immunofluorescence image of NPCs at day 11 of differentiation for PTA markers. (c) Immunofluorescence image of differentiating culture from day 12 to 18 for markers of early MET and basement membrane formation. Representative markers used for each cell type – LMN1 for basement membrane; SIX1, SIX2 and WT1 for early NPCs; JAG1 for MET and DAPI for nuclei.

Next, these NPCs were subjected to different culture formats to assess their propensity to undergo nephrogenesis (Figure 3.8a). By expanding compatible systems to utilize these NPCs, it will be possible to expand the versatility of this approach to suit the needs of the research question.

Here, several key strategies to use these NPCs were described (Figure 3.8a). A popular approach is to culture them under low attachment conditions to generate 3D kidney organoids.

These 3D kidney organoids can be generated without the need for Matrigel-embedding, which was previously shown to be required in other organoid systems (Sato et al., 2009). As a well of differentiating culture can be used to generate many kidney organoids, several approaches such as shaking, or bioreactor cultures may be adopted for large-scale preparations. Similarly, nephron structures can also be generated by seeding NPCs back onto a 2D surface, enabling high throughput approaches on 96 or 384 multi-well plate. In agreement with the Takasato protocol, liquid-air interface culture was beneficial for kidney organoid maturation (Takasato et al., 2015). On liquid-air interfaces, kidney organoids exhibited more defined structures and better nutrient accessibility.

Figure 3.8. Broadening the applications of hPSC-derived kidney organoids

(a) Schematic of NPC-compatible cell culture formats that can employed to generate kidney organoids at different scales for various applications.

3.1.5. Characterization of cellular populations within kidney organoids

At day 24 of differentiation, kidney organoids cultured on liquid-air interfaces harboured clearly defined structures and plateauing growth rates. On gross investigation, they are flat,

disc-like and typically measure 2 to 3 mm across (Figure 3.9a). Numerous “tubule-like” structures may be identified, providing a visual indication that kidney organoids were successfully generated.

Immunofluorescence microscopy was employed for primary analysis. Using this approach, several kidney segment-specific markers could be evaluated at once. Additionally, the relative location of these markers could be illuminated. This is important as various markers may be co-expressed in differential nephron segments and cell states. For instance, the presence of *lotus tetragonolobus* (LTL) and CDH1 double positive tubules indicate an immature tubular state. The separation of these two markers indicate differentiation into the proximal or distal tubules (Przepiorski et al., 2018).

The kidney organoid is made up of mostly nephron-like structures which were densely packed and surrounded by a rich and elaborated vasculature network (Figure 3.9b). Nephron-like structures in the organoid were segmented in a proximal-to-distal pattern and connected to a common origin (Figure 3.9c). Glomeruli marked by WT1 and NPHS1 were organized in clusters and located at the proximal end of the nephron-like structures. These glomeruli were connected to proximal tubules which were positive for LTL, Loop of Henle (or medial segment) expressing Jagged-1 (JAG1) and distal tubules expressing CDH1. All tubules in the kidney organoid expressed the pan-tubular marker CK8⁺ and were connected to a CALB1⁺ origin at the distal end (Figure 3.9c). Vasculature networks were almost always established in close proximity to the podocytes. However, there was no vascular invasion and formation of a glomerular capillary tuft *in vitro*, likely due to the absence of hemodynamics or flow (Figure 3.9d)(Homan et al., 2019). Nonetheless, these avascular glomeruli formed glomerular basement membrane indicated by the presence of laminin within podocyte clusters (LMN1). LMN1 was also found to be surrounding the glomeruli and basolateral end of proximal tubule, altogether indicative of organized polarity (Figure 3.9e). In addition, immunostaining with

additional markers was performed to validate the identity of the different segments within the tubules. Proximal tubules were positive for the endocytosis-receptor LRP2 and K-cadherin (CDH6)(Figure 3.9f). Uromodulin (UMOD), a marker for medial tubule or Loop of Henle cells, was detected in between LTL⁺ proximal tubules and CDH1⁺ distal tubules (Figure 3.9g). Distal tubules expressed aquaporin-2 (AQP2) in addition to CDH1. Renal interstitial cells (MEIS1⁺FOXD1⁺WT1⁺) and vascular endothelial cells (CD31⁺CD34⁺CD144⁺) were found to be distributed throughout the whole kidney organoid, indicative of multilineage communication and co-development (Figure 3.9a,b). Together, the detection of additional cell-type specific marker provides additional evidence of the identity of these cells.

Figure 3.9. Characterization of cell types within hPSC-derived kidney organoids.

(a) Brightfield image of a single kidney organoid. (b) Whole organoid immunofluorescence analysis of kidney organoids. Whole mount immunofluorescence analysis of (c) nephron-like structures, (d) glomerulus, (e) basement membrane, (f) proximal tubules, (g) loop of Henle, (h) distal tubules, (i) vasculature and (j) stromal and interstitial cells. Representative markers used for each segment – NPHS1 and WT1 for glomeruli/ podocytes; LTL, LRP2 and CDH6 for proximal tubules; UMOD and JAG1 for Loop of Henle; CDH1, AQP2 and CALB1 for distal tubules; LMN1 for basement membrane; CD31, CD34, CD144 for vascular endothelial cells; FOXD1 and MEIS1 for stromal cells and DAPI for nuclei.

3.1.6. Transcriptomics profiling of kidney organoids

Immunofluorescence analysis is a robust approach to validate the expression of kidney markers at a protein level while also providing insights into structure and localization. However, the number of markers that can be interrogated at once is limited. Hence, to profile the global transcriptomics landscape in our kidney organoids, bulk RNA sequencing was performed on day 24 kidney organoids.

We sought help from our collaborators at Genome Institute of Singapore – Dr Elaine Chew, Miss Michelle Lian and Asst Prof Jia Nee Foo, who will be helping us with the bulk RNA sequencing computational analysis.

We compared the Fragments Per Kilobase of Transcript per Million (FPKM) values with samples representing hPSCs and NPCs at day 0 and 10, respectively. The reads for each gene

were determined using the Fragments Per Kilobase of Transcript per Million mapped reads (FPKM) method and compared to samples representing hPSC (day 0) and NPCs (day 10).

We pulled out specific genes corresponding to various kidney development stages and segment for analysis (Brunskill et al., 2008; Lindström et al., 2018a). Expectedly, cells at day 10 of differentiation, expressed the highest amount of NPC-specific markers, such as *CITED1*, *EYA1*, *ITGA8*, *SALL1*, *SIX1* and *SIX2* (Figure 3.10a). These NPCs also expressed several markers of differentiation, including *HEY1*, *LYPD1*, *PAX8*, *BMP* and *ID* genes, indicating that they had likely initiated nephrogenesis (Figure 3.10b). Importantly, while these markers were robustly expressed in the NPC stage, many of these markers were downregulated in the kidney organoids at day 24 of differentiation. Mesenchyme-to-epithelialization (MET) markers, which suggests the formation of nascent nephrons, were not detected at day 10 of differentiation, but were detected in day 24 (Figure 3.10c). This is expected, as our results indicate that MET usually happens between day 12 to 16.

Profiling of the day 24 kidney organoids revealed that they expressed many markers indicative of nephron segmentation. We observed an upregulation of genes that correspond to relatively established nephron components, including glomeruli, proximal tubules, Loop of Henle and distal tubules at day 24 (Figure 3.10d,e,f,g). Most of these genes were neither detected in day 0 nor day 10, suggesting that the expression of these markers is specific and indicative of maturation. However, the expression profile of aquaporins (AQPs) and solute carrier family (SLCs) of genes painted a different story. These two classes of transporters represent an array of functional transporters that enable solute uptake in the kidney. While we detected the expression of *AQP1*, *SLC12A1*, *SLC27A1*, *SLC23A1*, *SLC24A2* and *SLC3A1* in the kidney tubules, there were little to no detectable expression of *AQP2*, *SLC22A6*, *SLC13A3* and *SLC7A13* (Figure 3.10d,e,f,g). Hence, these results indicate that kidney organoids displayed a renal identity and maturation, but only to a certain extent. The absence of these transport

proteins could be due to a limitation of *in vitro* culture and/or a lack of signals that regulate their expression.

Finally, we sought to validate the existence of collecting ducts in our kidney organoids. We detected the expression of *KRT8*, which was a collecting duct marker in mouse but a pan-tubular marker in human kidney (Figure 3.10h). However, *AQP2* nor *GATA3* which would be more representative of collecting ducts, were not detected. Hence, while immunostainings have shown that nephron tubules converged into a single branch point, we were unable to confirm the presence of collecting duct cells in our kidney organoids.

3.1.7. *In vitro* functional characterization of kidney organoids

The kidney mediates the removal of metabolic waste from blood plasma by filtration and reabsorption, conferred to by intricate structures created by specific cell placements and extracellular matrix production. With the absence of physical parameters, such as hydrostatic pressures and draining networks, these functions are difficult to recapitulate *in vitro* (Riegel, 1986). During filtration, tubular epithelial cells transport solutes and water across their cell body by active transport and deposit them back into circulation using the peritubular capillaries on the basal edge (Aronson and Giebisch, 1997; Taggart, 1958). Hence, the cellular organization and placement is critical to kidney function.

Kidney organoids contain kidney-specific cell types that were organized into rudimentary nephron-like structures. The functionality of these kidney organoids were assessed using a well-established *in vitro* cellular uptake assay (Freedman et al., 2015; Xinari et al., 2016b, 2012). To begin, day 24 kidney organoids were treated with fluorescently labelled dextran for 6 hours. Within 6 hours, proximal tubular cells should be able to internalize this dextran if they are responsive. Fresh medium without dextran was replenished and organoids were incubated for an additional 24 hours. This step removes residual fluorescence that were not internalized and allows us to detect any dextran that were internalized.

Following fixation, kidney organoids were labelled for LRP2, which is a receptor on the proximal tubules which mediates endocytosis (Christensen and Birn, 2002). As expected, 70 kDa fluorescently labelled dextrans were accumulated and co-localizes together with these LRP2⁺ regions (Figure 3.11a, upper panels). The co-localization of dextrans with LRP2 suggests that it is likely that uptake of dextran was mediated by endocytosis. However, when challenged with a significantly larger 2,000 kDa dextran, there was no fluorescence detected (Figure 3.11a, lower panels). These results indicate size-dependent uptake of dextran molecules, and large molecules which were not taken up were lost following media change.

Apart from the uptake of dextran, which is an uncharged sugar molecule, kidney organoids were also challenged with albumin, which is a protein molecule. Using the same approach, kidney organoids were treated with FITC-labelled albumin for 6 hours, before washing them out with a 24-hour incubation using fresh media. Indeed, FITC-labelled albumin was detected within LRP2⁺ proximal tubules, which is indicative of their ability to scavenge albumin molecules (Figure 3.11b). These results showed that proximal tubule in our kidney organoids were capable of both size-specific dextran and albumin reabsorption.

Figure 3.11. Functionality assessment of in vitro kidney organoids.

(a) Immunofluorescence analysis of day 24 kidney organoids treated with 4 hours of either 70 kDa or 2,000 kDa fluorescently labelled dextran (chase), and 24 hours of incubation with fresh

medium (chase). (b) Immunofluorescence analysis of day 24 kidney organoids treated 4 hours of fluorescently labelled albumin, followed by 24 hours of incubation with fresh medium (chase). Representative markers used for each cell type – LRP2 for proximal tubules and DAPI for nuclei.

3.2. Precise cell fate control in hPSC-derived nephron-like structures *via* temporal modulation of canonical WNT signalling

3.2.1. Identification of renal vesicle stage during kidney organoid differentiation

Kidney organoids at day 14 of differentiation are comprised of numerous renal vesicles that formed in about 3 to 4 days following single cell dissociation and reaggregation. Under brightfield microscopy, these structures are distinguished by small, rounded structures within kidney organoids (Figure 3.12a). Histological analysis revealed that these vesicles contained mostly polarized epithelial cells, with distinct lumen formation (Figure 3.12b).

Figure 3.12. Renal vesicle formation in 3D kidney organoids at day 15 of differentiation. (a) Brightfield images of multiple 3D kidney organoids in suspension culture. (b) H&E section of a 3D kidney organoid.

Next, immunofluorescence analysis was performed on these early kidney organoids. Almost all the cells were positive for WT1, indicating that cells were still at a NPC. In later stages, WT1 expression is restricted to only the podocytes and stromal cells, and is downregulated in tubular epithelia. Laminin staining surrounding early renal vesicles indicates cellular polarization and the formation of a basement membrane (Figure 3.13a). PAX8, which were expressed in developing nephrons was detected within renal vesicles but not in the surrounding stromal. Some larger renal vesicles expressed the MET marker JAG1, indicative that they are undergoing nascent nephrogenesis (Figure 3.13b).

Figure 3.13. Characterization of 3D kidney organoids at day 15 of differentiation.

(a) Immunofluorescence analysis of LMN1 and WT1 in kidney organoids. (b) Immunofluorescence analysis of PAX8 and JAG1 in kidney organoids. Representative markers used for each cell type – LMN1 for basement membrane; WT1 for NPC; PAX8 for differentiating NPCs; JAG1 for MET and DAPI for nuclei.

3.2.2. Coordinating proximal-distal cellular populations by canonical WNT signaling

During kidney development, inductive signals from UB recruit the MM and orchestrate nephrogenesis through the activity of sWnt9b. Duration of exposure to UB-derived signals were identified to direct the lineage specification of NPCs into either podocytes, or proximal and distal tubules (Lindström et al., 2018a). Administration of CHIR99021 was able to induce ectopic nephrogenesis in human kidney organoids and *ex vivo* mouse kidney cultures (Carroll et al., 2005; Karner et al., 2011; Morizane et al., 2015; Takasato et al., 2015). Hence, it appears that Wnt9b likely acts through the canonical Wnt signalling axis and this activation can be recapitulated by CHIR99021 (Figure 3.14a).

Hence, I sought to investigate if it was possible to influence the differentiation of renal vesicles by providing a low concentration of CHIR99021 (1 μ M). The patterning signal is applied on day 14 of differentiation, the critical duration of which kidney organoids harbour numerous early renal vesicles. The treatment will be conducted for 1 day (day 14 to 15), 3 days (day 14 to 17) or throughout the rest of the differentiation (day 14 to 24)(Figure 3.14b).

Figure 3.14. Renal vesicle patterning using CHIR99021.

(a) Schematic of *in vivo* nephron patterning during kidney development (left) and *in vitro* nephron patterning in kidney organoids (right). Green “T”-shaped structure on the left schematic depicts the early ureteric bud, pink structures depicts the renal vesicles and yellow structures depict an activation of Wnt signalling using CHIR99021. (b) Proposed timeframe and duration for administration of the patterning signal.

3.2.3. Effects of patterning signal on kidney organoids

3.2.3.1. Increased proliferation and size of kidney organoids

Kidney organoids that were subjected to the patterning signal displayed higher growth rates. On liquid-air interface cultures, kidney organoids that were not subjected to patterning CHIR99021 were smaller as compared to kidney organoids that were subjected to growth under constant patterning signal (Figure 3.15a). Kidney organoids that were not treated with the patterning signal had an average diameter and area coverage of around 600 µm and 250 mm², as compared to around 750 µm and 400 mm² for kidney organoids that were subjected to

patterning (Figure 3.15b). This was in agreement with the involvement of Wnt/ β -catenin in cell growth and proliferation, and was earlier reflected in kidney *ex vivo* and organoid models (Clevers, 2006; Lindström et al., 2015; Morizane et al., 2015; Nusse and Clevers, 2017).

Figure 3.15. Patterning CHIR99021 increased kidney organoid size.

(a) Representative brightfield microscopy of 3D kidney organoids on liquid-air interface at day 24 of differentiation with and without patterning CHIR99021 treatment. (b) Measurements and quantification of physical parameters of kidney organoids using diameter and area coverage. Data were presented as mean $\pm$ SEM ($n = 3$ independent experiments). Scale bars, 200 μ m. Statistical analysis was performed using unpaired Student's t-test, **** $p < 0.0001$.

3.2.3.2. Time-dependent multilineage patterning using CHIR99021

It was observed that using our protocol, differentiating NPCs preferentially adopt a proximal differentiation trajectory. Histological analysis showed that 3D kidney organoids contain a

large number of podocytes, with few tubules (Figure 3.16a). It is likely that this could be due to the timing of the 2nd CHIR99021 treatment (3 μ M) which was administered at day 7 to 9 of differentiation. Having an early 2nd CHIR99021 treatment specifies and primes the NPCs towards nephrogenesis, whilst not influencing proximal-to-distal cell fate decisions which are occurring at a later timepoint. Hence, administration of a 1 μ M CHIR99021 in our system was able to modulate the differentiation trajectory of NPCs towards a tubular epithelium lineage instead of a podocyte lineage. The patterning effects were more profound following 10 days of CHIR99021 treatment as kidney organoids contained mostly tubules, marked by their distinctive cuboidal epithelial morphology under histological analysis (Figure 3.16b). Strikingly, nephron patterning could be performed in a time-dependent manner, to achieve the preferred glomerulus to tubule ratio. Kidney organoids were patterned for 1, 3 and 10 days and the cellular composition was analysed using segment specific markers. Accordingly, kidney organoids which were not subjected to patterning CHIR99021 contained a high number of glomeruli, which were positive for NPHS1 (Figure 3.16c,d). Proximal tubules labelled by LTL and distal tubules labelled by CDH1 were observed at a lesser frequency. With increasing duration of CHIR99021 treatment, the amount of NPHS1⁺ glomerulus was diminished, while the amount of both LTL⁺ and CDH1⁺ segments increased (Figure 3.16c,d).

The vasculature within kidney organoids is established and maintained by podocyte-derived VEGF-A (this will be described in detail in the next subchapter). Accordingly, kidney organoids which not subjected to patterning CHIR99021 contained a higher number of glomeruli and a rich vasculature. On contrary, patterned kidney organoids lesser number of glomeruli and correspondingly less vascular cells. Profiling the amount of secreted VEGF-A revealed a dramatic reduction of VEGF-A production (> 3-fold) upon long-term patterning (Figure 3.16e). Hence, these results revealed that patterning CHIR99021 increases the

population of tubular cells and reduces the population of glomeruli and associated vascular endothelial cells.

Figure 3.16. Time-dependent multilineage patterning using CHIR99021.

(a) Representative H&E section of 3D kidney organoid at day 24 of differentiation. (b) Representative H&E section of a 3D kidney organoid at day 24 of differentiation that was

subjected to 10 days of patterning CHIR99021 signal. (c) Immunofluorescence analysis of kidney-specific markers (NPHS1, LTL, CDH1)(top and bottom panels) and vasculature marker (CD31)(bottom panels) at day 24 of differentiation. (d) Quantification of area coverage of specific nephron segment from whole mount immunofluorescence images. Data are presented as mean $\pm$ SEM ($n = 2$ independent experiments with 3 technical replicates) (analysis performed by Miss Nan Zheng). (e) VEGF-A protein secretion by 3D kidney organoids at day 24 following different exposure lengths to patterning CHIR99021 signal. Data were presented as mean $\pm$ SEM ($n = 3$ independent experiments).

3.2.4. Canonical WNT activation does not influence established nephron structures and vasculature

While our results indicated that long-term patterning *via* canonical Wnt has indirect implications in vascular formation, Wnt signalling may also be implicated in vascular development and angiogenesis (Dejana Elisabetta and Kühl Michael, 2010). To rule out that vascular perturbations upon long-term patterning is a consequence of decreased podocyte population, and not a direct negative effect of CHIR99021 on endothelial cells, the patterning signal was introduced at a later time point, when all the nephron structures and vasculature are established. Expectedly, application of the patterning CHIR99021 from day 20 or 24 of differentiation for 8 and 4 days, respectively, did not result in any nephron patterning, nor was the vasculature compromised (Figure 3.17a,b). Indeed, these results indicated that the timing of patterning CHIR99021 was paramount to achieve nephron patterning, and that the decreased vasculature content in long-term patterning was not a consequence of prolonged CHIR99021 treatment.

Figure 3.17. Late patterning CHIR99021 does not affect established nephrons and vasculature.

(a) Proposed timeframe for administration of the late patterning CHIR99021 treatment. (b) Immunofluorescence analysis of kidney-specific markers (NPHS1, CDH1) and vasculature marker (CD31) at day 28 of differentiation.

3.3. Characterization of the cellular properties and origin of renal vasculature in hPSC-derived kidney organoids

3.3.1. Temporal kinetics of vasculogenesis during kidney organoid differentiation

To unravel the kinetics of vasculogenesis, the differentiating cultures were sequentially terminated to identify the timing of podocyte and vascular progenitor emergence. The VEGF receptor 2/ kinase domain receptor (KDR) was an early marker for vascular progenitors, and could appear before the emergence of CD31, which represent a mature endothelial state.

At day 10 of differentiation, no KDR, CD31 and NPHS1 positive cells were detected. A KDR⁺ population of cells emerged at day 11 and were scattered throughout the culture. Hence, day

11 was identified as the window for vascular progenitor emergence. At day 14 of differentiation, these KDR⁺ population acquire and co-express CD31, indicative of their maturation into vascular endothelial cells. Co-staining with CD31 and NPHS1 would enable us to identify the specification of mature endothelium and podocytes, respectively. The acquisition of CD31 coincides with the emergence of NPHS1⁺ podocytes, suggesting a possible relationship between the two cell types (Figure 3.18a).

Figure 3.18. Emergence of KDR⁺ vascular progenitor cells at day 11 of differentiation.

(a) Immunofluorescence analysis for markers of vascular progenitor (KDR), mature endothelial cells (CD31) and podocytes (NPHS1) from day 10 to 14 of differentiation.

3.3.2. Quantitative analysis of VEGF-A production during kidney organoid differentiation

Vascular endothelial growth factor A (VEGF-A) is a potent inducer of vasculogenesis and angiogenesis in development, homeostasis and disease. In the kidneys, podocytes in the glomeruli secrete large amounts of VEGF-A to establish and maintain the glomerular capillaries (Eremina et al., 2006, 2003; Woolf et al., 2009). Apart from the glomerulus, tubular

epithelial cells were also found to express VEGF-A, which drives peritubular vascularization (Kang et al., 2001). Hence, these lines of evidence prompted us to investigate the temporal kinetics and cellular origin of VEGF-A in our kidney organoids.

Cells and media were collected from the differentiating culture at specific timepoints representing various progenitor stages. Using qRT-PCR, the relative expression of *VEGF-A* transcripts was measured. Throughout the course of differentiation, VEGF-A gene expression gradually increased, with sharp increases between day 10 and 14, and plateauing after day 21 onwards (Figure 3.19a). Expectedly, increasing amounts of VEGF-A protein in the cell culture medium increased over time and closely correlated with *VEGF-A* gene expression levels (Figure 3.19b).

Figure 3.19. VEGF-A expression increases throughout differentiation.

(a) Time course analysis of *VEGF-A* gene expression measured using qRT-PCR. (b) Time course analysis of VEGF-A protein secretion in culture medium measured using ELISA. Data are presented as mean $\pm$ SEM ($n = 2$ independent experiments with 3 technical replicates).

3.3.3. VEGF-A/VEGFR signalling axis drives kidney organoid vasculogenesis

To validate that VEGF-A was the primary signal driving vascular formation in our kidney organoids, the effects of three FDA-approved VEGF receptor inhibitors on the vasculature network were examined. If the chemical blockade of VEGF receptors result in the demise of the vascular endothelial network, it would confirm that vascular development in hPSC-derived kidney organoids was dependent on VEGF-A. Treatment of the kidney organoids with low doses (10 nM) of either Axitinib, Cediranb or Tivozanib severely compromised the vasculature network in 1 or 3 days (Figure 3.20a). Nephron structures within were unaffected, as evaluated with NPHS1 and CDH1 staining. These results demonstrated that vascularization of kidney organoids is directly dependent on VEGF-A.

Figure 3.20. Inhibition of kidney organoid using VEGF receptor inhibitors.

(a) Immunostaining analysis of day 24 kidney organoids treated with three different VEGF receptor inhibitors for 1 day (top panels) or 3 days (bottom panels). Representative markers used for each segment – NPHS1 for podocytes; CD31 for vascular endothelial cells; CDH1 for distal tubules and DAPI for nuclei.

3.3.4. Identification of podocytes as the major source of VEGF-A

Podocytes secrete VEGF-A throughout development and homeostasis to maintain the glomerular vasculature (Eremina et al., 2006). To confirm if podocytes in these hPSC-derived kidney organoids are the main producers of VEGF-A, as their *in vivo* counterparts, we dissociated kidney organoids into single cells and isolated them using an anti-podocalyxin (PODXL) antibody. Up to 3.1% of all the cells within kidney organoids were PODXL⁺, which was a modest number as the tight junctions between these cells makes dissociation challenging (Figure 3.21a). Gene expression was compared between the PODXL⁺ podocytes and PODXL⁻ population, which comprised of all other cell types. The PODXL⁺ fraction was highly enriched in *PODXL*, *NPHS1* and podocin (*NPHS2*), confirming that podocytes were successfully enriched and extracted from the rest of the population. VEGF-A expression in the PODXL⁺ fraction was 10-fold higher than the PODXL⁻ fraction, confirming that podocytes are the major producers of VEGF-A to drive kidney organoid vasculogenesis (Figure 3.21b).

Figure 3.21. Podocyte-derived VEGF-A drives kidney organoid vasculogenesis.

(a) Enrichment of PODXL⁺ cellular population from kidney organoid using FACS. (b) Gene expression analysis of podocyte-specific genes and VEGF-A from the enriched and residual population using qRT-PCR. Data are presented as mean $\pm$ SEM ($n = 2$ independent experiments with 3 technical replicates).

3.3.5. Unveiling an unappreciated lineage potential of nephron progenitor cells

Vasculature development commences from the *de novo* formation of angioblasts that originate from the yolk sac. Migration and innervation of these vessels through the fetus elaborates the entire vasculature system (Coffin et al., 1991; Hatzopoulos et al., 1998; Risau, 1997). Within

each organ, these primitive vascular plexuses undergo significant remodelling to establish hierarchical network with tissue-specific functionality (Adams and Alitalo, 2007; Carmeliet, 2003; Eichmann et al., 2005; le Noble et al., 2004). Recent studies have shown that vasculature in different organs may be contributed by resident progenitors residing in within, explaining for the complexity of various tissue-specific endothelial phenotypes (Cheung et al., 2012; Goldman et al., 2014; Marcu et al., 2018, 2018; Mugford et al., 2008b; Peng et al., 2013).

Having identified day 11 as the window for vascular progenitor emergence, co-staining of KDR and several NPC-specific markers was performed. Surprisingly, KDR⁺ cells almost always co-express NPC markers, including SIX1, SIX2 and SALL1 (Figure 3.22a). Using flow cytometry, single cells from day 11 to 14 of differentiation were profiled to uncover the kinetics of vascular lineage specification. SIX1⁺ NPCs were induced at high efficiencies (>80%) with acquisition of KDR, but not CD31 at day 11 of differentiation. Progressively, SIX1⁺ KDR⁺ cells acquire CD31⁺ from day 13 onwards (Figure 3.22b,c). Strikingly, these novel population of cells make up around 1 to 3% of the total cell population (Figure 3.22c). Together, these evidences showed that a subpopulation of NPCs may acquire a vascular progenitor identity to generate the vascular network within hPSC-derived kidney organoid. In the later part of the thesis, we named these cells “renal vascular progenitors” for ease of explanation.

Figure 3.22. Lineage specification kinetics of a novel renal vascular progenitor cell population.

(a) Immunofluorescence analysis of day 11 differentiating cells for NPC-specific markers and KDR. (b) Flow cytometry analysis of day 11 to 14 differentiating cells for SIX1, KDR and CD31 (analysis performed with help from Miss Tian Zhang). (c) Quantification of the flow cytometry data presented in (b).

3.2.6. Single-cell transcriptomics of early nephrogenesis

The population of these renal vascular progenitor cells is about 1-3%, making it challenging to isolate a sufficient number of viable cells from the whole population for analysis. Routine bulk transcriptomics analysis such as qRT-PCR and RNA sequencing analysis would not work as the transcriptomics of the minority cell population would be diluted out by the rest of the cells. A viable approach to study this population of cells would be to employ the use of single-cell RNA sequencing (scRNA-Seq) analysis. Empowered with the ability profile the transcriptomics from each cell, this technology will enable the study of rare cell populations in (Gawad et al., 2016; Pennisi, 2018). We sought help from our collaborators at Genome Institute of Singapore – Dr Elaine Chew, Miss Michelle Lian and Asst Prof Jia Nee Foo, who will be helping us with the scRNA-Seq experiment and computational analysis.

To study the kinetics of vascular endothelial cell fate acquisition from NPCs, we sequenced a total of 62,506 cells from day 10, 12 and 14 of differentiation, respectively. Cells underwent strict quality control measures, of which entries with excessively high transcripts counts and mitochondria genes were removed. Unsupervised clustering of these pooled dataset followed by *t*-distributed stochastic neighbour embedding (*t*-SNE) analysis revealed 11 distinct clusters (Figure 3.23a). These 11 clusters were annotated into 6 main classes of cells that were recently defined in other studies including NPC, early NPC, nascent nephron, stromal, proliferating cells, and endothelial cells (Figure 3.23b)(Lindström et al., 2018a; Menon et al., 2018; Park et al., 2018; Phipson et al., 2019; Wu et al., 2018b). Strikingly, cluster 10 was distinctively separated from the rest of the population. This cluster was highly enriched in proliferative and endothelial markers, including CD34, CD31, SOX17, KDR. We postulated that cluster 10 is likely to be the nascent endothelial cluster (Figure 3.23a,b). Using cell counts, we counted the percentage of cells that co-expressed SIX1, KDR and CD31. We detected increasing number of cells that expressed both SIX1 and KDR over time (Figure 3.23c). A SIX1⁺KDR⁺CD31⁺

triple population, which likely represented maturing vascular progenitors emerged at day 12. Interestingly, this population of cells always reside within 1 to 3% of the total cell number, in agreement with our flow cytometry data. To understand the differentiation of trajectory of these differentiating NPCs, we ordered cells along pseudotime using Monocle 2. Cells were accurately ordered along pseudotime, with day 10 cells at the root and day 14 cells bifurcating at the adjacent end (Figure 3.23d, e). These results indicate that cells likely originate from a common progenitor before adopting a divergent differentiation trajectory. Lastly, we examined differentially expressed genes (DEGs) during each bifurcation point along pseudotime. Gene ontology (GO) analysis on the DEGs were associated with kidney development. Of note, we identified that branchpoint 2 was highly associated with GO terms associated with blood vessel formation, suggesting a relatively early lineage bifurcating event. (Figure 3.23f).

Figure 3.23. Single cell RNA sequencing analysis of cells undergoing early nephrogenesis.

(a) tSNE plot from cells at day 10, 12 and 14 of differentiation identified 11 clusters representing distinct transcriptional states (analysis performed by Dr Elaine Chew, Miss

Michelle Lian and Asst Prof Jia Nee Foo). (b) Violin plots showing expression of representative marker genes. (c) Cell counts based on co-expression of SIX1, KDR and CD31 (analysis performed by Dr Elaine Chew, Miss Michelle Lian and Asst Prof Jia Nee Foo). (d) and (e) Pseudotime ordering of cells using Monocle 2 identifies 4 branchpoints (analysis performed by Dr Elaine Chew, Miss Michelle Lian and Asst Prof Jia Nee Foo). (f) Gene ontology analysis of branchpoint 2 highlight DEGs involved in blood vessel formation (analysis performed by Dr Elaine Chew, Miss Michelle Lian and Asst Prof Jia Nee Foo).

3.4. Engraftment and functional maturation of hPSC-derived kidney organoids following *in vivo* implantation

3.4.1. Implantation of hPSC-derived kidney organoids into the renal capsule of NSG mice

The NOD SCID gamma (NSG) mice is an immunodeficient mouse model that lack several features of a functional innate and adaptive immune system. The absence of cytokines, B cells, NK cells and mature T cells permits the engraftment of a wide range of foreign cells and materials to investigate their phenotype *in vivo* (Shultz et al., 2007). The renal capsule was selected as an ideal transplantation site due to its rich vascular bed and ease of surgical manipulation (Sharmin et al., 2015; Takebe et al., 2013). To investigate the influence of an *in vivo* niche on the functionality and maturation of hPSC-derived kidney organoids, day 24 kidney organoids were implanted underneath the renal capsule of NSG mice (Figure 3.24a).

A total of up to 8-12 kidney organoids were implanted underneath each kidney capsule. Kidney organoids were small and flat, and the renal capsule could hold up to 12 organoids securely with every organoid contacting the vascular bed. Implanted kidney organoids engrafted rapidly, with extensive neovascularization observed in 7 days (Figure 3.24b). To access the viability of the graft and to trace its locality, kidney organoids generated from H1-embryonic stem cells stably expressing the Firefly luciferase transgene (H1-Luc) was used. Mice that received these implants were luminescent when the substrate luciferin was systemically administered, confirming that these grafts were viable and remained in the expected locality (Figure 3.24c).

In 4 weeks, the grafts grew larger in size, and a visible microcapillary network was observed throughout the implant (Figure 3.24a). Additionally, the addition of a Firefly luciferase transgene into H1 hESCs had no detectable impact on pluripotency or the potential of cells to undergo kidney differentiation. Characterization of kidney organoids derived from H1-Luciferase exhibit similar differentiation efficiency as wildtype hESCs (Figure 3.24d,e). Implanted H1-Luc-derived kidney organoids formed vascularized glomeruli, Bowman's capsule-like space and maintained the expression of segment-specific tubular markers (Figure 3.24f, g).

Figure 3.24. Successful engraftment and survivability of hPSC-derived kidney organoids.

(a) Workflow and timeframe for the implantation of kidney organoids into NSG mice. (b) Gross examination of graft following 7 days of implantation. (c) In vivo luciferase assay on mice receiving hPSC-derived kidney organoids expressing Firefly luciferase. (d) Brightfield image of kidney organoids derived from H1-Luc. (e) Immunofluorescence analysis of kidney organoids at day 24 of differentiation. (f) Immunofluorescence analysis of a vascularized glomerulus 4 weeks following implantation. (g) Immunofluorescence analysis of tubular structures 4 weeks following implantation. Representative markers used for each cell type – NPHS1 for podocytes; LTL for proximal tubules; CDH1 for distal tubules; hCD31 for human-specific vascular endothelial cells and DAPI for nuclei.

3.4.2. Histological analysis of recovered grafts 4 weeks post implantation

For routine transplantations, kidney organoids generated from H9-ESCs were employed. Kidney organoid implants were collected 4 weeks after implantation for histological analysis. Nephron-like structures were seen scattered throughout the whole graft . A substantial volume of the graft was comprised of fibroblasts, which proliferated extensively as compared to the renal cells (Figure 3.25a). Interestingly, blastema or teratoma formation was absent in our system as compared to previous reports, indicative that the kidney organoids were well-differentiated (Bantounas et al., 2018; van den Berg et al., 2018). Within the grafts, contiguous nephron segments were observed, with a glomerulus connected to adjacent tubules (Figure 3.25b). Tubular epithelium was identified by their simple cuboidal epithelium morphology and a distinctive lumen (Figure 3.25c). Importantly, the glomerulus undergoes significant structural maturation, including Bowman's capsule-like epithelium formation and glomerular vascularization. Red blood cells were seen to perfuse throughout the glomerulus, indicating that avascular glomeruli were able to draw in vasculature to form the glomerular capillary tuft following implantation (Figure 3.25d).

Figure 3.25. Histological analysis of kidney organoids following 4 weeks implantation. Representative H&E section of (a) whole graft, (b) a contiguous nephron, (c) tubular epithelium and (d) vascularized glomeruli with red blood cells.

3.4.3. Evaluation of implant maturation using immunofluorescence staining

As the filtration apparatus of the kidney, maturation of glomeruli following implantation was important towards achieving functionality. Immunofluorescence analysis was performed on 4-week old grafts using glomerular-specific markers. In the implanted glomerulus, the distribution of NPHS1, were localized to podocyte-podocyte contacts. This staining pattern was distinctive from NPHS1 distribution in cultures, which adopted a ubiquitous expression pattern (Figure 3.26a). Earlier studies have shown that vasculature from the host are able to vascularize implanted cellular constructs *via* angiogenesis (Bantounas et al., 2018; Takebe et al., 2013; van den Berg et al., 2018). Using a human-specific CD31 antibody, the presence of human-derived endothelial cells within the glomeruli were detected (Figure 3.26b). This suggests that with the introduction of blood flow, hPSC-derived vascular endothelial cells may

undergo remodelling and glomerular invasion to form the glomerular capillaries. Furthermore, PDGFR β ⁺ mesangial cells and parietal epithelium cells were observed within and around the glomerulus, respectively (Figure 3.26c). Altogether, these results indicate that an *in vivo* environment facilitates the structural maturation of glomerulus in kidney organoids.

Figure 3.26. Characterization of glomerular maturation following 4-weeks transplantation.

Immunofluorescence analysis of (a) silt-diaphragm structures marked by NPHS1, (b) human-derived vascular endothelial cells marked by human-specific CD31 (hCD31) and (c) mesangial cells marked by PDGFR β . WT1 labels the podocytes and DAPI labels the nuclei..

3.4.4. Ultrastructural analysis of 4 weeks implants

Several cellular features of the kidneys are not resolved using histological or immunohistochemical techniques. Many of these features, which include looking at cell projections, fenestrae or basement membranes require the use of electron microscopy. To assess for features of both structural and functional maturation, both transmission and scanning electron microscopy (TEM and SEM, respectively) was performed on 4-week old implanted kidney organoids.

The formation of a Bowman's capsule space was cross validated using SEM imaging of a dissected glomeruli. Within the Bowman's capsule, podocytes with interdigitating foot processes were observed (Figure 3.27a,b). The organization of these cells mirror that of the native glomerulus, with the podocytes lining the Bowman's capsule-like space (Figure 3.27a). Using TEM analysis on ultrathin sections, we were able to identify that podocytes harboured interdigitating cellular projections resembling primary and secondary foot processes (Figure 3.27c).

Figure 3.27. Ultrastructural analysis of glomeruli within implanted kidney organoids.

Representative SEM images of (a) an exposed Bowman's capsule-like space and glomerulus; (b) podocytes with interdigitating foot processes. (c) Representative TEM images of primary and secondary foot processes between podocytes. "P" denotes cell body of the podocytes (analysis performed with help from the Advanced Bioimaging Facility at SingHealth Duke-NUS Academic Medical Centre or the Electron Microscopy Unit at Yong Loo Lin School of Medicine, National University of Singapore).

Next, tubular compartments within the implants were visualized using SEM. Cross section of a tubule harboured a distinctive lumen and cuboidal epithelium. Numerous microvilli within the apical lumen of the tubule, which are important for expanding the surface area for reabsorption were observed (Figure 3.28a, b). There were also distinctively long cilia among the microvilli in the tubular lumen, which are essential for mechano-sensing and signal

transduction (Figure 3.28a). Between proximal tubules, tight junctions bridged cell-cell contacts, and were visualized as electron-dense structures using TEM (Figure 3.28c).

Figure 3.28. Ultrastructural analysis of tubules within implanted kidney organoids.

(a) Representative SEM images of a cross section of a tubule showing numerous microvilli and cilia. Representative TEM images of proximal tubules showing (b) brush borders and (c) electron-dense tight junctions. “T” denotes proximal tubule cell body (analysis performed with help from the Advanced Bioimaging Facility at SingHealth Duke-NUS Academic Medical Centre or the Electron Microscopy Unit at Yong Loo Lin School of Medicine, National University of Singapore).

Finally, the presence of the glomerular filtration barrier, which is a critical for filtration, was examined. I started this process by locating red blood cells within the implants, which were indicative of perfused microcapillaries. In some instances, it was possible to find a glomerular basement membrane deposition and a podocyte on the adjacent side (Figure 3.29a,b). These

structures suggests that there is an establishment of a glomerular filtration barrier formation. There were also small gaps present on the endothelial layer, resembling pore-like features in fenestrated glomerular capillary cells. Altogether, these suggests that implantation of hPSC-derived kidney organoids promotes the maturation of several structural features otherwise unobserved *in vitro*.

Figure 3.29. Ultrastructural analysis of the glomerular filtration barrier within implanted kidney organoids.

(a) Representative TEM image of glomerular filtration barrier-like formation showing fenestrated endothelium, basement membrane and the putative glomerular filtration barrier. (b) Representative TEM image of glomerular filtration barrier-like formation showing fenestrated endothelium and basement membrane formation. “R” denotes red blood cells, “BM” denotes basement membrane, “P” denotes podocyte cell body and arrowheads indicate endothelial fenestrations (analysis performed with help from the Advanced Bioimaging Facility at SingHealth Duke-NUS Academic Medical Centre or the Electron Microscopy Unit at Yong Loo Lin School of Medicine, National University of Singapore).

3.4.5. *In vivo* dextran handling assay by 4 weeks implants

The glomerulus performs selective filtration based on two key principles – charge and size. Sugar moieties on the surface of the fenestrated endothelial cells repel most of the negatively charged protein molecules, such as albumin, which is abundant in the blood plasma. Silt-diaphragms formed by interdigitating foot processes forms a size-selective filtration mechanism, retaining large molecules while allowing the smaller ones to pass through. With the formation of a rudimentary filtration barrier, it is possible that these glomerular structures were capable of filtration.

I employed a well-established *in vivo* dextran assay to assess the filtration and reabsorption capability of vascularized implanted tissue constructs (Bantounas et al., 2018; Xinaris et al., 2016a, 2012). Phycoerythrin-labelled dextran molecules of two different sizes – 70 kDa representing a small molecular weight and 2,000 kDa representing a large molecular weight – were systemically administered into mice bearing 4-week-old implants. The mice were sacrificed two hours later, and the implants were harvested for analysis. The 70 kDa dextran was detected in blood vessels and within the glomerular capillary tufts (Figure 3.30a). Some of the dextran was observed to bypass the glomerular filtration membrane and accumulated in the Bowman's capsule-like space and proximal tubules. Interestingly, intracellular accumulation of dextrans in small puncta within LTL⁺ proximal tubules resemble endosomes (Figure 3.30a). In contrast, the significantly larger 2,000 kDa dextrans were not detected in the LTL⁺ proximal tubules (Figure 3.30b). However, they were detected within blood vessel lumen, which provided additional evidence of successful anastomosis. These findings were cross validated with dextran injections in the adult mouse kidney. Expectedly, dextran handling profiles were similar between implanted kidney organoids and the embryonic kidneys, with accumulation of 70 kDa dextrans within proximal tubules but not the 2,000 kDa dextran (Figure

3.30c). Together, these results indicated that the implanted kidney organoids undergo significant structural maturation which enabled rudimentary filtration and reabsorption.

Figure 3.30. Filtration and reabsorption in mouse using fluorescently labelled dextran.

(a) Immunohistochemistry images of 4-week-old implants receiving low molecular weight dextran and (b) large molecular weight dextran. (c) Immunohistochemistry images of adult mouse kidney receiving low molecular weight dextran (top panels) and high molecular weight

dextran (bottom panels). Asterisks denote glomeruli and arrows indicate dextran accumulation in the proximal tubules of implanted organoids. Representative marker used for each segment – LTL for proximal tubules and DAPI for nuclei.

3.4.6. Rudimentary urinary filtrate production and accumulation

The grafts were carefully examined for features of functional maturation. In many cases, dilated tubules with an enlarged lumen could be seen. These lumens contained eosin-positive precipitation, which may imply the accumulation of urinary filtrate (Figure 3.31a). I hypothesize that these are histological artefacts generated by the dehydration of likely proteinaceous filtrate, which acquired a pinkish eosin staining pattern. These artefacts were first reported by the Nishinakamura group when they implanted podocytes into an immunocompromised animal (Sharmin et al., 2015). To provide assurance that this phenotype was representative of filtrate staining, histological analysis using the same approach was performed on mouse E19.5 kidneys, which are likely to have initiated filtration. Expectedly, precipitation within the tubules of these kidneys was observed, in a manner that was similar to that in the implants (Figure 3.31b). Immunofluorescence analysis revealed that these structures were positive for dolichos biflorus agglutinin (DBA) and CDH1, which were markers of the distal tubule. Interestingly, these structures were negative for LTL, a marker of proximal tubule, and indicated that these filtrate were trapped within the distal end of implanted nephrons (Figure 3.31c)

Figure 3.31. Rudimentary filtrate accumulation in dilated tubules.

(a) H&E sections of 4 weeks-old implants. (b) H&E sections of a mouse embryonic kidney. (c) Immunohistochemistry images of dilated cysts for proximal (LTL) and distal tubular (CDH1 and DBA) markers.

These findings indicate that implanted kidney organoids mature in structure, which include glomerular vascularization, tubular maturation, and Bowman's capsule-like space formation. In turn, these structural maturations conferred rudimentary kidney functions such as size-specific dextran handling and filtrate formation, both of which were otherwise unachievable *in vitro*.

3.5. Extensions of the protocol

3.5.1. Cryopreservation of hPSC-derived NPCs

The duration required to produce kidney organoids from differentiation of hPSCs is about 3-4 weeks across all the protocols that were reported (Freedman et al., 2015; Low et al., 2019; Morizane et al., 2015; Przepiorski et al., 2018; Takasato et al., 2015). This is excluding the time, cost and skills required for hPSC and hiPSC maintenance. The development of

approaches to reduce the amount of time required for kidney organoid generation would be extremely useful to accelerate the research process.

One possible option would be to cryopreserve hPSC-derived NPCs at around day 10 of differentiation. These NPCs can then be revived, and the differentiation can be resumed to generate kidney organoids. When applied to our protocol, this would remove more than 10 days off the 24 days that were required, enabling kidney organoids to be generated in as little as 14 days. However, there are currently no reports of successful hPSC-derived NPC cryopreservation. An optimal freezing protocol should achieve a high survivability and retain the multipotential of NPCs.

At day 10-12 of differentiation, NPCs were gently dissociated into single cells using Accutase treatment. Cells were cryopreserved in basal differentiation media containing 10 μ M Y-27632, 50 ng/mL FGF9, and 10% DMSO (Figure 3.32a). We reasoned that Y-27632 promotes cell survival following single-cell dissociation, and DMSO functions to prevent the formation of intracellular ice crystal formation.

Revived NPCs exhibited a high cell viability (>80%) as assessed using Trypan Blue staining (Figure 3.32b) and can be revived on Matrigel-coated plates with the addition of 10 μ M Y-27632 for 24 hours. Live cells, but not dead cells, should attach to the Matrigel-coated plates. Kidney organoids can then be generated from these revived NPCs, following the original differentiation protocol and timeframe. In 3 days, NPCs formed pre-tubular aggregates, providing further assurance that NPCs were successfully revived and indicate the onset of nephrogenesis (Figure 3.32c). At day 14 post thawing, kidney organoids with numerous nephron-like structures are established (Figure 3.32d). Immunofluorescence analysis revealed that all segments of the nephrons were formed – including NPHS1⁺PODXL⁺ podocytes, LTL⁺ proximal tubules and CDH1⁺ distal tubules (Fig. 3e). In addition, the formation of CD31⁺

vasculature provides further assures that NPCs retained their multipotency following freeze-and-thaw cycle (Figure 3.32e).

Figure 3.32. Cryopreservation of hPSC-derived NPCs

(a) Schematic of workflow and timeline for cryopreservation and revival. (b) Assessment of cell viability using Trypan blue staining. (c) Brightfield analysis of revived cultures 3 days post thawing showing pre-tubular aggregate formation. (d) Brightfield of revived cultures 14 days post thawing showing formation of nephron-like structures. (e) Immunofluorescence analysis of nephron-like structures generated using revived NPCs showing nephron-like

structures and vasculature. Representative marker used for each cell type – NPHS1 for podocytes; LTL for proximal tubules; CDH1 for distal tubules; CD31 for vascular endothelial cells and DAPI for nuclei.

3.5.2. High-content screening platform using hPSC-derived kidney organoids

Our current protocol for generating kidney organoids necessitates the bifurcations of cell culture approaches, with a 3D intermediate stage before liquid-air interface culture (Low et al., 2019). It is labour intensive and unamenable for high-throughput screening (HTS) approaches, which are invaluable to accelerate drug discovery. With this motivation in mind, efforts were focused on trying to develop a high-throughput workflow and platform for generating kidney organoids.

At around day 10-12 of differentiation, single NPCs were replated onto Matrigel-coated 96- or 384-multiwell plates. Differentiation was continued for another 14 days for the formation of kidney organoids (Figure 3.33a). Notably, for self-organization and nephrogenesis to take place on 2D surfaces (on 96- or 384-well plates), an optimal cell number is required. At sub-optimal confluency, NPCs formed a fibroblast-like morphology and differentiation was ceased. A minimum of 75,000 NPCs is required for each 96-well plate (which equates to a cell density of $\sim 234,375$ cells/cm²) for self-organization to take place, with a high starting cell number generating a higher number of nephron structures (Figure 3.33b). Drug treatment, if any, may be performed once the nephron structures are established at around day 20 of differentiation (Figure 3.33a). Using immunofluorescence analysis, various segment-specific regions of the nephrons were identified. Podocytes marked by NPHS1 are organized in small clusters and are located next to LTL⁺ proximal tubules. These proximal tubules are connected to adjacent CDH1⁺ distal tubules, representative of segmented nephrons (Figure 3.33c). Each well contains up to hundreds of nephron structures, providing enough sample size for statistical analysis.

Images acquired on a high content analyser can be automatically processed using commercial programmes, such as Harmony (Perkin Elmer) or Investigator (GE INCell) software. Alternatively, images can also be acquired on a confocal microscope and analysed manually using ImageJ software (Figure 3.33d).

Figure 3.33. High content analysis platform using hPSC-derived kidney organoids

(a) Schematic of workflow for high content screening. (b) Brightfield imaging of the optimal cell number required for self-organization and nephrogenesis on multi-well plate surface. (c) Whole well immunofluorescence analysis of kidney organoids at day 24 showing (>100s nephron-like structures per well) in a single 384-well (left) and 96-well (right). (d) An example

of a manual image analysis pipeline using an open source image processing software, ImageJ. Representative marker used for each segment – NPHS1 for podocytes; LTL for proximal tubules; CDH1 for distal tubules; CD31 for vascular endothelial cells and DAPI for nuclei.

Organoids represent an excellent in vitro human organ model and may better predict drug responses as compared to traditional 2D human cell lines, of which critical cell-to-cell and cell-to-matrix interactions were absent. The establishment of a high-throughput screening platform based on kidney organoids would enable large-scale screenings. However, this has been challenging to achieve due to specialized requirements for morphogenesis to happen. Here, I have shown that as a proof-of-concept that it is possible to generate kidney organoids in large-format multi-well plates and subject them to high content analysis. With this approach, it may be possible to carry out large scale assays and predict segment-specific alterations in a shorter amount of time.

4. Discussion

4.1. Stepwise and efficient directed differentiation of hPSCs into kidney organoids

In this thesis, I describe the generation of vascularized 3D kidney organoids from hPSCs, performed an extensive characterization of its cellular composition, structural features, and functionality. The protocol results in a high induction of NPCs that expresses SIX1, SIX2, SALL1 and CITED1, and is comparable to previously published protocols (Morizane et al., 2015). In both 2D and 3D formats, NPCs spontaneously undergoes MET to form nephrons, while transiting past intermediate stages resembling RV, CSB and SSBs. These early structures undergo further morphogenesis into nephron-like structures. A thorough investigation of these cells using highly established markers confirmed the presence of podocytes and various tubular cell types. Importantly, markers attributing to a particular segment were not seen to be overlapping into other segments, which indicates that tubules have acquired a certain maturity status (Morizane et al., 2015; Przepiorski et al., 2018). For instance, the LTL expression was always separated from CDH1 expression, indicative of a clear separation between the two tubular segments. Additionally, the expression of AQP2, a marker of the distal convoluted tubule, was always localized to CDH1⁺, but not LTL⁺ segments. In addition, a suite of kidney-relevant supportive cell types was also co-differentiated in parallel, such as the vasculature, stromal and neurons. The presence of these cells suggests that the kidney organoid microenvironment is a niche permissive for the specification and maintenance of these cells. Additionally, having these cells in our kidney organoid expands their application. For instance, neurons within the kidneys are emerging as a new paradigm for understanding hypertension (DiBona, 2002; Wyss et al., 1992). Hence, kidney organoids with an established neuronal network may provide a new reductionist approach to investigate the function of these cells in

the kidney. The discovery of stromal cells within kidney organoids can be used to study myofibroblast differentiation and renal fibrosis (LeBleu et al., 2013; Mack and Yanagita, 2015; Przepiorski et al., 2018). Importantly, these cells collectively make up just a small population of the organoid, as kidney-specific cell types and nephron-like structures still constitute majority of the cells within these organoids.

Both our protocol and the Morizane protocol were able to induce a high efficiency of NPCs from hPSCs, despite differences in protocol. It was possible to omit the 3 days of Activin A treatment from day 4 to 7, which was previously shown to be necessary to induce HOXD11 expression (Morizane et al., 2015). I demonstrated that this supplementation was not required, and immunostaining of our cells at day 7 expressed HOXD11 without the need for Activin A.

How different are the NPCs generated using the Morizane protocol compared to ours? Evidently, NPCs from both protocols were able to self-organize and form segmented nephron-like structures. Hence, in principle, they should be relatively similar. Results from our bulk transcriptomics results indicate that an upregulation of cardiac and lung signatures were detected in the Morizane protocol, which I hypothesized might arise due to the use of Activin A. Two possibilities might explain this difference. Firstly, assuming that NPCs are similar in terms of gene expression profile, it could be possible that the DEGs are a consequence of the 10% of uncharacterized cell types that were different between the two protocols. These gene signatures are detected using RNA sequencing and present as DEGs. Secondly, there is a difference in the type, quality, or state of NPCs generated by the two protocols. The concept of different NPC “quality” was first proposed, to explain the different routes that were possible to generate kidney organoids and the progenitor cell types (Nishinakamura, 2019). A possible way to answer this question would be to use single cell transcriptomics. At a single cell level, all cells in the differentiating culture, including NPCs and off-target cells can be identified, mapped, and compared.

4.2. Generating kidney organoids with both UB and MM

I acknowledge the lack of UB-derived cells and associated collecting duct structures within the kidney organoids generated using our protocol. Specifically, our protocol generates NPCs at around 90% efficiency. These NPCs were able to undergo self-organization and morphogenesis into segmented nephron-like structures in the absence of UB-derivatives.

In the mouse, *Osr1*⁺ IM cells gives rise to both the UB and MM (Mugford et al., 2008b). In this regard, the IM is a common intermediate and should be able to generate both the UB and MM. However, genetic lineage tracing work by the Nishinakamura group have suggested that there is a need to revisit IM specification. In the mouse, the anterior IM is formed at E8.5 and generates the nephric duct and UB. The nascent IM at the posterior end of the embryo is specified at E8.5, and differentiates into the posterior IM at E9.5 (Nishinakamura, 2019; Taguchi et al., 2014). Importantly, these two IM populations are distinct from each other and in both space and time. Together, these results would indicate that the IM likely bifurcated early in development, and separate protocols may be required generate pure populations of either the UB or MM.

This information, or hypothesis, may be important for the refining of current directed differentiation protocols. In theory, if the two populations had bifurcated early in development, an efficient directed differentiation protocol should only be able to induce a specific lineage, whilst restricting the other (Morizane et al., 2015; Taguchi et al., 2014; Taguchi and Nishinakamura, 2017). Conversely, if the two population share a common IM progenitor, it would be possible to induce both populations in a single protocol (Takasato et al., 2015; Takasato and Little, 2015).

The Takasato protocol was reported to be able to generate both nephrons and ureteric epithelium by co-differentiating MM and UB in a single protocol (Takasato et al., 2015).

However, emerging evidence have suggested that the GATA3⁺CDH1⁺ presumptive ureteric epithelium population are MM-derived distal tubules. Indeed, these CDH1 cells were not reported to express additional collecting duct markers, such as AQP2 or AQP4 when subjected to analysis using single cell RNA sequencing (Wu et al., 2018b). By benchmarking kidney organoids from the two protocols, these CALB1⁺GATA3⁺ presumptive collecting ducts were also observed in kidney organoids produced using the Morizane protocol (Wu et al., 2018b). Hence, this study indicates that GATA3 is may be expressed in distal tubules and suggests that the presumptive GATA3⁺CDH1⁺ UB population from the Takasato protocol may be MM-derived.

4.3. Patterning nephron-segments within hPSC-derived kidney organoids

The nephron is a contiguous tube structure that harbours different epithelial cells lining the lumen, with segments named after their anatomical location relative to the glomerulus. The differentiation of these specialized cells are dependent on regional cell signalling and transcription (Kopan et al., 2007). Hence, ‘patterning’ these segments refers to the ability to modulate the different cell types along these nascent nephrons. Patterning has been successfully achieved using *ex vivo* culture studies, usually by the culture of an embryonic organ, or embryonic cell-derived organoid with pathway modulators (Costantini and Kopan, 2010; Davies, 2008; Davies et al., 2014; del Moral and Warburton, 2010; Lindström et al., 2015; Mills et al., 2017).

A comprehensive appreciation of how nephrons are segmentally patterned will aid in the reconstruction of functional nephrons from hPSCs. A series of *ex vivo* culture experiments have shown that β -catenin is indispensable throughout nephrogenesis, and regulates segmentation by cross-interactions with PTEN/PI3K, Notch and BMP signalling pathways (Lindström et al., 2015). Using TCF/Lef::H2B-GFP reporter mice, highest levels of Wnt activation were detected

at the distal regions of the nephron which is postulated to be induced by UB-derived Wnt9b (Carroll et al., 2005; Karner et al., 2011; Lindström et al., 2015). At the proximal end, while β -catenin expression was also detected, high levels of the phosphorylated form indicates that these proteins are likely targeted for breakdown and harbour no activity. At the medial end of the nephron BMP/PTEN/PI3K activity was detected by the phospho-SMAD staining and negatively regulates β -catenin. A low β -catenin activity coupled with Notch signalling plays a key role in proximal tubule specification. Pharmacological inhibition of Notch resulted in elevated β -catenin, which led to impaired proximal tubule formation (Cheng et al., 2007; Lindström et al., 2015). Collectively, these results highlight the role of β -catenin as a key regulator of proximal nephron patterning, of which its regulation is influenced by other signalling pathways. However, these were lesser explored in a hPSC-derived kidney organoid system, owing to differences in the property of differentiated cells and technical difficulties.

I postulated that the timing of a 3 μ M CHIR99021 treatment at day 7 to 9 in our protocol (as compared to day 9 to 11 in the Morizane protocol) did not affect NPC proximal-distal specification, which was happening at a later time point (Morizane et al., 2015). Hence, it is then possible to proximal-distal patterning events using a 1 μ M CHIR99021 treatment when kidney organoids are mostly at the RV stage of development at around day 13 to 15. In comparison to the Takasato protocol, a short, but high concentration CHIR99021 pulse was observed to induce distal specification which were closer to the liquid interface (Takasato et al., 2015). Using our protocol, 3D kidney organoids at day 14 of differentiation contain renal vesicles that are responsive to patterning signals. At this stage, a 1 μ M CHIR99021 pulse may be administered to increase the amount of distal compartments, while inhibiting the proximal compartments. The effects of this patterning signal are profound, with obvious cell fate modulation detected with 1 day of patterning. Hence, the duration of the timing, and to a lesser extent the dose, is critical for the extent of patterning. Importantly, using just a single drug

treatment, it is possible to also adjust the vasculature and the stromal population within kidney organoids. This is likely due to multilineage communication within the kidney organoids, with VEGF-A-derived from the podocytes driving vasculogenesis. Stromal outgrowth could be discernible using brightfield imaging, contributing to a larger organoid area and coverage. Expectedly, the patterning signal does not have an effect when the nephron segments and vasculature are already specified after more than 20 days of differentiation. Again, this confirms that the developmental window for patterning to take place is transient, and this is similar to early nephron development *in vivo*.

An important consideration was that hPSC-derived NPC cells may not be equivalent to embryonic-derived NPCs cells. However, they exhibit several features of bona fide NPCs that make them useful for studying nephron development, such as the capacity to self-organize and undergo nephrogenesis (Morizane et al., 2015). Several groups and ours have postulated that both embryonic-derived and hPSC-derived NPCs follow a default podocyte differentiation trajectory unless influenced by other signalling pathways (Lindström et al., 2018a; Low et al., 2019). Hence, these suggests that the quality of NPCs generated from hPSCs are getting closer to their embryonic counterparts.

4.4. Potential applications of this patterning approach

The ability to modulate the differentiation trajectory of multiple cell types within a kidney organoid simultaneously using a low dose of Wnt/ β -catenin activator is easy to carry out. Additionally, the absence of UB presents an avenue to investigate nephron development in the absence of exogenous influences, allowing the dissection of pathways regulating segmentation and patterning. Furthermore, this approach is amenable to time-dependent dosing, where a shorter duration of treatment offers more proximal cell types as compared to more distal cell

types with a longer duration of treatment. In synchrony, the amount of vasculature is indirectly affected by the podocytes population and can be modulated as such.

This technique may facilitate more targeted disease modelling approaches, enabling cell populations to be tailored according to the needs of the research objective. For instance, kidney organoids which are not patterned would be useful to study glomeruli-associated diseases (e.g. nephrotic syndrome and diabetic nephropathy), as they contain mostly podocytes and vascular endothelial cells. Patterned kidney organoids containing mostly proximal and distal tubules may be harnessed to study tubular-associated diseases (e.g. polycystic kidney disease and tubulopathies). Hence, this approach expands the repertoire and versatility of applications for hPSC-derived kidney organoids.

There are several limitations of this technique. The current approach of using a treatment as a patterning signal induces a “global” activation or inhibition of the specific pathway, in which all the renal vesicles are exposed to the signal in all directions. Nephron patterning is a complex developmental event, governed by various signalling pathways and cell-cell interactions. Together, these contribute localized and precise delivery of signals to govern the differentiation of cells and proliferation of a particular segment. An interplay of additional signalling including BMP, PTEN, TGF β and Notch are required for essential for proper axis specification (Lindström et al., 2015). There are several pathways that were uncovered in *ex vivo* studies have yet to be explored on hPSC-derived kidney organoid models.

Additionally, time-dependent cell-cell interactions with either the UB, stromal or other nephron-derived cell types may influence its differentiation trajectory (Das et al., 2013; Fetting et al., 2014; Lindström et al., 2018a). These interactions may act as localized sources of paracrine signalling, following the specification of its identity. The lack of, or small population

of UB cells within our kidney organoids offer an opportunity to study Wnt-related signals in nephron patterning in the absence of UB-derived signals.

4.5. A “human”-specific model for understanding renal vasculature development

The difficulty of studying human development lies in shortage of fetal material and the inaccessibility of knocking in genetic reporters. The main source of fetal materials are from discarded products of conception (POCs), of which availability is uncertain and scarce. Kidney organoids can bridge the gap, enabling the study of human kidney development without the need for human samples. Hence, these models have enabled an unprecedented opportunity to study human kidney vasculature development in an *in vitro* context. A key finding presented in this thesis was that vascular progenitor cells expressed nephron progenitor markers, suggesting that they share a common renal progenitor origin. This population of cells makes up 1 to 3% of the total NPC population and appears at day 11 of differentiation in multiple hPSC lines. In response to VEGF-A, which is secreted by the nascent podocytes emerging at around day 12 to 14 of differentiation, these cells acquire CD31⁺ expression and gradually lose their NPC signatures. Importantly, VEGF signalling was shown to driving vascular formation in these kidney organoids, as chemical inhibition of VEGF receptors obliterated the vascular network. To confirm the existence of this cells, immunofluorescence analysis, flow cytometry analysis and sc-RNA-seq were performed on cells from day 11 to 14, which were actively undergoing early nephrogenesis and vasculogenesis. Using Seurat and Monocle 2 analysis, we identified the vascular progenitor population, which express the NPC marker SIX1⁺.

In the mouse, it is known that vascular progenitors invade into the glomerulus at the proximal end of developing nephrons to generate the capillary tufts, but the origin of these cells were elusive (Lopez and Gomez, 2011). Hence, it may be possible that *de novo* formation of these vascular progenitors from a subset of MM/NPCs may contribute to this event (Figure 4.1a).

This hypothesis is also favourable due to the anatomical location of differentiating MM/NPCs in relative proximity to the developing proximal nephron. However, the caveat is that MM/NPCs were not considered to be able to acquire a vascular identity (Gao et al., 2005; Howden et al., 2019; Mugford et al., 2008b). This may be discussed in three separate viewpoints. Firstly, this may represent a possible species-specific difference between mouse and human. Traditionally, kidney vascular development was largely studied using mice models, and this was likely undiscovered due to intrinsic differences in gene-regulatory pathways between the two species. In support of this hypothesis, vascular development mechanics in another organ system, the pancreas, is divergent in human and mice (Dolenšek et al., 2015; Jennings et al., 2015; Jørgensen et al., 2007). Secondly, lineage tracing experiments performed using reporter lines in hPSCs may not be sensitive enough to identify these renal vascular progenitors, which consisted of only a small population of cells (Howden et al., 2019). Thirdly, it may be possible that these renal vascular progenitors are a consequence of culture conditions, which is not representative of *in vivo* kidney development. This question can be partially answered if this population of NPC-derived vascular progenitors may be detected in human fetal kidneys. Indeed, with single cell assays gaining traction and advancing in accessibility and sensitivity, human-derived materials may be able to provide a clearer picture of the whole developmental landscape (Hochane et al., 2019; Klein and Treutlein, 2019). In a particular study, Hochane and colleagues have shown that endothelial cells in human fetal kidneys were also found to express *CITED1*, a marker for NPC. Using flow cytometry approaches, we have also detected the co-expression of SIX1 with KDR in a small population of cells in human fetal kidneys. Together, results from others and our group have provided early evidences for the existence of this elusive population (Hochane et al., 2019; Low et al., 2019). As the sequencing technology progresses, along with emerging human kidney datasets, we will be able to paint a

more complete picture of the kidney vasculature development landscape and confirm this finding.

Figure 4.1. A proposed mechanism for kidney vasculogenesis and maturation from NPCs.

A subpopulation of NPCs acquires the expression of KDR, which enables their response to podocyte VEGF-A. Upon maturation into CD31⁺ vascular endothelial cells, they lose NPC marker expression at a later stage.

4.6. Kidney organoids complementing the limitations associated with model organisms

Human development is enigmatic and is a difficult question to approach directly. Model organisms, especially rodent models, have yielded substantial amount of knowledge underpinning kidney development. Mouse models, together with *ex vivo* culture and its manipulations, have provided an unprecedented approach to study mammalian organogenesis. This information have been crucial in allowing us to recapitulate these conditions *in vitro* to generate kidney organoids from hPSCs (Little et al., 2016). However, we do not actually have a complete picture of embryonic development yet. While organoids are limited models that may not recapitulate the full spectrum of developmental phenotype, they may be useful in bridging species-specific differences. Kidney organoids harbour the multicellular complexity found in developing kidneys and enable the study of mechanisms governing early development (Figure 4.2a,b). In this version of kidney organoids, structures such as the UB and nephrogenic niche were absent, but are still useful for the study of mechanisms governing early development

The discovery of a possible novel vascular progenitor in the kidney an example of using organoids to bridge knowledge gaps left behind from studies on model organisms. For example, nephrogenesis creases pre-natal in humans but continue up to day 5 post-natal in the mouse. This difference are associated with a difference in genetics and the activity of nephron progenitor cell-associated genes, but many of these differences have not been uncovered yet (McMahon, 2016; O'Brien et al., 2016).

Figure 4.2. Parallels in cellular complexity and relative organization between *in vitro* human kidney organoids and embryonic mouse kidney.

(a) Representative immunofluorescence analysis of mouse embryonic kidneys (this panel was kindly provided by Dr Alexander N Combes from Monash University, Australia). (b) Representative immunofluorescence analysis of human kidney organoids. Representative markers used for each segment – NPHS1, NPHS2 and PODXL for glomeruli/ podocytes; LTL for proximal tubules; JAG1 for Loop of Henle; CDH1 for distal tubules; SLC12A1, GATA3 and CDH1 for collecting ducts; CD31 and CD34 for vascular endothelial cells; SIX2 for nephron progenitor cells.

Organoids offer a complex, yet reductionist platform to study early organogenesis that is difficult to interrogate in animal models. Human organoids are usually expandable and can be produced for large scale studies, while human fetal organs are difficult to obtain. With the emergence of single-cell technologies, it is now possible to interrogate complex developmental landscapes (Klein and Treutlein, 2019; Kumar et al., 2017; Potter, 2018). The power of single-cell transcriptomics, combined with powerful computational approaches are uncovering novel genes and regulatory mechanisms underpinning organogenesis in fetal organs without the need for traditional lineage tracers (Hochane et al., 2019; Lindström et al., 2018b). Taken together, organoids represent a new paradigm to approach human developmental biology. However, novel insights uncovered using human organoid models should be cross validated in both native mouse and human embryonic organs.

On the other end of the spectrum, emerging *in vitro* human models such as synthetic embryos, gastruloids and assembloids, are uncovering more information on early human embryonic development otherwise unrealizable (Beccari et al., 2018; Deglincerti et al., 2016; Li et al., 2019; Paşca, 2019; Shahbazi et al., 2016; Simunovic and Brivanlou, 2017). It will be exciting to see what novel insights these *in vitro* models may be able to uncover.

4.7. Maturation of hPSC-derived kidney organoids *in vivo*

The generation of functional organ constructs from hPSCs *in vitro* could represent an alternative source of tissue, or organ for replacement therapies. This has been particularly

crucial in organs such as the kidney, which have very limited capacity for self-repair (Little, 2006; Little et al., 2016; Miao et al., 2020). In the kidney, functionality is conferred by the arrangements of specialized cells that were built to perform a specific function. An example of such a feature is specialized glomerular endothelial cells that harbour fenestrations on their cell body and are on the opposing side to interdigitating podocytes on the other side of the GBM. Similar features are also present in the peritubular capillaries and tubules to enable reabsorption. Hence, maturation in the context of the kidney is not just dependent on cell status and transcriptomics, but also the formation of complex microanatomy.

To date, several groups have shown that implantation of hPSC-derived kidney cells or tissue into an *in vivo* niche was able to facilitate maturation. Upon implantation to a CAM model, kidney organoids could reach a transcriptomic profile reminiscent of 2nd trimester fetal kidney (Garreta et al., 2019). Implantation of NPCs or kidney organoids into immunocompromised mice enhanced structural and functional maturation (Bantounas et al., 2018; Low et al., 2019; van den Berg et al., 2018). Grafts engrafted well whether implanted subcutaneously or underneath the renal capsule, with neovascularization originating from the host vasculature. Importantly, the formation of a glomerular capillary tufts from graft-derived highlights the plasticity of hPSC-derived vascular endothelial cells and their capacity for glomerular invasion (Bantounas et al., 2018; Low et al., 2019). Importantly, these studies shared a common feature, which is the introduction of kidney organoids to a host vascular network, which invades into the graft *via* angiogenesis.

Intravenous administration of fluorescently-labelled dextran is a popular approach to investigate the functionality of both *in vitro* and implanted kidney organoids (Bantounas et al., 2018; Przepiorski et al., 2018; Xinari et al., 2016a). The accumulation of dextran within tubules indicates that the glomerular vascularization and, as blood flow is required to bring these molecules into the graft and subsequently tubules. This will be an indirect indicator of

filtration, likely through a glomerular barrier. Work from our group as well as others showed that small molecular weight dextran in the 10 to 70 kDa range could be accumulated within proximal tubules (Bantounas et al., 2018; Low et al., 2019). This experiment was expanded with the injection of a 2,000 kDa dextran. Assuming the successful formation of a glomerular filtration barrier, this large dextran would be excluded from the tubules as it is unable to bypass the GBM. Indeed this was the case, as both the implanted kidney organoids and native mouse kidney were not able to allow the 2,000 kDa molecule to bypass (Low et al., 2019).

The first report of filtrate generation was reported by Sharmin and colleagues by the implantation of iPSC-derived podocytes (Sharmin et al., 2015). Technically, it is difficult to observe filtrate formation since they happen in minuscule amounts. However, if the whole implant was processed *in toto*, proteinaceous filtrate accumulations will be dehydrated and infiltrated with paraffin. These artefacts are labelled by eosin staining under H&E staining. Hence, the accumulation of eosin-rich precipitations within distal tubules likely indicate the production of filtrate which had accumulated due to the lack of an exit. Similarly, similar accumulation patterns in E19.5 mouse kidneys was observed.

Currently, it is unclear how the *in vivo* niche can promote the maturation of the implanted kidney organoids. With nutrients coming from the blood plasma, there may be uncharacterized factors that may drive the further differentiation. There were attempts to achieve maturation without *in vivo* implantation by subjecting kidney organoids to fluid flow. Using microfluidics, kidney organoids were subjected to a constant fluid flow, which mimics hemodynamics and fluid shear forces (Homan et al., 2019). This approach resulted in higher VEGF-A production and more vascular endothelial cells. In addition, more complex features, such as vascular invasion into the glomerulus were observed (Homan et al., 2019). Hence, these studies indicate that fluid dynamics may be a physical parameter in improving the maturity status of hPSC-derived kidney organoids and may inspire further developments to existing tissue culture systems.

4.8. Limitations of *in vivo* implantation

The uninhibited proliferation of fibroblast-like cells within the graft was reported in every attempt to implant hPSC-derived kidney organoids (Bantounas et al., 2018; Sharmin et al., 2015; Subramanian et al., 2019; van den Berg et al., 2018). Notably, in many instances, reports showed an increase in graft size over time, that was attributed to this population. This issue seems to be more pronounced in mesoderm-derivatives rather than in endoderm-derivatives (Ang et al., 2018; Hohwieler et al., 2017; Takebe et al., 2013). It is likely that cells from different germ layers may exhibit different characteristics upon re-introduction to an *in vivo* niche. While the exact mechanism remains unknown, there is a need to devise strategies to reduce, or prevent the proliferation of these cells to maximize the efficiency of the graft.

In this study, I demonstrated that implanted human kidney organoids could achieve rudimentary filtration formation, but all of these were trapped within the distal tubules. Hence, to channel the filtrate out of the implant, there is a need to engineer a collecting duct system to channel them into a single output for disposal. Furthermore, the accumulation of filtrate at the distal tubules highlights the importance of the collecting duct network, which is formed by branching morphogenesis. Several organs, especially the lung or kidney, contains an extensive branching network with a unified entry/ exit point. Currently, branching morphogenesis in hPSC-derived organoids have been difficult to recapitulate *in vitro*. One of the features that is urgently required in kidney organoids is to engineer a collecting duct network. Such a network should be amenable to fusion to the native mouse collecting duct or bladder and allow urinary passage. However, it is unlikely for these to occur normally and may require the integration of bioengineering, bio-fabricating or surgical approaches.

Similar to the need for a collecting duct system to lead the filtrate out using a common exit, a common entry is required. In the kidney, the large renal arteries and veins collectively handle 20 - 30% of the cardiac output and distribute this amount across the entire kidney in a system

of highly organized and structured vessels. Small vessels form neovascularization upon transplantation, but these pales in comparison to the volume and architecture of the actual kidney. In the E12.5 mouse, the renal artery buds out of the aorta and starts invasion into the kidney mesenchyme. At the same period, the kidneys are connected to multiple points on the iliac artery. A pruning mechanism that is believed to be regulated by macrophages, restrict the vascular plexus into a single renal artery and vein at E15.5 (Munro et al., 2017; Murakami et al., 2019). It appears that *de novo* vasculogenesis in kidney organoids is not sufficient and require the construction of larger vessels from an exogenous origin. Hence, efforts are required to construct higher order vessels for the management of blood flow to the implant.

5. Conclusion and future work

5.1. Conclusion

In conclusion, I present the development of a novel protocol to generate kidney organoids from hPSCs. Using our protocol, kidney organoids contained mostly nephron-like structures, with stromal and vascular endothelial cells. However, there was little to no UB-induction, hence lacking a collecting duct system. Using this platform, the critical role of podocyte-derived VEGF-A in driving vascular formation was validated and is similar to kidney development *in vivo*. The discovery of this population is suggestive of a difference in development between mice and humans and provides evidence for kidney vasculogenesis in humans.

Kidney organoids generated using this model were amenable to nephron patterning using a canonical Wnt agonist. It is possible to modulate the differentiation of multiple cell populations within the kidney organoids, with important implications for developmental biology and bioengineering. In principle, more glomeruli induction should translate to more filtration and more tubular induction should translate to more reabsorption. Modulate the differentiation trajectories of NPCs will aid in the derivation of different cell types required to build kidney-mimetics in the future.

Implantation of these kidney organoids into an immunocompromised host exposes it to an *in vivo* environment and enables further maturation. Successful vascularization of the glomeruli by both intrinsic and exogenous vasculature is indicative of vascular plasticity, enabling remodelling and glomerular invasion. The formation of fenestrated endothelium and Bowman's capsule in glomeruli; and microvilli within tubules are indicative of functional maturation, enabling rudimentary filtration and reabsorption. The accumulation of urinary filtrate in dilated tubules demonstrated the promise of hPSC-derived kidney organoids for renal replacement therapeutics.

Collectively, I present a robust and reproducible platform to generate vascularized, patternable and functional kidney organoids from hPSCs, which may be utilized for modelling human kidney physiopathology, drug screening and regenerative medicine.

5.2. Bridging the gap between hPSC-derived kidney organoids and post-natal kidney

A long-standing problem with of hPSC-derived organoids, as well as other hPSC-derived organoids, is the failure to undergo maturation towards a postnatal transcriptomics *in vitro*. In the context of kidney organoids, the transcriptomics of the hPSC-derived kidney organoids may be recapitulate that of second trimester kidney at most (Garreta et al., 2019). Innovative ways have been postulated by many groups, whom have explored the use of organ scaffolding, microfluidics and biomaterials (Garreta et al., 2019; Homan et al., 2019; Song et al., 2013). However, none of these methods seem to be successful in achieving a post-natal maturation status. Here, I describe two complementing strategies that aid in this progress.

5.2.1. Pivotal role of single cell technologies

The development of cutting edge techniques, including multi-dimensional imaging and single cell techniques have offered deep insights into development using human fetal samples (Lindström et al., 2018b, 2018d, 2018a, 2018c). The finding that human and mice shared similar and differential mechanisms in kidney development is expected, but to tease out these differences in greater detail is challenging. Some of these findings included differences in anatomical structure, nephron patterning dynamics and uncovering more progenitor lineage markers. The identification of many differences in nephron segment markers and transporters also prompts us to re-evaluate many of the tools and markers that are currently in use benchmark our kidney organoids. For example, the expression of cytokeratin 8 is exclusive to the collecting ducts in the mouse, but broadly expressed in all tubular epithelium in humans

(Lindström et al., 2018c). Several other genes, such as *Foxd1* were also differentially regulated between the mouse and human stromal compartments. In this aspect, single cell approaches will be extremely useful to profile of multiple markers in each cell type.

Many groups have recognized the value of performing single cell analysis on the different variations of kidney samples that they are able to obtain. These include human fetal kidney (Hochane et al., 2019; Lindström et al., 2018b), human adult kidney (Liao et al., 2020; Wilson et al., 2019; Wu et al., 2018a), human kidney organoids (Low et al., 2019; Phipson et al., 2019; Subramanian et al., 2019; Westerling-Bui et al., 2019; Wu et al., 2018a) and mouse kidneys (Combes et al., 2019; Miao et al., 2020; Park et al., 2018). Currently, single cell transcriptomic sequencing has enabled us to benchmark kidney organoids based on their reproducibility, cellular composition, quality, and maturation. Comparison across different laboratories, protocols and iPSC lines will also enable us to identify intrinsic differences and mitigate these differences. For example, many groups have reported the presence of unfavourable cell types, such as neurons, melanocytes or muscle, in the kidney organoids (Subramanian et al., 2019; Wu et al., 2018a). By using a BDNF inhibitor, Wu and colleagues were able to translate these findings and remove the undesired neuronal population (Wu et al., 2018a). The cellular composition of kidney organoids also varies significantly between many groups. These were more profound in the stromal and endothelial populations. The composition of stromal cells was reported to make up a large percentage of total cells within the kidney organoids in many instances. Similarly, there were difficulties reproducing the extensive endothelial population by another independent laboratory (Phipson et al., 2019; Subramanian et al., 2019; Westerling-Bui et al., 2019; Wu et al., 2018a). These challenges, previously unresolved by immunofluorescence and bulk RNA sequencing techniques, were illuminated using single cell approaches.

As the current generation of human kidney organoid differentiation protocols were based mainly using knowledge from mouse development, this information will serve as useful references in advancing the state of the technology. The ensemble of these single cell datasets would enable the construction of a multiparametric transcriptomics atlas that incorporates information regarding the cell diversity, time, and species. This may enable the identification of novel cell types, markers or signals that will be invaluable to guide the refinement of the current kidney organoid differentiation protocols.

5.2.2. Recapitulation of complex cellular interplay

The developmental niche is made up of a highly complex and dynamic interplay of cells, including supplementary cells such as the vasculature, neurons and immune cells. Within an *in vitro* system, these components are absent, and may play previously unrealized roles in organoid development. Currently, the incorporation of these additional modules can be achieved by two different approaches. Firstly, building organoids by co-culture approach may be used to integrate desired cell types into the organoid system (Cakir et al., 2019; Jung et al., 2018; Takebe et al., 2015, 2013). Secondly, it may be possible to refine the differentiation protocol, and provide instructive growth factors to promote the differentiation of the supportive cell types (Czerniecki et al., 2018; Low et al., 2019; Neal et al., 2018).

The vasculature network coordinates organ development, and closely follows the branching network of the organ. Almost all the time, ramifying organs will have an aligned arterial and venous network (Drake and Fleming, 2000). Vascular co-development in the kidney is well-characterized, but its underlying mechanism is poorly understood. Hence, it is increasingly appreciated that apart from bringing in nutrients and oxygen, the vasculature may have a role in sculpting the sub-anatomical features of the target organ (Gao et al., 2005; Lammert et al., 2001; Matsumoto et al., 2001). The exogenous blood vessels migrate into the MM at almost

the same time as the UB and co-develops through kidney organogenesis (Lopez and Gomez, 2011; Munro et al., 2017). Munro and colleagues reported that the bifurcation of the UB is always coordinated by an angiogenic vascular sprout that cuts across at the branchpoint. These blood vessels initially appear as a vascular plexus around the nascent UB and cap mesenchyme, and are always carrying erythrocytes, indicative of their connection to the main circulation. This discovery indicates that apart from paracrine secretions, the vasculature may also physically regulate the development of organ systems.

Apart from blood flow, oxygen and nutrients, the vasculature also brings immune cells into the developing organ. The immune system is made up of a plethora of cells, with various subclasses of macrophages, lymphocytes, and leukocytes. These cells play crucial roles in organ development, by acting directly on the stem cell compartment or remodelling of the niche. For instance, lymphocytes play a key role in intestinal development, largely by IL-2 secretion. When hPSC-derived intestinal organoids were co-culture with T cells or IL-2, the expression of adult small intestine markers and functional enzymes were significantly upregulated. In addition, these organoids displayed a higher propensity for angiogenesis when implanted into an immunodeficient mouse model (Jung et al., 2018). The function of immune cells in kidney development was very recently revisited by Munro and colleagues. It was discovered that macrophages prune the developing vasculature by the phagocytosis of dying erythrocytes and endothelial cells. These macrophages likely originate from the yolk and follow the early blood vessel ingrowth into the kidney (Ide et al., 2020). The depletion of macrophages using an anti-Csf1r neutralizing antibody results in smaller kidneys with isolated and disconnected endothelial networks (Munro et al., 2019). Taken together, these evidences suggests that cells of the immune system survey and “clean up” the early developing organs, play critical roles in proper development. It may be possible to introduce these cells into the organoids by doing a co-culture. However, it remains challenging to provide the appropriate immune cell subset at

the correct place and time *in vitro*. The proper activation of immune cells may require their activation in secondary immune sites, and complex interaction with other immune subsets (Chaplin, 2010; Janeway et al., 2001; Mantovani et al., 2011).

The kidney is innervated by the sympathetic nervous system, which regulates filtration capacity and regulates the overall blood pressure (Mount and Power, 2006; Weiss et al., 2001). While the role of neurons during kidney development is unknown, it is likely they also respond to MM-derived GDNF (Kopp, 2011; Reidy and Rosenblum, 2009). In *Gdnf* mutant mice, both the UB and neurons fail to form, and results in kidney agenesis (Reidy and Rosenblum, 2009). This mechanism could explain the driving force behind neurogenesis in kidney organoids, but more work is required to elucidate if the relationship is reciprocal (Wu et al., 2018b). In other organs, such as the intestine and its corresponding organoid system, the enteric nerves were found to play a role in maturation (Holloway et al., 2019). Successful modelling of this neuro-intestinal relationship *in vitro* was achieved by the co-culture of neural crest cells (NCCs) with human intestinal organoids (HIOs) (Workman et al., 2017). These NCCs integrated into the organoids matured into neurons and glial cells, enhancing the overall transcriptomics and proliferative capacity within intestinal crypts (Workman et al., 2017). Collectively, these results indicate that neuro-epithelial interactions may be advantageous in the maturation of hPSC derivatives. Such a relationship remains to be elucidated in kidney development and may be aid in the maturation or functionality of hPSC-derived kidney organoids.

Altogether, these studies demonstrate that development of an organ necessitates a sophisticated interplay between different cell types. The supplementary cell types that contribute to organogenesis may be derived from a completely germ layer, or anatomical location. Hence, the reductionist approach that has made *in vitro* tissue culture so successful, may be a limitation in achieving more sophisticated laboratory grown tissue constructs.

5.3. Applications in disease modelling

5.3.1. Modelling genetic kidney disease

Genome-wide association studies (GWAS) studies had uncovered many genetic mutations that are implicated in CKD and CAKUT. Development of models that recapitulate the disease phenotype caused by these genes will enable the validation of its specific role in pathogenesis, as well as identify novel biomarkers or interventions. Traditionally, the study of these diseases employed the use of engineered cell lines and/or animal models such as zebrafishes or mice (Susztak et al., 2008; Yang et al., 2010).

Kidney organoids generated from iPSCs offers an unprecedented approach to model these genetic kidney diseases in a patient-specific manner. The genetic background of iPSCs is identical to the patient that it is derived from and harbours the same genetic mutations that drives pathogenesis in the patient. By using these iPSCs to generate kidney organoids, we now have an excellent opportunity to study genetic kidney disease, its disease progression mechanisms, and screen for personalized drugs (Sharma et al., 2020). The amenability for iPSCs to undergo genome editing using CRISPR/Cas9 allows the mutation to be corrected, generating isogenic lines for controls. In theory, these isogenic controls share similar genetic background apart from the mutation, reducing confounding effects and offer one of the most ideal controls. Fluorescence tags or lineage reporters may also be inserted, enabling phenotypic and subcellular investigations into the activity of the affected gene (Howden et al., 2019; Sharmin et al., 2015). Conversely, if patient samples are not accessible, genome editing using CRISPR/Cas9 offers the ability to engineer precise additions, deletions or tags into human ESCs (Freedman et al., 2015). These insights may culminate into novel insights that enable personalized drug screening. For instance, it was found that in patients with polycystic kidney disease (PKD), the microenvironment could be implicated in cystic progression. In the same

research group, it was found that blockade of myosin activity may ameliorate cystogenesis (Cruz et al., 2017; Czerniecki et al., 2018).

Altogether, kidney organoids generated from iPSCs may offer a reductionist and translational approach to interrogate genetic kidney defects, and enable the screening of therapeutic agents that may mitigate the pathogenic phenotype.

5.3.2. Modelling kidney injury

Kidney organoids generated from healthy or “wildtype” hPSCs may serve as normal kidney tissue for nephrotoxicity. Organoids serve as physiological models, bridging the gap between routine cell culture and animal models (Fatehullah et al., 2016; Li and Belmonte, 2019). Specific drug transporters, mitochondria activity and cellular metabolism unique to a particular kidney cell mediate their susceptibility to toxicity (Faria et al., 2019). In the kidney, drug-induced damage may affect the glomerulus, tubules, vasculature or stromal populations (Choudhury and Ahmed, 2006; Małyszko et al., 2017; Vervaeke et al., 2017). Most of these populations are present in hPSC-derived kidney organoids and are perfectly suited for this process.

The establishment and maturation of these cell types may be essential for the screening process. In particular, the proximal tubules are the most susceptible to nephrotoxic drugs. Transport proteins on the luminal surface of tubules facilitate the entry of drugs into the tubular cells, where they are rapidly metabolized by the high mitochondria activity. These transporter proteins are diverse, including the SLCs, AQP, organic anion transporters (OAT) and adenosine triphosphate-binding cassette (ABC) transporters. We and many other groups have shown that tubules within hPSC-derived kidney organoids do not express the full suite of transporters, and this may hamper their utility in the compound screening process (Low et al., 2019; Phipson et al., 2019; Wu et al., 2018a).

Assuring, the effects of several well-established nephrotoxic drugs were recapitulated in kidney organoids, and results in the injury of a particular region in the nephron. These drugs include cisplatin and gentamycin, which were toxic to the kidney tubules, and adriamycin, which was known to cause glomeruli injury (Czerniecki et al., 2018; Kumar et al., 2019; Morizane et al., 2015; Takasato et al., 2015). These results demonstrate a proof-of-concept in the utility of hPSC-derived kidney organoids in the compound development process.

References

12. Glomerular Filtration Barrier - Talbot Flashcards [WWW Document], n.d. . Quizlet. URL <https://quizlet.com/163475429/12-glomerular-filtration-barrier-talbot-flash-cards/> (accessed 11.1.20).
- Abrahamson, D.R., Robert, B., 2003. Derivation and differentiation of glomerular endothelial cells. *Nephrol. Dial. Transplant.* 18 Suppl 6, vi2-7. <https://doi.org/10.1093/ndt/gfg1056>
- Abrahamson, D.R., Robert, B., Hyink, D.P., St John, P.L., Daniel, T.O., 1998. Origins and formation of microvasculature in the developing kidney. *Kidney Int. Suppl.* 67, S7-11. <https://doi.org/10.1046/j.1523-1755.1998.06702.x>
- Acosta-Ochoa, I., Bustamante-Munguira, J., Mendiluce-Herrero, A., Bustamante-Bustamante, J., Coca-Rojo, A., 2019. Impact on Outcomes across KDIGO-2012 AKI Criteria According to Baseline Renal Function. *J Clin Med* 8. <https://doi.org/10.3390/jcm8091323>
- Adams, R.H., Alitalo, K., 2007. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat. Rev. Mol. Cell Biol.* 8, 464–478. <https://doi.org/10.1038/nrm2183>
- Ang, L.T., Tan, A.K.Y., Autio, M.I., Goh, S.H., Choo, S.H., Lee, K.L., Tan, J., Pan, B., Lee, J.J.H., Lum, J.J., Lim, C.Y.Y., Yeo, I.K.X., Wong, C.J.Y., Liu, M., Oh, J.L.L., Chia, C.P.L., Loh, C.H., Chen, A., Chen, Q., Weissman, I.L., Loh, K.M., Lim, B., 2018. A Roadmap for Human Liver Differentiation from Pluripotent Stem Cells. *Cell Rep* 22, 2190–2205. <https://doi.org/10.1016/j.celrep.2018.01.087>
- Aronson, P.S., Giebisch, G., 1997. Mechanisms of chloride transport in the proximal tubule. *Am. J. Physiol.* 273, F179-192. <https://doi.org/10.1152/ajprenal.1997.273.2.F179>
- Aubert, J., Dunstan, H., Chambers, I., Smith, A., 2002. Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. *Nat. Biotechnol.* 20, 1240–1245. <https://doi.org/10.1038/nbt763>
- Auerbach, R., Grobstein, C., 1958. Inductive interaction of embryonic tissues after dissociation and reaggregation. *Exp. Cell Res.* 15, 384–397. [https://doi.org/10.1016/0014-4827\(58\)90039-9](https://doi.org/10.1016/0014-4827(58)90039-9)
- Bantounas, I., Ranjzad, P., Tengku, F., Silajdžić, E., Forster, D., Asselin, M.-C., Lewis, P., Lennon, R., Plagge, A., Wang, Q., Woolf, A.S., Kimber, S.J., 2018. Generation of Functioning Nephrons by Implanting Human Pluripotent Stem Cell-Derived Kidney Progenitors. *Stem Cell Reports* 10, 766–779. <https://doi.org/10.1016/j.stemcr.2018.01.008>
- Barak, H., Huh, S.-H., Chen, S., Jeanpierre, C., Martinovic, J., Parisot, M., Bole-Feysot, C., Nitschké, P., Salomon, R., Antignac, C., Ornitz, D.M., Kopan, R., 2012. FGF9 and FGF20 maintain the stemness of nephron progenitors in mice and man. *Dev. Cell* 22, 1191–1207. <https://doi.org/10.1016/j.devcel.2012.04.018>
- Bard, J.B., Gordon, A., Sharp, L., Sellers, W.I., 2001. Early nephron formation in the developing mouse kidney. *J. Anat.* 199, 385–392. <https://doi.org/10.1046/j.1469-7580.2001.19940385.x>
- Barnes, J.D., Crosby, J.L., Jones, C.M., Wright, C.V., Hogan, B.L., 1994. Embryonic expression of Lim-1, the mouse homolog of Xenopus Xlim-1, suggests a role in lateral mesoderm differentiation and neurogenesis. *Dev. Biol.* 161, 168–178. <https://doi.org/10.1006/dbio.1994.1018>
- Beccari, L., Moris, N., Girgin, M., Turner, D.A., Baillie-Johnson, P., Cossy, A.-C., Lutolf, M.P., Duboule, D., Arias, A.M., 2018. Multi-axial self-organization properties of mouse embryonic stem cells into gastruloids. *Nature* 562, 272–276. <https://doi.org/10.1038/s41586-018-0578-0>

- Ben-Haim, N., Lu, C., Guzman-Ayala, M., Pescatore, L., Mesnard, D., Bischofberger, M., Naef, F., Robertson, E.J., Constam, D.B., 2006. The Nodal Precursor Acting via Activin Receptors Induces Mesoderm by Maintaining a Source of Its Convertases and BMP4. *Developmental Cell* 11, 313–323. <https://doi.org/10.1016/j.devcel.2006.07.005>
- Benjamin, O., Lappin, S.L., 2020. End-Stage Renal Disease, in: StatPearls. StatPearls Publishing, Treasure Island (FL).
- Beppu, H., Kawabata, M., Hamamoto, T., Chytil, A., Minowa, O., Noda, T., Miyazono, K., 2000. BMP Type II Receptor Is Required for Gastrulation and Early Development of Mouse Embryos. *Developmental Biology* 221, 249–258. <https://doi.org/10.1006/dbio.2000.9670>
- Bertocchini, F., Stern, C.D., 2002. The Hypoblast of the Chick Embryo Positions the Primitive Streak by Antagonizing Nodal Signaling. *Developmental Cell* 3, 735–744. [https://doi.org/10.1016/S1534-5807\(02\)00318-0](https://doi.org/10.1016/S1534-5807(02)00318-0)
- Biben, C., Stanley, E., Fabri, L., Kotecha, S., Rhinn, M., Drinkwater, C., Lah, M., Wang, C.C., Nash, A., Hilton, D., Ang, S.L., Mohun, T., Harvey, R.P., 1998. Murine cerberus homologue mCer-1: a candidate anterior patterning molecule. *Dev. Biol.* 194, 135–151. <https://doi.org/10.1006/dbio.1997.8812>
- Biga, L.M., Dawson, S., Harwell, A., Hopkins, R., Kaufmann, J., LeMaster, M., Matern, P., Morrison-Graham, K., Quick, D., Runyeon, J., 2019. 25.2 Microscopic Anatomy of the Kidney: Anatomy of the Nephron, in: *Anatomy & Physiology*. OpenStax/Oregon State University.
- Boron, W.F., Boulpaep, E.L., 2012. *Medical Physiology*, 2e Updated Edition E-Book. Elsevier Health Sciences.
- Bouchard, M., 2004. Transcriptional control of kidney development. *Differentiation* 72, 295–306. <https://doi.org/10.1111/j.1432-0436.2004.07207001.x>
- Bouchard, M., Souabni, A., Mandler, M., Neubüser, A., Busslinger, M., 2002. Nephric lineage specification by Pax2 and Pax8. *Genes Dev* 16, 2958–2970. <https://doi.org/10.1101/gad.240102>
- Boyle, S., Misfeldt, A., Chandler, K.J., Deal, K.K., Southard-Smith, E.M., Mortlock, D.P., Baldwin, H.S., de Caestecker, M., 2008. Fate mapping using Cited1-CreERT2 mice demonstrates that the cap mesenchyme contains self-renewing progenitor cells and gives rise exclusively to nephronic epithelia. *Dev. Biol.* 313, 234–245. <https://doi.org/10.1016/j.ydbio.2007.10.014>
- Brenner, B.M., Mackenzie, H.S., 1997. Nephron mass as a risk factor for progression of renal disease. *Kidney Int Suppl* 63, S124–127.
- Brodbeck, S., Englert, C., 2004. Genetic determination of nephrogenesis: the Pax/Eya/Six gene network. *Pediatr Nephrol* 19, 249–255. <https://doi.org/10.1007/s00467-003-1374-z>
- Brunskill, E.W., Aronow, B.J., Georgas, K., Rumballe, B., Valerius, M.T., Aronow, J., Kaimal, V., Jegga, A.G., Grimmond, S., McMahon, A.P., Patterson, L.T., Little, M.H., Potter, S.S., 2008. Atlas of Gene Expression in the Developing Kidney at Microanatomic Resolution. *Developmental Cell* 15, 781–791. <https://doi.org/10.1016/j.devcel.2008.09.007>
- Bulbul, M.C., Dagel, T., Afsar, B., Ulus, N.N., Kuwabara, M., Covic, A., Kanbay, M., 2018. Disorders of Lipid Metabolism in Chronic Kidney Disease. *BPU* 46, 144–152. <https://doi.org/10.1159/000488816>
- Butler, A., Hoffman, P., Smibert, P., Papalexi, E., Satija, R., 2018. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* 36, 411–420. <https://doi.org/10.1038/nbt.4096>
- Cakir, B., Xiang, Y., Tanaka, Y., Kural, M.H., Parent, M., Kang, Y.-J., Chapeton, K., Patterson, B., Yuan, Y., He, C.-S., Raredon, M.S.B., Dengelegi, J., Kim, K.-Y., Sun, P., Zhong,

- M., Lee, S., Patra, P., Hyder, F., Niklason, L.E., Lee, S.-H., Yoon, Y.-S., Park, I.-H., 2019. Engineering of human brain organoids with a functional vascular-like system. *Nat Methods* 16, 1169–1175. <https://doi.org/10.1038/s41592-019-0586-5>
- Carmeliet, P., 2003. Angiogenesis in health and disease. *Nat. Med.* 9, 653–660. <https://doi.org/10.1038/nm0603-653>
- Carone, F.A., Peterson, D.R., Oparil, S., Pullman, T.N., 1979. Renal tubular transport and catabolism of proteins and peptides. *Kidney International* 16, 271–278. <https://doi.org/10.1038/ki.1979.129>
- Carroll, T.J., Park, J.-S., Hayashi, S., Majumdar, A., McMahon, A.P., 2005. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev. Cell* 9, 283–292. <https://doi.org/10.1016/j.devcel.2005.05.016>
- Chaplin, D.D., 2010. Overview of the Immune Response. *J Allergy Clin Immunol* 125, S3-23. <https://doi.org/10.1016/j.jaci.2009.12.980>
- Charles A Janeway, J., Travers, P., Walport, M., Shlomchik, M.J., 2001. Principles of innate and adaptive immunity. *Immunobiology: The Immune System in Health and Disease*. 5th edition.
- Chen, Y., Schier, A.F., 2002. Lefty proteins are long-range inhibitors of squint-mediated nodal signaling. *Curr. Biol.* 12, 2124–2128. [https://doi.org/10.1016/s0960-9822\(02\)01362-3](https://doi.org/10.1016/s0960-9822(02)01362-3)
- Cheng, H.-T., Kim, M., Valerius, M.T., Surendran, K., Schuster-Gossler, K., Gossler, A., McMahon, A.P., Kopan, R., 2007. Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. *Development* 134, 801–811. <https://doi.org/10.1242/dev.02773>
- Cheung, C., Bernardo, A.S., Trotter, M.W.B., Pedersen, R.A., Sinha, S., 2012. Generation of human vascular smooth muscle subtypes provides insight into embryological origin-dependent disease susceptibility. *Nat Biotechnol* 30, 165–173. <https://doi.org/10.1038/nbt.2107>
- Choudhury, D., Ahmed, Z., 2006. Drug-associated renal dysfunction and injury. *Nature Clinical Practice Nephrology* 2, 80–91. <https://doi.org/10.1038/ncpneph0076>
- Christensen, E.I., Birn, H., 2002. Megalin and cubilin: multifunctional endocytic receptors. *Nature Reviews Molecular Cell Biology* 3, 258–267. <https://doi.org/10.1038/nrm778>
- Clevers, H., 2006. Wnt/ β -Catenin Signaling in Development and Disease. *Cell* 127, 469–480. <https://doi.org/10.1016/j.cell.2006.10.018>
- Coffin, J.D., Harrison, J., Schwartz, S., Heimark, R., 1991. Angioblast differentiation and morphogenesis of the vascular endothelium in the mouse embryo. *Dev. Biol.* 148, 51–62. [https://doi.org/10.1016/0012-1606\(91\)90316-u](https://doi.org/10.1016/0012-1606(91)90316-u)
- Colbert, G.B., Elrggal, M.E., Gaur, L., Lerma, E.V., 2020. Update and review of adult polycystic kidney disease. *Disease-a-Month, Update and Review of Adult Polycystic Kidney Disease* 66, 100887. <https://doi.org/10.1016/j.disamonth.2019.100887>
- Combes, A.N., Phipson, B., Lawlor, K.T., Dorison, A., Patrick, R., Zappia, L., Harvey, R.P., Oshlack, A., Little, M.H., 2019. Single cell analysis of the developing mouse kidney provides deeper insight into marker gene expression and ligand-receptor crosstalk. *Development* 146. <https://doi.org/10.1242/dev.178673>
- Costantini, F., Kopan, R., 2010. Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development. *Dev Cell* 18, 698–712. <https://doi.org/10.1016/j.devcel.2010.04.008>
- Cruz, N.M., Song, X., Czerniecki, S.M., Gulieva, R.E., Churchill, A.J., Kim, Y.K., Winston, K., Tran, L.M., Diaz, M.A., Fu, H., Finn, L.S., Pei, Y., Himmelfarb, J., Freedman, B.S., 2017. Organoid cystogenesis reveals a critical role of microenvironment in human

- polycystic kidney disease. *Nat Mater* 16, 1112–1119. <https://doi.org/10.1038/nmat4994>
- Cui, C., Yang, X., Chuai, M., Glazier, J.A., Weijer, C.J., 2005. Analysis of tissue flow patterns during primitive streak formation in the chick embryo. *Dev. Biol.* 284, 37–47. <https://doi.org/10.1016/j.ydbio.2005.04.021>
- Czerniecki, S.M., Cruz, N.M., Harder, J.L., Menon, R., Annis, J., Otto, E.A., Gulieva, R.E., Islas, L.V., Kim, Y.K., Tran, L.M., Martins, T.J., Pippin, J.W., Fu, H., Kretzler, M., Shankland, S.J., Himmelfarb, J., Moon, R.T., Paragas, N., Freedman, B.S., 2018. High-Throughput Screening Enhances Kidney Organoid Differentiation from Human Pluripotent Stem Cells and Enables Automated Multidimensional Phenotyping. *Cell Stem Cell* 22, 929–940.e4. <https://doi.org/10.1016/j.stem.2018.04.022>
- D'Amour, K.A., Bang, A.G., Eliazar, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M.A., Kroon, E., Carpenter, M.K., Baetge, E.E., 2006. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nature Biotechnology* 24, 1392–1401. <https://doi.org/10.1038/nbt1259>
- Das, A., Tanigawa, S., Karner, C.M., Xin, M., Lum, L., Chen, C., Olson, E.N., Perantoni, A.O., Carroll, T.J., 2013. Stromal-epithelial crosstalk regulates kidney progenitor cell differentiation. *Nature cell biology* 15, 1035. <https://doi.org/10.1038/ncb2828>
- Davies, J.A., 2008. Synthetic morphology: prospects for engineered, self-constructing anatomies. *J Anat* 212, 707–719. <https://doi.org/10.1111/j.1469-7580.2008.00896.x>
- Davies, J.A., Hohenstein, P., Chang, C.-H., Berry, R., 2014. A self-avoidance mechanism in patterning of the urinary collecting duct tree. *BMC Dev Biol* 14, 35. <https://doi.org/10.1186/s12861-014-0035-8>
- Davis, R.P., Ng, E.S., Costa, M., Mossman, A.K., Sourris, K., Elefanty, A.G., Stanley, E.G., 2008. Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors. *Blood* 111, 1876–1884. <https://doi.org/10.1182/blood-2007-06-093609>
- Davis, S., Cooper, J.E., 2019. No Time to Wait: Optimizing Use of Deceased Donor Kidneys. *CJASN* 14, 1560–1561. <https://doi.org/10.2215/CJN.10820919>
- Davis, T.K., Hoshi, M., Jain, S., 2014. To Bud or not to Bud: The RET perspective in CAKUT. *Pediatr Nephrol* 29, 597–608. <https://doi.org/10.1007/s00467-013-2606-5>
- Deglincerti, A., Croft, G.F., Pietila, L.N., Zernicka-Goetz, M., Siggia, E.D., Brivanlou, A.H., 2016. Self-organization of the in vitro attached human embryo. *Nature* 533, 251–254. <https://doi.org/10.1038/nature17948>
- Dejana Elisabetta, Kühl Michael, 2010. The Role of Wnt Signaling in Physiological and Pathological Angiogenesis. *Circulation Research* 107, 943–952. <https://doi.org/10.1161/CIRCRESAHA.110.223750>
- del Moral, P.-M., Warburton, D., 2010. Explant Culture of Mouse Embryonic Whole Lung, Isolated Epithelium, or Mesenchyme Under Chemically Defined Conditions as a System to Evaluate the Molecular Mechanism of Branching Morphogenesis and Cellular Differentiation. *Methods Mol Biol* 633, 71–79. https://doi.org/10.1007/978-1-59745-019-5_5
- DiBona, G.F., 2002. Sympathetic nervous system and the kidney in hypertension. *Curr. Opin. Nephrol. Hypertens.* 11, 197–200. <https://doi.org/10.1097/00041552-200203000-00011>
- Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. <https://doi.org/10.1093/bioinformatics/bts635>

- Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W., Kemler, R., 1985. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *Development* 87, 27–45.
- Dolenšek, J., Rupnik, M.S., Stožer, A., 2015. Structural similarities and differences between the human and the mouse pancreas. *Islets* 7. <https://doi.org/10.1080/19382014.2015.1024405>
- Drake, C.J., Fleming, P.A., 2000. Vasculogenesis in the day 6.5 to 9.5 mouse embryo. *Blood* 95, 1671–1679.
- Dressler, G.R., Deutsch, U., Chowdhury, K., Nornes, H.O., Gruss, P., 1990. Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system. *Development* 109, 787–795.
- Eichmann, A., Yuan, L., Moyon, D., Lenoble, F., Pardanaud, L., Breant, C., 2005. Vascular development: from precursor cells to branched arterial and venous networks. *Int. J. Dev. Biol.* 49, 259–267. <https://doi.org/10.1387/ijdb.041941ae>
- Eremina, V., Cui, S., Gerber, H., Ferrara, N., Haigh, J., Nagy, A., Ema, M., Rossant, J., Jothy, S., Miner, J.H., Quaggin, S.E., 2006. Vascular Endothelial Growth Factor A Signaling in the Podocyte-Endothelial Compartment Is Required for Mesangial Cell Migration and Survival. *JASN* 17, 724–735. <https://doi.org/10.1681/ASN.2005080810>
- Eremina, V., Sood, M., Haigh, J., Nagy, A., Lajoie, G., Ferrara, N., Gerber, H.-P., Kikkawa, Y., Miner, J.H., Quaggin, S.E., 2003. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. *J Clin Invest* 111, 707–716. <https://doi.org/10.1172/JCI17423>
- Faria, J., Ahmed, S., Gerritsen, K.G.F., Mihaila, S.M., Masereeuw, R., 2019. Kidney-based in vitro models for drug-induced toxicity testing. *Arch Toxicol* 93, 3397–3418. <https://doi.org/10.1007/s00204-019-02598-0>
- Fassett, R.G., Venuthurupalli, S.K., Gobe, G.C., Coombes, J.S., Cooper, M.A., Hoy, W.E., 2011. Biomarkers in chronic kidney disease: a review. *Kidney International* 80, 806–821. <https://doi.org/10.1038/ki.2011.198>
- Fatehullah, A., Tan, S.H., Barker, N., 2016. Organoids as an in vitro model of human development and disease. *Nat. Cell Biol.* 18, 246–254. <https://doi.org/10.1038/ncb3312>
- Fetting, J.L., Guay, J.A., Karolak, M.J., Iozzo, R.V., Adams, D.C., Maridas, D.E., Brown, A.C., Oxburgh, L., 2014. FOXD1 promotes nephron progenitor differentiation by repressing decorin in the embryonic kidney. *Development* 141, 17–27. <https://doi.org/10.1242/dev.089078>
- Fleming, B.M., Yelin, R., James, R.G., Schultheiss, T.M., 2013. A role for Vg1/Nodal signaling in specification of the intermediate mesoderm. *Development* 140, 1819–1829. <https://doi.org/10.1242/dev.093740>
- Florens, N., Calzada, C., Lyasko, E., Juillard, L., Soulage, C.O., 2016. Modified Lipids and Lipoproteins in Chronic Kidney Disease: A New Class of Uremic Toxins. *Toxins (Basel)* 8. <https://doi.org/10.3390/toxins8120376>
- Freedman, B.S., Brooks, C.R., Lam, A.Q., Fu, H., Morizane, R., Agrawal, V., Saad, A.F., Li, M.K., Hughes, M.R., Werff, R.V., Peters, D.T., Lu, J., Baccei, A., Siedlecki, A.M., Valerius, M.T., Musunuru, K., McNagny, K.M., Steinman, T.I., Zhou, J., Lerou, P.H., Bonventre, J.V., 2015. Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. *Nat Commun* 6, 8715. <https://doi.org/10.1038/ncomms9715>
- Fujii, H., Kono, K., Nishi, S., 2019. Characteristics of coronary artery disease in chronic kidney disease. *Clin. Exp. Nephrol.* 23, 725–732. <https://doi.org/10.1007/s10157-019-01718-5>

- Fujii, T., Pichel, J.G., Taira, M., Toyama, R., Dawid, I.B., Westphal, H., 1994. Expression patterns of the murine LIM class homeobox gene *lim1* in the developing brain and excretory system. *Dev. Dyn.* 199, 73–83. <https://doi.org/10.1002/aja.1001990108>
- Funa, N.S., Schachter, K.A., Lerdrup, M., Ekberg, J., Hess, K., Dietrich, N., Honoré, C., Hansen, K., Semb, H., 2015. β -Catenin Regulates Primitive Streak Induction through Collaborative Interactions with SMAD2/SMAD3 and OCT4. *Cell Stem Cell* 16, 639–652. <https://doi.org/10.1016/j.stem.2015.03.008>
- Ganeva, V., Unbekandt, M., Davies, J.A., 2011. An improved kidney dissociation and reaggregation culture system results in nephrons arranged organotypically around a single collecting duct system. *Organogenesis* 7, 83–87. <https://doi.org/10.4161/org.7.2.14881>
- Gansevoort, R.T., Correa-Rotter, R., Hemmelgarn, B.R., Jafar, T.H., Heerspink, H.J.L., Mann, J.F., Matsushita, K., Wen, C.P., 2013. Chronic kidney disease and cardiovascular risk: epidemiology, mechanisms, and prevention. *Lancet* 382, 339–352. [https://doi.org/10.1016/S0140-6736\(13\)60595-4](https://doi.org/10.1016/S0140-6736(13)60595-4)
- Gao, X., Chen, X., Taglienti, M., Rumballe, B., Little, M.H., Kreidberg, J.A., 2005. Angioblast-mesenchyme induction of early kidney development is mediated by Wt1 and Vegfa. *Development* 132, 5437–5449. <https://doi.org/10.1242/dev.02095>
- Garcia-Sanz, A., Rodriguez-Barbero, A., Bentley, M.D., Ritman, E.L., Romero, J.C., 1998. Three-Dimensional Microcomputed Tomography of Renal Vasculature in Rats. *Hypertension* 31, 440–444. <https://doi.org/10.1161/01.HYP.31.1.440>
- Garreta, E., Prado, P., Tarantino, C., Oria, R., Fanlo, L., Martí, E., Zalvidea, D., Trepát, X., Roca-Cusachs, P., Gavalda-Navarro, A., Cozzuto, L., Campistol, J.M., Izpisua Belmonte, J.C., Hurtado Del Pozo, C., Montserrat, N., 2019. Fine tuning the extracellular environment accelerates the derivation of kidney organoids from human pluripotent stem cells. *Nat Mater* 18, 397–405. <https://doi.org/10.1038/s41563-019-0287-6>
- Gawad, C., Koh, W., Quake, S.R., 2016. Single-cell genome sequencing: current state of the science. *Nat. Rev. Genet.* 17, 175–188. <https://doi.org/10.1038/nrg.2015.16>
- Georgas, K., Rumballe, B., Valerius, M.T., Chiu, H.S., Thiagarajan, R.D., Lesieur, E., Aronow, B.J., Brunskill, E.W., Combes, A.N., Tang, D., Taylor, D., Grimmond, S.M., Potter, S.S., McMahon, A.P., Little, M.H., 2009. Analysis of early nephron patterning reveals a role for distal RV proliferation in fusion to the ureteric tip via a cap mesenchyme-derived connecting segment. *Dev. Biol.* 332, 273–286. <https://doi.org/10.1016/j.ydbio.2009.05.578>
- Glomerular capillaries [WWW Document], n.d. . TheFreeDictionary.com. URL <https://medical-dictionary.thefreedictionary.com/Glomerular+capillaries> (accessed 11.1.20).
- Go, A.S., Chertow, G.M., Fan, D., McCulloch, C.E., Hsu, C., 2004. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N. Engl. J. Med.* 351, 1296–1305. <https://doi.org/10.1056/NEJMoa041031>
- Goldman, O., Han, S., Hamou, W., Jodon de Villeroche, V., Uzan, G., Lickert, H., Gouon-Evans, V., 2014. Endoderm Generates Endothelial Cells during Liver Development. *Stem Cell Reports* 3, 556–565. <https://doi.org/10.1016/j.stemcr.2014.08.009>
- Gong, K.-Q., Yallowitz, A.R., Sun, H., Dressler, G.R., Wellik, D.M., 2007. A Hox-Eya-Pax complex regulates early kidney developmental gene expression. *Mol. Cell. Biol.* 27, 7661–7668. <https://doi.org/10.1128/MCB.00465-07>
- Grobstein, C., 1955. Tissue disaggregation in relation to determination and stability of cell type. *Ann. N. Y. Acad. Sci.* 60, 1095–1107. <https://doi.org/10.1111/j.1749-6632.1955.tb40091.x>

- Grote, D., Boualia, S.K., Souabni, A., Merkel, C., Chi, X., Costantini, F., Carroll, T., Bouchard, M., 2008. Gata3 acts downstream of beta-catenin signaling to prevent ectopic metanephric kidney induction. *PLoS Genet.* 4, e1000316. <https://doi.org/10.1371/journal.pgen.1000316>
- Grote, D., Souabni, A., Busslinger, M., Bouchard, M., 2006. Pax2/8-regulated Gata3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney. *Development* 133, 53–61. <https://doi.org/10.1242/dev.02184>
- Hatzopoulos, A.K., Folkman, J., Vasile, E., Eiselen, G.K., Rosenberg, R.D., 1998. Isolation and characterization of endothelial progenitor cells from mouse embryos. *Development* 125, 1457–1468.
- Herzlinger, D., Koseki, C., Mikawa, T., al-Awqati, Q., 1992. Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction. *Development* 114, 565–572.
- Hill, N.R., Fatoba, S.T., Oke, J.L., Hirst, J.A., O’Callaghan, C.A., Lasserson, D.S., Hobbs, F.D.R., 2016. Global Prevalence of Chronic Kidney Disease - A Systematic Review and Meta-Analysis. *PLoS ONE* 11, e0158765. <https://doi.org/10.1371/journal.pone.0158765>
- Hiraoka, M., 2003. Medical management of congenital anomalies of the kidney and urinary tract. *Pediatr Int* 45, 624–633.
- Hirst, C.E., Ng, E.S., Azzola, L., Voss, A.K., Thomas, T., Stanley, E.G., Elefanty, A.G., 2006. Transcriptional profiling of mouse and human ES cells identifies SLAIN1, a novel stem cell gene. *Developmental Biology* 293, 90–103. <https://doi.org/10.1016/j.ydbio.2006.01.023>
- Hochane, M., Berg, P.R. van den, Fan, X., Bérenger-Currias, N., Adegeest, E., Bialecka, M., Nieveen, M., Menschaart, M., Lopes, S.M.C. de S., Semrau, S., 2019. Single-cell transcriptomics reveals gene expression dynamics of human fetal kidney development. *PLOS Biology* 17, e3000152. <https://doi.org/10.1371/journal.pbio.3000152>
- Hohwieler, M., Illing, A., Hermann, P.C., Mayer, T., Stockmann, M., Perkhofer, L., Eiseler, T., Antony, J.S., Müller, M., Renz, S., Kuo, C.-C., Lin, Q., Sendler, M., Breunig, M., Kleiderman, S.M., Lechel, A., Zenker, M., Leichsenring, M., Rosendahl, J., Zenke, M., Sainz, B., Mayerle, J., Costa, I.G., Seufferlein, T., Kormann, M., Wagner, M., Liebau, S., Kleger, A., 2017. Human pluripotent stem cell-derived acinar/ductal organoids generate human pancreas upon orthotopic transplantation and allow disease modelling. *Gut* 66, 473–486. <https://doi.org/10.1136/gutjnl-2016-312423>
- Holloway, E.M., Capeling, M.M., Spence, J.R., 2019. Biologically inspired approaches to enhance human organoid complexity. *Development* 146. <https://doi.org/10.1242/dev.166173>
- Homan, K.A., Gupta, N., Kroll, K.T., Kolesky, D.B., Skylar-Scott, M., Miyoshi, T., Mau, D., Valerius, M.T., Ferrante, T., Bonventre, J.V., Lewis, J.A., Morizane, R., 2019. Flow-enhanced vascularization and maturation of kidney organoids in vitro. *Nat. Methods* 16, 255–262. <https://doi.org/10.1038/s41592-019-0325-y>
- Howden, S.E., Vanslambrouck, J.M., Wilson, S.B., Tan, K.S., Little, M.H., 2019. Reporter-based fate mapping in human kidney organoids confirms nephron lineage relationships and reveals synchronous nephron formation. *EMBO Rep.* 20. <https://doi.org/10.15252/embr.201847483>
- Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* 4, 44–57. <https://doi.org/10.1038/nprot.2008.211>

- Hughson, M., Farris, A.B., Douglas-Denton, R., Hoy, W.E., Bertram, J.F., 2003. Glomerular number and size in autopsy kidneys: the relationship to birth weight. *Kidney Int.* 63, 2113–2122. <https://doi.org/10.1046/j.1523-1755.2003.00018.x>
- Hyink, D.P., Tucker, D.C., St John, P.L., Leardkamolkarn, V., Accavitti, M.A., Abrass, C.K., Abrahamson, D.R., 1996. Endogenous origin of glomerular endothelial and mesangial cells in grafts of embryonic kidneys. *Am. J. Physiol.* 270, F886–899. <https://doi.org/10.1152/ajprenal.1996.270.5.F886>
- Ide, S., Yahara, Y., Kobayashi, Y., Strausser, S.A., Ide, K., Watwe, A., Xu-Vanpala, S., Privratsky, J.R., Crowley, S.D., Shinohara, M.L., Alman, B.A., Souma, T., 2020. Yolk-sac-derived macrophages progressively expand in the mouse kidney with age. *eLife* 9, e51756. <https://doi.org/10.7554/eLife.51756>
- Igarashi, P., Somlo, S., 2007. Polycystic Kidney Disease. *JASN* 18, 1371–1373. <https://doi.org/10.1681/ASN.2007030299>
- Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., Benvenisty, N., 2000. Differentiation of Human Embryonic Stem Cells into Embryoid Bodies Comprising the Three Embryonic Germ Layers. *Mol Med* 6, 88–95. <https://doi.org/10.1007/BF03401776>
- Jain, S., 2009. The many faces of RET dysfunction in kidney. *Organogenesis* 5, 177–190.
- James, R.G., Schultheiss, T.M., 2005. Bmp signaling promotes intermediate mesoderm gene expression in a dose-dependent, cell-autonomous and translation-dependent manner. *Dev. Biol.* 288, 113–125. <https://doi.org/10.1016/j.ydbio.2005.09.025>
- James, R.G., Schultheiss, T.M., 2003. Patterning of the avian intermediate mesoderm by lateral plate and axial tissues. *Dev. Biol.* 253, 109–124. <https://doi.org/10.1006/dbio.2002.0863>
- Jennings, R.E., Berry, A.A., Strutt, J.P., Gerrard, D.T., Hanley, N.A., 2015. Human pancreas development. *Development* 142, 3126–3137. <https://doi.org/10.1242/dev.120063>
- Johansson, B.M., Wiles, M.V., 1995. Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. *Mol Cell Biol* 15, 141–151. <https://doi.org/10.1128/mcb.15.1.141>
- Jørgensen, M.C., Ahnfelt-Rønne, J., Hald, J., Madsen, O.D., Serup, P., Hecksher-Sørensen, J., 2007. An Illustrated Review of Early Pancreas Development in the Mouse. *Endocr Rev* 28, 685–705. <https://doi.org/10.1210/er.2007-0016>
- Jung, K.B., Lee, H., Son, Y.S., Lee, M.-O., Kim, Y.-D., Oh, S.J., Kwon, O., Cho, S., Cho, H.-S., Kim, D.-S., Oh, J.-H., Zilbauer, M., Min, J.-K., Jung, C.-R., Kim, J., Son, M.-Y., 2018. Interleukin-2 induces the in vitro maturation of human pluripotent stem cell-derived intestinal organoids. *Nature Communications* 9, 3039. <https://doi.org/10.1038/s41467-018-05450-8>
- Kaimori, J., Germino, G.G., 2008. ARPKD and ADPKD: First Cousins or More Distant Relatives? *JASN* 19, 416–418. <https://doi.org/10.1681/ASN.2008010033>
- Kalluri, H.V., Hardinger, K.L., 2012. Current state of renal transplant immunosuppression: Present and future. *World J Transplant* 2, 51–68. <https://doi.org/10.5500/wjt.v2.i4.51>
- Kang, D.H., Joly, A.H., Oh, S.W., Hugo, C., Kerjaschki, D., Gordon, K.L., Mazzali, M., Jefferson, J.A., Hughes, J., Madsen, K.M., Schreiner, G.F., Johnson, R.J., 2001. Impaired angiogenesis in the remnant kidney model: I. Potential role of vascular endothelial growth factor and thrombospondin-1. *J. Am. Soc. Nephrol.* 12, 1434–1447.
- Karner, C.M., Das, A., Ma, Z., Self, M., Chen, C., Lum, L., Oliver, G., Carroll, T.J., 2011. Canonical Wnt9b signaling balances progenitor cell expansion and differentiation during kidney development. *Development* 138, 1247–1257. <https://doi.org/10.1242/dev.057646>

- Katz, A.I., Lindheimer, M.D., 1977. Actions of Hormones on the Kidney. *Annual Review of Physiology* 39, 97–133. <https://doi.org/10.1146/annurev.ph.39.030177.000525>
- Kaufman, D.P., Basit, H., Knohl, S.J., 2020. Physiology, Glomerular Filtration Rate (GFR), in: *StatPearls*. StatPearls Publishing, Treasure Island (FL).
- Kidneys, Bladder and Prostate Scans, n.d. . Ultrasound Services. URL <https://ultrasoundservices.co.uk/services/kidney-bladder/> (accessed 11.1.20).
- Kim, D., Dressler, G.R., 2005. Nephrogenic Factors Promote Differentiation of Mouse Embryonic Stem Cells into Renal Epithelia. *JASN* 16, 3527–3534. <https://doi.org/10.1681/ASN.2005050544>
- Kim, Y.-H., Kwon, T.-H., Frische, S., Kim, J., Tisher, C.C., Madsen, K.M., Nielsen, S., 2002. Immunocytochemical localization of pendrin in intercalated cell subtypes in rat and mouse kidney. *American Journal of Physiology-Renal Physiology* 283, F744–F754. <https://doi.org/10.1152/ajprenal.00037.2002>
- Klein, A.M., Treutlein, B., 2019. Single cell analyses of development in the modern era. *Development* 146. <https://doi.org/10.1242/dev.181396>
- Kobayashi, A., Kwan, K.-M., Carroll, T.J., McMahon, A.P., Mendelsohn, C.L., Behringer, R.R., 2005. Distinct and sequential tissue-specific activities of the LIM-class homeobox gene *Lim1* for tubular morphogenesis during kidney development. *Development* 132, 2809–2823. <https://doi.org/10.1242/dev.01858>
- Kobayashi, A., Mugford, J.W., Krautzberger, A.M., Naiman, N., Liao, J., McMahon, A.P., 2014. Identification of a Multipotent Self-Renewing Stromal Progenitor Population during Mammalian Kidney Organogenesis. *Stem Cell Reports* 3, 650–662. <https://doi.org/10.1016/j.stemcr.2014.08.008>
- Kobayashi, A., Valerius, M.T., Mugford, J.W., Carroll, T.J., Self, M., Oliver, G., McMahon, A.P., 2008. *Six2* defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development. *Cell Stem Cell* 3, 169–181. <https://doi.org/10.1016/j.stem.2008.05.020>
- Kobayashi, T., Tanaka, H., Kuwana, H., Inoshita, S., Teraoka, H., Sasaki, S., Terada, Y., 2005. Wnt4-transformed mouse embryonic stem cells differentiate into renal tubular cells. *Biochem Biophys Res Commun* 336, 585–595. <https://doi.org/10.1016/j.bbrc.2005.08.136>
- Kohda, Y., Murakami, H., Moe, O.W., Star, R.A., 2000. Analysis of segmental renal gene expression by laser capture microdissection. *Kidney International* 57, 321–331. <https://doi.org/10.1046/j.1523-1755.2000.00824.x>
- Kokko, J.P., 1987. The role of the collecting duct in urinary concentration. *Kidney International* 31, 606–610. <https://doi.org/10.1038/ki.1987.41>
- Kopan, R., Cheng, H.-T., Surendran, K., 2007. Molecular insights into segmentation along the proximal-distal axis of the nephron. *J Am Soc Nephrol* 18, 2014–2020. <https://doi.org/10.1681/ASN.2007040453>
- Kopp, U.C., 2011. *Neuroanatomy, Neural Control of Renal Function*. Morgan & Claypool Life Sciences.
- Kramer, J., Steinhoff, J., Klinger, M., Fricke, L., Rohwedel, J., 2006. Cells differentiated from mouse embryonic stem cells via embryoid bodies express renal marker molecules. *Differentiation* 74, 91–104. <https://doi.org/10.1111/j.1432-0436.2006.00062.x>
- Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D., Jaenisch, R., 1993. *WT-1* is required for early kidney development. *Cell* 74, 679–691. [https://doi.org/10.1016/0092-8674\(93\)90515-r](https://doi.org/10.1016/0092-8674(93)90515-r)
- Ku, E., Lee, B.J., Wei, J., Weir, M.R., 2019. Hypertension in CKD: Core Curriculum 2019. *American Journal of Kidney Diseases* 74, 120–131. <https://doi.org/10.1053/j.ajkd.2018.12.044>

- Kubo, A., Shinozaki, K., Shannon, J.M., Kouskoff, V., Kennedy, M., Woo, S., Fehling, H.J., Keller, G., 2004. Development of definitive endoderm from embryonic stem cells in culture. *Development* 131, 1651–1662. <https://doi.org/10.1242/dev.01044>
- Kumar, P., Tan, Y., Cahan, P., 2017. Understanding development and stem cells using single cell-based analyses of gene expression. *Development* 144, 17–32. <https://doi.org/10.1242/dev.133058>
- Kumar, S.V., Er, P.X., Lawlor, K.T., Motazedian, A., Scurr, M., Ghobrial, I., Combes, A.N., Zappia, L., Oshlack, A., Stanley, E.G., Little, M.H., 2019. Kidney micro-organoids in suspension culture as a scalable source of human pluripotent stem cell-derived kidney cells. *Development* 146. <https://doi.org/10.1242/dev.172361>
- Kyte, D., Bishop, J., Brettell, E., Calvert, M., Cockwell, P., Dutton, M., Eddington, H., Hadley, G., Ives, N.J., Jackson, L.J., Stringer, S., Valente, M., 2018. Use of an electronic patient-reported outcome measure in the management of patients with advanced chronic kidney disease: the RePROM pilot trial protocol. *BMJ Open* 8, e026080. <https://doi.org/10.1136/bmjopen-2018-026080>
- Lam, N.N., Lentine, K.L., Levey, A.S., Kasiske, B.L., Garg, A.X., 2015. Long-term medical risks to the living kidney donor. *Nature Reviews Nephrology* 11, 411–419. <https://doi.org/10.1038/nrneph.2015.58>
- Lammert, E., Cleaver, O., Melton, D., 2001. Induction of pancreatic differentiation by signals from blood vessels. *Science* 294, 564–567. <https://doi.org/10.1126/science.1064344>
- Lancaster, M.A., Knoblich, J.A., 2014. Organogenesis in a dish: modeling development and disease using organoid technologies. *Science* 345, 1247125. <https://doi.org/10.1126/science.1247125>
- le Noble, F., Moyon, D., Pardanaud, L., Yuan, L., Djonov, V., Matthijsen, R., Bréant, C., Fleury, V., Eichmann, A., 2004. Flow regulates arterial-venous differentiation in the chick embryo yolk sac. *Development* 131, 361–375. <https://doi.org/10.1242/dev.00929>
- LeBleu, V.S., Taduri, G., O’Connell, J., Teng, Y., Cooke, V.G., Woda, C., Sugimoto, H., Kalluri, R., 2013. Origin and Function of Myofibroblasts in Kidney Fibrosis. *Nat Med* 19, 1047–1053. <https://doi.org/10.1038/nm.3218>
- Li, M., Belmonte, J.C.I., 2019. Organoids — Preclinical Models of Human Disease. *New England Journal of Medicine*.
- Li, R., Zhong, C., Yu, Y., Liu, H., Sakurai, M., Yu, L., Min, Z., Shi, L., Wei, Y., Takahashi, Y., Liao, H.-K., Qiao, J., Deng, H., Nuñez-Delicado, E., Esteban, C.R., Wu, J., Belmonte, J.C.I., 2019. Generation of Blastocyst-like Structures from Mouse Embryonic and Adult Cell Cultures. *Cell* 179, 687–702.e18. <https://doi.org/10.1016/j.cell.2019.09.029>
- Liao, J., Yu, Z., Chen, Y., Bao, M., Zou, C., Zhang, H., Liu, D., Li, T., Zhang, Q., Li, J., Cheng, J., Mo, Z., 2020. Single-cell RNA sequencing of human kidney. *Scientific Data* 7, 4. <https://doi.org/10.1038/s41597-019-0351-8>
- Lindström, N.O., De Sena Brandine, G., Tran, T., Ransick, A., Suh, G., Guo, J., Kim, A.D., Parvez, R.K., Ruffins, S.W., Rutledge, E.A., Thornton, M.E., Grubbs, B., McMahon, J.A., Smith, A.D., McMahon, A.P., 2018a. Progressive Recruitment of Mesenchymal Progenitors Reveals a Time-Dependent Process of Cell Fate Acquisition in Mouse and Human Nephrogenesis. *Dev. Cell* 45, 651–660.e4. <https://doi.org/10.1016/j.devcel.2018.05.010>
- Lindström, N.O., Guo, J., Kim, A.D., Tran, T., Guo, Q., De Sena Brandine, G., Ransick, A., Parvez, R.K., Thornton, M.E., Baskin, L., Grubbs, B., McMahon, J.A., Smith, A.D., McMahon, A.P., 2018b. Conserved and Divergent Features of Mesenchymal Progenitor Cell Types within the Cortical Nephrogenic Niche of the Human and Mouse Kidney. *J. Am. Soc. Nephrol.* 29, 806–824. <https://doi.org/10.1681/ASN.2017080890>

- Lindström, N.O., Lawrence, M.L., Burn, S.F., Johansson, J.A., Bakker, E.R., Ridgway, R.A., Chang, C.-H., Karolak, M.J., Oxburgh, L., Headon, D.J., Sansom, O.J., Smits, R., Davies, J.A., Hohenstein, P., 2015. Integrated β -catenin, BMP, PTEN, and Notch signalling patterns the nephron. *eLife* 4, e04000. <https://doi.org/10.7554/eLife.04000>
- Lindström, N.O., McMahon, J.A., Guo, J., Tran, T., Guo, Q., Rutledge, E., Parvez, R.K., Saribekyan, G., Schuler, R.E., Liao, C., Kim, A.D., Abdelhalim, A., Ruffins, S.W., Thornton, M.E., Basking, L., Grubbs, B., Kesselman, C., McMahon, A.P., 2018c. Conserved and Divergent Features of Human and Mouse Kidney Organogenesis. *JASN* 29, 785–805. <https://doi.org/10.1681/ASN.2017080887>
- Lindström, N.O., Tran, T., Guo, J., Rutledge, E., Parvez, R.K., Thornton, M.E., Grubbs, B., McMahon, J.A., McMahon, A.P., 2018d. Conserved and Divergent Molecular and Anatomic Features of Human and Mouse Nephron Patterning. *J. Am. Soc. Nephrol.* 29, 825–840. <https://doi.org/10.1681/ASN.2017091036>
- Little, M.H., 2006. Regrow or Repair: Potential Regenerative Therapies for the Kidney. *JASN* 17, 2390–2401. <https://doi.org/10.1681/ASN.2006030218>
- Little, M.H., Combes, A.N., Takasato, M., 2016. Understanding kidney morphogenesis to guide renal tissue regeneration. *Nat Rev Nephrol* 12, 624–635. <https://doi.org/10.1038/nrneph.2016.126>
- Little, M.H., McMahon, A.P., 2012. Mammalian Kidney Development: Principles, Progress, and Projections. *Cold Spring Harb Perspect Biol* 4. <https://doi.org/10.1101/cshperspect.a008300>
- Liu, X., Li, N., Xu, T., Sun, F., Li, R., Gao, Q., Chen, L., Wen, C., 2017. Effect of renal perfusion and structural heterogeneity on shear wave elastography of the kidney: an in vivo and ex vivo study. *BMC Nephrology* 18, 265. <https://doi.org/10.1186/s12882-017-0679-2>
- Loh, Y.-H., Hartung, O., Li, H., Guo, C., Sahalie, J.M., Manos, P.D., Urbach, A., Heffner, G.C., Grskovic, M., Vigneault, F., Lensch, M.W., Park, I.-H., Agarwal, S., Church, G.M., Collins, J.J., Irion, S., Daley, G.Q., 2010. Reprogramming of T cells from human peripheral blood. *Cell Stem Cell* 7, 15–19. <https://doi.org/10.1016/j.stem.2010.06.004>
- Lopez, M.L.S.S., Gomez, R.A., 2011. Development of the Renal Arterioles. *JASN* 22, 2156–2165. <https://doi.org/10.1681/ASN.2011080818>
- Low, J.H., Li, P., Chew, E.G.Y., Zhou, B., Suzuki, K., Zhang, T., Lian, M.M., Liu, M., Aizawa, E., Esteban, C.R., Yong, K.S.M., Chen, Q., Campistol, J.M., Fang, M., Khor, C.C., Foo, J.N., Belmonte, J.C.I., Xia, Y., 2019. Generation of Human PSC-Derived Kidney Organoids with Patterned Nephron Segments and a De Novo Vascular Network. *Cell Stem Cell* 25, 373–387.e9. <https://doi.org/10.1016/j.stem.2019.06.009>
- Lu, C.C., Robertson, E.J., 2004. Multiple roles for Nodal in the epiblast of the mouse embryo in the establishment of anterior-posterior patterning. *Dev. Biol.* 273, 149–159. <https://doi.org/10.1016/j.ydbio.2004.06.004>
- Mack, M., Yanagita, M., 2015. Origin of myofibroblasts and cellular events triggering fibrosis. *Kidney International* 87, 297–307. <https://doi.org/10.1038/ki.2014.287>
- Małyszko, Jolanta, Kozłowska, K., Kozłowski, L., Małyszko, Jacek, 2017. Nephrotoxicity of anticancer treatment. *Nephrol Dial Transplant* 32, 924–936. <https://doi.org/10.1093/ndt/gfw338>
- Mantovani, A., Cassatella, M.A., Costantini, C., Jaillon, S., 2011. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nature Reviews Immunology* 11, 519–531. <https://doi.org/10.1038/nri3024>
- Marcu, R., Choi, Y.J., Xue, J., Fortin, C.L., Wang, Y., Nagao, R.J., Xu, J., MacDonald, J.W., Bammler, T.K., Murry, C.E., Muczynski, K., Stevens, K.R., Himmelfarb, J., Schwartz,

- S.M., Zheng, Y., 2018. Human Organ-Specific Endothelial Cell Heterogeneity. *iScience* 4, 20–35. <https://doi.org/10.1016/j.isci.2018.05.003>
- Matsumoto, K., Yoshitomi, H., Rossant, J., Zaret, K.S., 2001. Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* 294, 559–563. <https://doi.org/10.1126/science.1063889>
- McDonald, K.M., Miller, P.D., Anderson, R.J., Berl, T., Schrier, R.W., 1976. Hormonal control of renal water excretion. *Kidney International* 10, 38–45. <https://doi.org/10.1038/ki.1976.77>
- McMahon, A.P., 2016. Development of the Mammalian Kidney. *Curr Top Dev Biol* 117, 31–64. <https://doi.org/10.1016/bs.ctdb.2015.10.010>
- McMahon, A.P., Aronow, B.J., Davidson, D.R., Davies, J.A., Gaido, K.W., Grimmond, S., Lessard, J.L., Little, M.H., Potter, S.S., Wilder, E.L., Zhang, P., 2008. GUDMAP: The Genitourinary Developmental Molecular Anatomy Project. *JASN* 19, 667–671. <https://doi.org/10.1681/ASN.2007101078>
- Menon, R., Otto, E.A., Kokoruda, A., Zhou, J., Zhang, Z., Yoon, E., Chen, Y.-C., Troyanskaya, O., Spence, J.R., Kretzler, M., Cebrián, C., 2018. Single-cell analysis of progenitor cell dynamics and lineage specification in the human fetal kidney. *Development* 145. <https://doi.org/10.1242/dev.164038>
- Miao, Z., Balzer, M.S., Ma, Z., Liu, H., Wu, J., Shrestha, R., Aranyi, T., Kwan, A., Kondo, A., Pontoglio, M., Kim, J., Li, M., Kaestner, K.H., Susztak, K., 2020. Single cell resolution regulatory landscape of the mouse kidney highlights cellular differentiation programs and renal disease targets. *bioRxiv* 2020.05.24.113910. <https://doi.org/10.1101/2020.05.24.113910>
- Millman, J.R., Xie, C., Van Dervort, A., Gürtler, M., Pagliuca, F.W., Melton, D.A., 2016. Generation of stem cell-derived β -cells from patients with type 1 diabetes. *Nat Commun* 7. <https://doi.org/10.1038/ncomms11463>
- Mills, C.G., Lawrence, M.L., Munro, D.A.D., Elhendawi, M., Mullins, J.J., Davies, J.A., 2017. Asymmetric BMP4 signalling improves the realism of kidney organoids. *Scientific Reports* 7, 1–8. <https://doi.org/10.1038/s41598-017-14809-8>
- Mizutani, A., Koinuma, D., Tsutsumi, S., Kamimura, N., Morikawa, M., Suzuki, H.I., Imamura, T., Miyazono, K., Aburatani, H., 2011. Cell type-specific target selection by combinatorial binding of Smad2/3 proteins and hepatocyte nuclear factor 4 α in HepG2 cells. *J. Biol. Chem.* 286, 29848–29860. <https://doi.org/10.1074/jbc.M110.217745>
- Morizane, R., Lam, A.Q., Freedman, B.S., Kishi, S., Valerius, M.T., Bonventre, J.V., 2015. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. *Nat. Biotechnol.* 33, 1193–1200. <https://doi.org/10.1038/nbt.3392>
- Mount, P.F., Power, D.A., 2006. Nitric oxide in the kidney: functions and regulation of synthesis. *Acta Physiol (Oxf)* 187, 433–446. <https://doi.org/10.1111/j.1748-1716.2006.01582.x>
- Mudumana, S.P., Hentschel, D., Liu, Y., Vasilyev, A., Drummond, I.A., 2008. *odd skipped related1* reveals a novel role for endoderm in regulating kidney versus vascular cell fate. *Development* 135, 3355–3367. <https://doi.org/10.1242/dev.022830>
- Mugford, J.W., Sipilä, P., Kobayashi, A., Behringer, R.R., McMahon, A.P., 2008a. *Hoxd11* specifies a program of metanephric kidney development within the intermediate mesoderm of the mouse embryo. *Dev Biol* 319, 396–405. <https://doi.org/10.1016/j.ydbio.2008.03.044>
- Mugford, J.W., Sipilä, P., McMahon, J.A., McMahon, A.P., 2008b. *Osr1* expression demarcates a multi-potent population of intermediate mesoderm that undergoes

- progressive restriction to an *Osrl*-dependent nephron progenitor compartment within the mammalian kidney. *Dev. Biol.* 324, 88–98. <https://doi.org/10.1016/j.ydbio.2008.09.010>
- Munro, D.A., Wineberg, Y., Tarnick, J., Vink, C.S., Li, Z., Pridans, C., Dzierzak, E., Kalisky, T., Hohenstein, P., Davies, J.A., 2019. Macrophages restrict the nephrogenic field and promote endothelial connections during kidney development. *eLife* 8, e43271. <https://doi.org/10.7554/eLife.43271>
- Munro, D.A.D., Hohenstein, P., Davies, J.A., 2017. Cycles of vascular plexus formation within the nephrogenic zone of the developing mouse kidney. *Sci Rep* 7, 3273. <https://doi.org/10.1038/s41598-017-03808-4>
- Murakami, Y., Naganuma, H., Tanigawa, S., Fujimori, T., Eto, M., Nishinakamura, R., 2019. Reconstitution of the embryonic kidney identifies a donor cell contribution to the renal vasculature upon transplantation. *Scientific Reports* 9, 1–10. <https://doi.org/10.1038/s41598-018-37793-z>
- Murry, C.E., Keller, G., 2008. Differentiation of Embryonic Stem Cells to Clinically Relevant Populations: Lessons from Embryonic Development. *Cell* 132, 661–680. <https://doi.org/10.1016/j.cell.2008.02.008>
- Murugapoopathy, V., Gupta, I.R., 2020. A Primer on Congenital Anomalies of the Kidneys and Urinary Tracts (CAKUT). *CJASN* 15, 723–731. <https://doi.org/10.2215/CJN.12581019>
- Narlis, M., Grote, D., Gaitan, Y., Boualia, S.K., Bouchard, M., 2007. Pax2 and Pax8 Regulate Branching Morphogenesis and Nephron Differentiation in the Developing Kidney. *JASN* 18, 1121–1129. <https://doi.org/10.1681/ASN.2006070739>
- Neal, J.T., Li, X., Zhu, J., Giangarra, V., Grzeskowiak, C.L., Ju, J., Liu, I.H., Chiou, S.-H., Salahudeen, A.A., Smith, A.R., Deutsch, B.C., Liao, L., Zemek, A.J., Zhao, F., Karlsson, K., Schultz, L.M., Metzner, T.J., Nadauld, L.D., Tseng, Y.-Y., Alkhairy, S., Oh, C., Keskula, P., Mendoza-Villanueva, D., De La Vega, F.M., Kunz, P.L., Liao, J.C., Leppert, J.T., Sunwoo, J.B., Sabatti, C., Boehm, J.S., Hahn, W.C., Zheng, G.X.Y., Davis, M.M., Kuo, C.J., 2018. Organoid modeling of the tumor immune microenvironment. *Cell* 175, 1972–1988.e16. <https://doi.org/10.1016/j.cell.2018.11.021>
- Ng, E.S., Davis, R.P., Azzola, L., Stanley, E.G., Elefanty, A.G., 2005. Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. *Blood* 106, 1601–1603. <https://doi.org/10.1182/blood-2005-03-0987>
- Nie, X., Xu, J., El-Hashash, A., Xu, P.-X., 2011. Six1 regulates *Grem1* expression in the metanephric mesenchyme to initiate branching morphogenesis. *Dev. Biol.* 352, 141–151. <https://doi.org/10.1016/j.ydbio.2011.01.027>
- Nishikawa, M., Yanagawa, Naomi, Kojima, N., Yuri, S., Hauser, P.V., Jo, O.D., Yanagawa, Norimoto, 2012. Stepwise renal lineage differentiation of mouse embryonic stem cells tracing in vivo development. *Biochem Biophys Res Commun* 417, 897–902. <https://doi.org/10.1016/j.bbrc.2011.12.071>
- Nishikawa, S., Goldstein, R.A., Nierras, C.R., 2008. The promise of human induced pluripotent stem cells for research and therapy. *Nat. Rev. Mol. Cell Biol.* 9, 725–729. <https://doi.org/10.1038/nrm2466>
- Nishinakamura, R., 2019. Human kidney organoids: progress and remaining challenges. *Nat Rev Nephrol* 15, 613–624. <https://doi.org/10.1038/s41581-019-0176-x>
- Nolph, K.D., 2013. Peritoneal dialysis. Springer Science & Business Media.
- Nostro, M.C., Sarangi, F., Ogawa, S., Holtzinger, A., Corneo, B., Li, X., Micallef, S.J., Park, I.-H., Basford, C., Wheeler, M.B., Daley, G.Q., Elefanty, A.G., Stanley, E.G., Keller,

- G., 2011. Stage-specific signaling through TGF β family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. *Development* 138, 861–871. <https://doi.org/10.1242/dev.055236>
- Nusse, R., Clevers, H., 2017. Wnt/ β -Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* 169, 985–999. <https://doi.org/10.1016/j.cell.2017.05.016>
- Obara-Ishihara, T., Kuhlman, J., Niswander, L., Herzlinger, D., 1999. The surface ectoderm is essential for nephric duct formation in intermediate mesoderm. *Development* 126, 1103–1108.
- O'Brien, L.L., Guo, Q., Lee, Y., Tran, T., Benazet, J.-D., Whitney, P.H., Valouev, A., McMahon, A.P., 2016. Differential regulation of mouse and human nephron progenitors by the Six family of transcriptional regulators. *Development* 143, 595–608. <https://doi.org/10.1242/dev.127175>
- Osafune, K., Nishinakamura, R., Komazaki, S., Asashima, M., 2002. In vitro induction of the pronephric duct in *Xenopus* explants. *Dev Growth Differ* 44, 161–167. <https://doi.org/10.1046/j.1440-169x.2002.00631.x>
- Osafune, K., Takasato, M., Kispert, A., Asashima, M., Nishinakamura, R., 2006. Identification of multipotent progenitors in the embryonic mouse kidney by a novel colony-forming assay. *Development* 133, 151–161. <https://doi.org/10.1242/dev.02174>
- Osakada, F., Jin, Z.-B., Hiram, Y., Ikeda, H., Danjyo, T., Watanabe, K., Sasai, Y., Takahashi, M., 2009. In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. *J Cell Sci* 122, 3169–3179. <https://doi.org/10.1242/jcs.050393>
- Pagliuca, F.W., Millman, J.R., Gürtler, M., Segel, M., Van Dervort, A., Ryu, J.H., Peterson, Q.P., Greiner, D., Melton, D.A., 2014. Generation of Functional Human Pancreatic β Cells In Vitro. *Cell* 159, 428–439. <https://doi.org/10.1016/j.cell.2014.09.040>
- Park, J., Shrestha, R., Qiu, C., Kondo, A., Huang, S., Werth, M., Li, M., Barasch, J., Suszták, K., 2018. Single-cell transcriptomics of the mouse kidney reveals potential cellular targets of kidney disease. *Science* 360, 758–763. <https://doi.org/10.1126/science.aar2131>
- Park, S.E., Georgescu, A., Huh, D., 2019. Organoids-on-a-chip. *Science* 364, 960–965. <https://doi.org/10.1126/science.aaw7894>
- Paşca, S.P., 2019. Assembling human brain organoids. *Science* 363, 126–127. <https://doi.org/10.1126/science.aau5729>
- Pearce, D., Soundararajan, R., Trimpert, C., Kashlan, O.B., Deen, P.M.T., Kohan, D.E., 2015. Collecting Duct Principal Cell Transport Processes and Their Regulation. *Clin J Am Soc Nephrol* 10, 135–146. <https://doi.org/10.2215/CJN.05760513>
- Pedersen, A., Skjong, C., Shawlot, W., 2005. Lim 1 is required for nephric duct extension and ureteric bud morphogenesis. *Dev. Biol.* 288, 571–581. <https://doi.org/10.1016/j.ydbio.2005.09.027>
- Peng, T., Tian, Y., Boogerd, C.J., Lu, M.M., Kadzik, R.S., Stewart, K.M., Evans, S.M., Morrissey, E.E., 2013. Coordination of heart and lung co-development by a multipotent cardiopulmonary progenitor. *Nature* 500, 589–592. <https://doi.org/10.1038/nature12358>
- Pennisi, E., 2018. Chronicling embryos, cell by cell, gene by gene. *Science* 360, 367–367. <https://doi.org/10.1126/science.360.6387.367>
- Perea-Gomez, A., Vella, F.D.J., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H., Behringer, R.R., Ang, S.-L., 2002. Nodal Antagonists in the Anterior Visceral Endoderm Prevent the Formation of Multiple Primitive Streaks. *Developmental Cell* 3, 745–756. [https://doi.org/10.1016/S1534-5807\(02\)00321-0](https://doi.org/10.1016/S1534-5807(02)00321-0)

- Pham, P.-T., Pham, P.-A., Pham, P.-C., Parikh, S., Danovitch, G., 2010. Evaluation of adult kidney transplant candidates. *Semin Dial* 23, 595–605. <https://doi.org/10.1111/j.1525-139X.2010.00809.x>
- PhD, J.E.H., 2016. *Guyton and Hall Textbook of Medical Physiology, Jordanian Edition E-Book*. Elsevier Health Sciences.
- Phipson, B., Er, P.X., Combes, A.N., Forbes, T.A., Howden, S.E., Zappia, L., Yen, H.-J., Lawlor, K.T., Hale, L.J., Sun, J., Wolvetang, E., Takasato, M., Oshlack, A., Little, M.H., 2019. Evaluation of variability in human kidney organoids. *Nat. Methods* 16, 79–87. <https://doi.org/10.1038/s41592-018-0253-2>
- Potter, S.S., 2018. Single-cell RNA sequencing for the study of development, physiology and disease. *Nat Rev Nephrol* 14, 479–492. <https://doi.org/10.1038/s41581-018-0021-7>
- Przepiorski, A., Sander, V., Tran, T., Hollywood, J.A., Sorrenson, B., Shih, J.-H., Wolvetang, E.J., McMahon, A.P., Holm, T.M., Davidson, A.J., 2018. A Simple Bioreactor-Based Method to Generate Kidney Organoids from Pluripotent Stem Cells. *Stem Cell Reports* 11, 470–484. <https://doi.org/10.1016/j.stemcr.2018.06.018>
- Qiu, X., Mao, Q., Tang, Y., Wang, L., Chawla, R., Pliner, H.A., Trapnell, C., 2017. Reversed graph embedding resolves complex single-cell trajectories. *Nat. Methods* 14, 979–982. <https://doi.org/10.1038/nmeth.4402>
- Quaggin, S.E., Kreidberg, J.A., 2008. Development of the renal glomerulus: good neighbors and good fences. *Development* 135, 609–620. <https://doi.org/10.1242/dev.001081>
- Ranganathan, D., John, G.T., 2012. Nocturnal hemodialysis. *Indian J Nephrol* 22, 323–332. <https://doi.org/10.4103/0971-4065.103905>
- Reidy, K., Kang, H.M., Hostetter, T., Susztak, K., 2014. Molecular mechanisms of diabetic kidney disease. *J Clin Invest* 124, 2333–2340. <https://doi.org/10.1172/JCI72271>
- Reidy, K.J., Rosenblum, N.D., 2009. Cell and molecular biology of kidney development. *Semin Nephrol* 29, 321–337. <https://doi.org/10.1016/j.semnephrol.2009.03.009>
- Ren, X., Zhang, J., Gong, X., Niu, X., Zhang, Xuejin, Chen, P., Zhang, Xuejun, 2010. Differentiation of murine embryonic stem cells toward renal lineages by conditioned medium from ureteric bud cells in vitro. *Acta Biochim Biophys Sin (Shanghai)* 42, 464–471. <https://doi.org/10.1093/abbs/gmq046>
- Riegel, J.A., 1986. Hydrostatic pressures in glomeruli and renal vasculature of the hagfish, *Eptatretus stouti*. *J. Exp. Biol.* 123, 359–371.
- Risau, W., 1997. Mechanisms of angiogenesis. *Nature* 386, 671–674. <https://doi.org/10.1038/386671a0>
- Robert, B., St John, P.L., Abrahamson, D.R., 1998. Direct visualization of renal vascular morphogenesis in Flk1 heterozygous mutant mice. *Am. J. Physiol.* 275, F164–172. <https://doi.org/10.1152/ajprenal.1998.275.1.F164>
- Robert, B., St John, P.L., Hyink, D.P., Abrahamson, D.R., 1996. Evidence that embryonic kidney cells expressing flk-1 are intrinsic, vasculogenic angioblasts. *Am. J. Physiol.* 271, F744–753. <https://doi.org/10.1152/ajprenal.1996.271.3.F744>
- Robson, L., 2014. The kidney – an organ of critical importance in physiology. *J Physiol* 592, 3953–3954. <https://doi.org/10.1113/jphysiol.2014.279216>
- Ronaldson-Bouchard, K., Ma, S.P., Yeager, K., Chen, T., Song, L., Sirabella, D., Morikawa, K., Teles, D., Yazawa, M., Vunjak-Novakovic, G., 2018. Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature* 556, 239–243. <https://doi.org/10.1038/s41586-018-0016-3>
- Ronco, C., Crepaldi, C., Cruz, D.N., 2009. *Peritoneal Dialysis: From Basic Concepts to Clinical Excellence*. Karger Medical and Scientific Publishers.

- Roy, A., Al-bataineh, M.M., Pastor-Soler, N.M., 2015. Collecting Duct Intercalated Cell Function and Regulation. *Clin J Am Soc Nephrol* 10, 305–324. <https://doi.org/10.2215/CJN.08880914>
- Saran, R., Robinson, B., Abbott, K.C., Agodoa, L.Y.C., Albertus, P., Ayanian, J., Balkrishnan, R., Bragg-Gresham, J., Cao, J., Chen, J.L.T., Cope, E., Dharmarajan, S., Dietrich, X., Eckard, A., Eggers, P.W., Gaber, C., Gillen, D., Gipson, D., Gu, H., Hailpern, S.M., Hall, Y.N., Han, Y., He, K., Hebert, P., Helmuth, M., Herman, W., Heung, M., Hutton, D., Jacobsen, S.J., Ji, N., Jin, Y., Kalantar-Zadeh, K., Kapke, A., Katz, R., Kovesdy, C.P., Kurtz, V., Lavalley, D., Li, Y., Lu, Y., McCullough, K., Molnar, M.Z., Montez-Rath, M., Morgenstern, H., Mu, Q., Mukhopadhyay, P., Nallamothu, B., Nguyen, D.V., Norris, K.C., O'Hare, A.M., Obi, Y., Pearson, J., Pisoni, R., Plattner, B., Port, F.K., Potukuchi, P., Rao, P., Ratkowiak, K., Ravel, V., Ray, D., Rhee, C.M., Schaubel, D.E., Selewski, D.T., Shaw, S., Shi, J., Shieu, M., Sim, J.J., Song, P., Soohoo, M., Steffick, D., Streja, E., Tamura, M.K., Tentori, F., Tilea, A., Tong, L., Turf, M., Wang, D., Wang, M., Woodside, K., Wyncott, A., Xin, X., Zeng, W., Zepel, L., Zhang, S., Zho, H., Hirth, R.A., Shahinian, V., 2017. US Renal Data System 2016 Annual Data Report: Epidemiology of Kidney Disease in the United States. *American Journal of Kidney Diseases* 69, A7–A8. <https://doi.org/10.1053/j.ajkd.2016.12.004>
- Sariola, H., Ekblom, P., Lehtonen, E., Saxén, L., 1983. Differentiation and vascularization of the metanephric kidney grafted on the chorioallantoic membrane. *Dev. Biol.* 96, 427–435. [https://doi.org/10.1016/0012-1606\(83\)90180-x](https://doi.org/10.1016/0012-1606(83)90180-x)
- Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., Clevers, H., 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459, 262–265. <https://doi.org/10.1038/nature07935>
- Saxén, L., Lehtonen, E., 1987. Embryonic kidney in organ culture. *Differentiation* 36, 2–11. <https://doi.org/10.1111/j.1432-0436.1987.tb00176.x>
- Saxén, L., Sariola, H., 1987. Early organogenesis of the kidney. *Pediatr. Nephrol.* 1, 385–392. <https://doi.org/10.1007/bf00849241>
- Schedl, A., 2007. Renal abnormalities and their developmental origin. *Nat Rev Genet* 8, 791–802. <https://doi.org/10.1038/nrg2205>
- Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F., Pachnis, V., 1994. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* 367, 380–383. <https://doi.org/10.1038/367380a0>
- Segev, D.L., Simpkins, C.E., Thompson, R.E., Locke, J.E., Warren, D.S., Montgomery, R.A., 2008. Obesity impacts access to kidney transplantation. *J. Am. Soc. Nephrol.* 19, 349–355. <https://doi.org/10.1681/ASN.2007050610>
- Serluca, F.C., Drummond, I.A., Fishman, M.C., 2002. Endothelial signaling in kidney morphogenesis: a role for hemodynamic forces. *Curr. Biol.* 12, 492–497. [https://doi.org/10.1016/s0960-9822\(02\)00694-2](https://doi.org/10.1016/s0960-9822(02)00694-2)
- Shahbazi, M.N., Jedrusik, A., Vuoristo, S., Recher, G., Hupalowska, A., Bolton, V., Fogarty, N.M.E., Campbell, A., Devito, L.G., Ilic, D., Khalaf, Y., Niakan, K.K., Fishel, S., Zernicka-Goetz, M., 2016. Self-organization of the human embryo in the absence of maternal tissues. *Nature Cell Biology* 18, 700–708. <https://doi.org/10.1038/ncb3347>
- Shamim, H., Mason, I., 1999. Expression of Fgf4 during early development of the chick embryo. *Mech. Dev.* 85, 189–192. [https://doi.org/10.1016/s0925-4773\(99\)00093-3](https://doi.org/10.1016/s0925-4773(99)00093-3)
- Sharma, A., Sances, S., Workman, M.J., Svendsen, C.N., 2020. Multi-lineage Human iPSC-Derived Platforms for Disease Modeling and Drug Discovery. *Cell Stem Cell* 26, 309–329. <https://doi.org/10.1016/j.stem.2020.02.011>

- Sharmin, S., Taguchi, A., Kaku, Y., Yoshimura, Y., Ohmori, T., Sakuma, T., Mukoyama, M., Yamamoto, T., Kurihara, H., Nishinakamura, R., 2015. Human Induced Pluripotent Stem Cell-Derived Podocytes Mature into Vascularized Glomeruli upon Experimental Transplantation. *JASN*. <https://doi.org/10.1681/ASN.2015010096>
- Shawlot, W., Behringer, R.R., 1995. Requirement for *Lim1* in head-organizer function. *Nature* 374, 425–430. <https://doi.org/10.1038/374425a0>
- Shultz, L.D., Ishikawa, F., Greiner, D.L., 2007. Humanized mice in translational biomedical research. *Nature Reviews Immunology* 7, 118–130. <https://doi.org/10.1038/nri2017>
- Simunovic, M., Brivanlou, A.H., 2017. Embryoids, organoids and gastruloids: new approaches to understanding embryogenesis. *Development* 144, 976–985. <https://doi.org/10.1242/dev.143529>
- Singh, A., Satchell, S.C., Neal, C.R., McKenzie, E.A., Tooke, J.E., Mathieson, P.W., 2007. Glomerular Endothelial Glycocalyx Constitutes a Barrier to Protein Permeability. *JASN* 18, 2885–2893. <https://doi.org/10.1681/ASN.2007010119>
- Song, J.J., Guyette, J.P., Gilpin, S.E., Gonzalez, G., Vacanti, J.P., Ott, H.C., 2013. Regeneration and experimental orthotopic transplantation of a bioengineered kidney. *Nat. Med.* 19, 646–651. <https://doi.org/10.1038/nm.3154>
- Stokes, J.B., 1990. Sodium and potassium transport by the collecting duct. *Kidney International* 38, 679–686. <https://doi.org/10.1038/ki.1990.259>
- Subramanian, A., Sidhom, E.-H., Emani, M., Vernon, K., Sahakian, N., Zhou, Y., Kost-Alimova, M., Slyper, M., Waldman, J., Dionne, D., Nguyen, L.T., Weins, A., Marshall, J.L., Rosenblatt-Rosen, O., Regev, A., Greka, A., 2019. Single cell census of human kidney organoids shows reproducibility and diminished off-target cells after transplantation. *Nat Commun* 10, 5462. <https://doi.org/10.1038/s41467-019-13382-0>
- Sulzbacher, S., Schroeder, I.S., Truong, T.T., Wobus, A.M., 2009. Activin A-induced differentiation of embryonic stem cells into endoderm and pancreatic progenitors-the influence of differentiation factors and culture conditions. *Stem Cell Rev Rep* 5, 159–173. <https://doi.org/10.1007/s12015-009-9061-5>
- Susantitaphong, P., Koulouridis, I., Balk, E.M., Madias, N.E., Jaber, B.L., 2012. Effect of frequent or extended hemodialysis on cardiovascular parameters: a meta-analysis. *Am. J. Kidney Dis.* 59, 689–699. <https://doi.org/10.1053/j.ajkd.2011.12.020>
- Susztak, K., Bitzer, M., Meyer, T.W., Hostetter, T.H., 2008. Animal models of renal disease. *Kidney International* 73, 526–528. <https://doi.org/10.1038/sj.ki.5002724>
- Sweetman, D., Wagstaff, L., Cooper, O., Weijer, C., Münsterberg, A., 2008. The migration of paraxial and lateral plate mesoderm cells emerging from the late primitive streak is controlled by different Wnt signals. *BMC Developmental Biology* 8, 63. <https://doi.org/10.1186/1471-213X-8-63>
- Taggart, J.V., 1958. Mechanisms of renal tubular transport. *The American Journal of Medicine* 24, 774–784. [https://doi.org/10.1016/0002-9343\(58\)90380-2](https://doi.org/10.1016/0002-9343(58)90380-2)
- Taguchi, A., Kaku, Y., Ohmori, T., Sharmin, S., Ogawa, M., Sasaki, H., Nishinakamura, R., 2014. Redefining the in vivo origin of metanephric nephron progenitors enables generation of complex kidney structures from pluripotent stem cells. *Cell Stem Cell* 14, 53–67. <https://doi.org/10.1016/j.stem.2013.11.010>
- Taguchi, A., Nishinakamura, R., 2017. Higher-Order Kidney Organogenesis from Pluripotent Stem Cells. *Cell Stem Cell* 21, 730–746.e6. <https://doi.org/10.1016/j.stem.2017.10.011>
- Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. <https://doi.org/10.1016/j.cell.2006.07.024>
- Takasato, M., Er, P.X., Becroft, M., Vanslambrouck, J.M., Stanley, E.G., Elefanty, A.G., Little, M.H., 2014. Directing human embryonic stem cell differentiation towards a renal

- lineage generates a self-organizing kidney. *Nat Cell Biol* 16, 118–126. <https://doi.org/10.1038/ncb2894>
- Takasato, M., Er, P.X., Chiu, H.S., Maier, B., Baillie, G.J., Ferguson, C., Parton, R.G., Wolvetang, E.J., Roost, M.S., Chuva de Sousa Lopes, S.M., Little, M.H., 2015. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature* 526, 564–568. <https://doi.org/10.1038/nature15695>
- Takasato, M., Little, M.H., 2015. The origin of the mammalian kidney: implications for recreating the kidney in vitro. *Development* 142, 1937–1947. <https://doi.org/10.1242/dev.104802>
- Takebe, T., Enomura, M., Yoshizawa, E., Kimura, M., Koike, H., Ueno, Y., Matsuzaki, T., Yamazaki, T., Toyohara, T., Osafune, K., Nakauchi, H., Yoshikawa, H.Y., Taniguchi, H., 2015. Vascularized and Complex Organ Buds from Diverse Tissues via Mesenchymal Cell-Driven Condensation. *Cell Stem Cell* 16, 556–565. <https://doi.org/10.1016/j.stem.2015.03.004>
- Takebe, T., Sekine, K., Enomura, M., Koike, H., Kimura, M., Ogaeri, T., Zhang, R.-R., Ueno, Y., Zheng, Y.-W., Koike, N., Aoyama, S., Adachi, Y., Taniguchi, H., 2013. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* 499, 481–484. <https://doi.org/10.1038/nature12271>
- Takebe, T., Wells, J.M., 2019. Organoids by design. *Science* 364, 956–959. <https://doi.org/10.1126/science.aaw7567>
- Takemoto, T., Uchikawa, M., Yoshida, M., Bell, D.M., Lovell-Badge, R., Papaioannou, V.E., Kondoh, H., 2011. Tbx6-dependent Sox2 regulation determines neural or mesodermal fate in axial stem cells. *Nature* 470, 394–398. <https://doi.org/10.1038/nature09729>
- Tam, P.P.L., Loebel, D.A.F., 2007. Gene function in mouse embryogenesis: get set for gastrulation. *Nat. Rev. Genet.* 8, 368–381. <https://doi.org/10.1038/nrg2084>
- Tanigawa, S., Naganuma, H., Kaku, Y., Era, T., Sakuma, T., Yamamoto, T., Taguchi, A., Nishinakamura, R., 2019. Activin Is Superior to BMP7 for Efficient Maintenance of Human iPSC-Derived Nephron Progenitors. *Stem Cell Reports* 13, 322–337. <https://doi.org/10.1016/j.stemcr.2019.07.003>
- Teo, A.K.K., Ali, Y., Wong, K.Y., Chipperfield, H., Sadasivam, A., Poobalan, Y., Tan, E.K., Wang, S.T., Abraham, S., Tsuneyoshi, N., Stanton, L.W., Dunn, N.R., 2012. Activin and BMP4 synergistically promote formation of definitive endoderm in human embryonic stem cells. *Stem Cells* 30, 631–642. <https://doi.org/10.1002/stem.1022>
- Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147. <https://doi.org/10.1126/science.282.5391.1145>
- Torres, M., Gómez-Pardo, E., Dressler, G.R., Gruss, P., 1995. Pax-2 controls multiple steps of urogenital development. *Development* 121, 4057–4065.
- Tramonti, G., Kanwar, Y.S., 2013. Review and Discussion of Tubular Biomarkers in the Diagnosis and Management of Diabetic Nephropathy. *Endocrine* 43, 494–503. <https://doi.org/10.1007/s12020-012-9820-y>
- Trapnell, C., Cacchiarelli, D., Grimsby, J., Pokharel, P., Li, S., Morse, M., Lennon, N.J., Livak, K.J., Mikkelsen, T.S., Rinn, J.L., 2014. The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat. Biotechnol.* 32, 381–386. <https://doi.org/10.1038/nbt.2859>
- Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L., Pachter, L., 2013. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat. Biotechnol.* 31, 46–53. <https://doi.org/10.1038/nbt.2450>
- Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., Pachter, L., 2012. Differential gene and transcript expression analysis

- of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 7, 562–578. <https://doi.org/10.1038/nprot.2012.016>
- Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., Pachter, L., 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* 28, 511–515. <https://doi.org/10.1038/nbt.1621>
- Trujillo, C.A., Gao, R., Negraes, P.D., Gu, J., Buchanan, J., Preissl, S., Wang, A., Wu, W., Haddad, G.G., Chaim, I.A., Domissy, A., Vandenberghe, M., Devor, A., Yeo, G.W., Voytek, B., Muotri, A.R., 2019. Complex Oscillatory Waves Emerging from Cortical Organoids Model Early Human Brain Network Development. *Cell Stem Cell* 25, 558–569.e7. <https://doi.org/10.1016/j.stem.2019.08.002>
- Tsang, T.E., Shawlot, W., Kinder, S.J., Kobayashi, A., Kwan, K.M., Schughart, K., Kania, A., Jessell, T.M., Behringer, R.R., Tam, P.P., 2000. *Lim1* activity is required for intermediate mesoderm differentiation in the mouse embryo. *Dev. Biol.* 223, 77–90. <https://doi.org/10.1006/dbio.2000.9733>
- Unbekandt, M., Davies, J.A., 2010. Dissociation of embryonic kidneys followed by reaggregation allows the formation of renal tissues. *Kidney Int.* 77, 407–416. <https://doi.org/10.1038/ki.2009.482>
- van den Berg, C.W., Ritsma, L., Avramut, M.C., Wiersma, L.E., van den Berg, B.M., Leuning, D.G., Liewers, E., Koning, M., Vanslambrouck, J.M., Koster, A.J., Howden, S.E., Takasato, M., Little, M.H., Rabelink, T.J., 2018. Renal Subcapsular Transplantation of PSC-Derived Kidney Organoids Induces Neo-vasculogenesis and Significant Glomerular and Tubular Maturation In Vivo. *Stem Cell Reports* 10, 751–765. <https://doi.org/10.1016/j.stemcr.2018.01.041>
- Vervaet, B.A., D’Haese, P.C., Verhulst, A., 2017. Environmental toxin-induced acute kidney injury. *Clin Kidney J* 10, 747–758. <https://doi.org/10.1093/ckj/sfx062>
- Wall, S.M., Hassell, K.A., Royaux, I.E., Green, E.D., Chang, J.Y., Shipley, G.L., Verlander, J.W., 2003. Localization of pendrin in mouse kidney. *American Journal of Physiology-Renal Physiology* 284, F229–F241. <https://doi.org/10.1152/ajprenal.00147.2002>
- Wang, P., Chen, Y., Yong, J., Cui, Y., Wang, R., Wen, L., Qiao, J., Tang, F., 2018. Dissecting the Global Dynamic Molecular Profiles of Human Fetal Kidney Development by Single-Cell RNA Sequencing. *Cell Reports* 24, 3554–3567.e3. <https://doi.org/10.1016/j.celrep.2018.08.056>
- Wardle, F.C., Papaioannou, V.E., 2008. Teasing out T-box targets in early mesoderm. *Curr. Opin. Genet. Dev.* 18, 418–425. <https://doi.org/10.1016/j.gde.2008.07.017>
- Weckmann, G.F.C., Stracke, S., Haase, A., Spallek, J., Ludwig, F., Angelow, A., Emmelkamp, J.M., Mahner, M., Chenot, J.-F., 2018. Diagnosis and management of non-dialysis chronic kidney disease in ambulatory care: a systematic review of clinical practice guidelines. *BMC Nephrol* 19, 258. <https://doi.org/10.1186/s12882-018-1048-5>
- Weiss, M.L., Chowdhury, S.I., Patel, K.P., Kenney, M.J., Huang, J., 2001. Neural circuitry of the kidney: NO-containing neurons. *Brain Res.* 919, 269–282. [https://doi.org/10.1016/s0006-8993\(01\)03030-x](https://doi.org/10.1016/s0006-8993(01)03030-x)
- Westerling-Bui, A., Soare, T.W., Venkatachalan, S., DeRan, M., Fast, E.M., Fanelli, A.B., Kyrchenko, S., Hoang, H., Corriea, G.M., Zhang, W., Yu, M., Daniels, M., Malojcic, G., Pan-Zhou, X.-R., Ledeboer, M.W., Harmange, J.-C., Emani, M., Tibbitts, T.T., Reilly, J.F., Mundel, P., 2019. A translational kidney organoid system bolsters human relevance of clinical development candidate (preprint). *Genomics*. <https://doi.org/10.1101/2019.12.30.891440>

- Wijgerde, M., Karp, S., McMahon, J., McMahon, A.P., 2005. Noggin antagonism of BMP4 signaling controls development of the axial skeleton in the mouse. *Dev. Biol.* 286, 149–157. <https://doi.org/10.1016/j.ydbio.2005.07.016>
- Wiles, M.V., Johansson, B.M., 1999. Embryonic stem cell development in a chemically defined medium. *Exp. Cell Res.* 247, 241–248. <https://doi.org/10.1006/excr.1998.4353>
- Wilson, P.C., Wu, H., Kirita, Y., Uchimura, K., Ledru, N., Rennke, H.G., Welling, P.A., Waikar, S.S., Humphreys, B.D., 2019. The single-cell transcriptomic landscape of early human diabetic nephropathy. *PNAS* 116, 19619–19625. <https://doi.org/10.1073/pnas.1908706116>
- Winnier, G., Blessing, M., Labosky, P.A., Hogan, B.L., 1995. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* 9, 2105–2116. <https://doi.org/10.1101/gad.9.17.2105>
- Woolf, A.S., Gnudi, L., Long, D.A., 2009. Roles of Angiopoietins in Kidney Development and Disease: Figure 1. *JASN* 20, 239–244. <https://doi.org/10.1681/ASN.2008020243>
- Workman, M.J., Mahe, M.M., Trisno, S., Poling, H.M., Watson, C.L., Sundaram, N., Chang, C.-F., Schiesser, J., Aubert, P., Stanley, E.G., Elefanty, A.G., Miyaoka, Y., Mandegar, M.A., Conklin, B.R., Neunlist, M., Brugmann, S.A., Helmuth, M.A., Wells, J.M., 2017. Engineered human pluripotent-stem-cell-derived intestinal tissues with a functional enteric nervous system. *Nat. Med.* 23, 49–59. <https://doi.org/10.1038/nm.4233>
- Wu, H., Malone, A.F., Donnelly, E.L., Kirita, Y., Uchimura, K., Ramakrishnan, S.M., Gaut, J.P., Humphreys, B.D., 2018a. Single-Cell Transcriptomics of a Human Kidney Allograft Biopsy Specimen Defines a Diverse Inflammatory Response. *JASN* 29, 2069–2080. <https://doi.org/10.1681/ASN.2018020125>
- Wu, H., Uchimura, K., Donnelly, E.L., Kirita, Y., Morris, S.A., Humphreys, B.D., 2018b. Comparative Analysis and Refinement of Human PSC-Derived Kidney Organoid Differentiation with Single-Cell Transcriptomics. *Cell Stem Cell* 23, 869–881.e8. <https://doi.org/10.1016/j.stem.2018.10.010>
- Wyss, J.M., Oparil, S., Sriparojthikoon, W., 1992. Neuronal control of the kidney: contribution to hypertension. *Can. J. Physiol. Pharmacol.* 70, 759–770. <https://doi.org/10.1139/y92-100>
- Xia, Y., Izpisua Belmonte, J.C., 2019. Design Approaches for Generating Organ Constructs. *Cell Stem Cell* 24, 877–894. <https://doi.org/10.1016/j.stem.2019.05.016>
- Xia, Y., Nivet, E., Sancho-Martinez, I., Gallegos, T., Suzuki, K., Okamura, D., Wu, M.-Z., Dubova, I., Esteban, C.R., Montserrat, N., Campistol, J.M., Izpisua Belmonte, J.C., 2013. Directed differentiation of human pluripotent cells to ureteric bud kidney progenitor-like cells. *Nat. Cell Biol.* 15, 1507–1515. <https://doi.org/10.1038/ncb2872>
- Xinaris, C., Benedetti, V., Novelli, R., Abbate, M., Rizzo, P., Conti, S., Tomasoni, S., Corna, D., Pozzobon, M., Cavallotti, D., Yokoo, T., Morigi, M., Benigni, A., Remuzzi, G., 2016a. Functional Human Podocytes Generated in Organoids from Amniotic Fluid Stem Cells. *J. Am. Soc. Nephrol.* 27, 1400–1411. <https://doi.org/10.1681/ASN.2015030316>
- Xinaris, C., Benedetti, V., Novelli, R., Abbate, M., Rizzo, P., Conti, S., Tomasoni, S., Corna, D., Pozzobon, M., Cavallotti, D., Yokoo, T., Morigi, M., Benigni, A., Remuzzi, G., 2016b. Functional Human Podocytes Generated in Organoids from Amniotic Fluid Stem Cells. *JASN* 27, 1400–1411. <https://doi.org/10.1681/ASN.2015030316>
- Xinaris, C., Benedetti, V., Rizzo, P., Abbate, M., Corna, D., Azzollini, N., Conti, S., Unbekandt, M., Davies, J.A., Morigi, M., Benigni, A., Remuzzi, G., 2012. In vivo maturation of functional renal organoids formed from embryonic cell suspensions. *J. Am. Soc. Nephrol.* 23, 1857–1868. <https://doi.org/10.1681/ASN.2012050505>

- Xu, P.-X., Zheng, W., Huang, L., Maire, P., Laclef, C., Silvius, D., 2003. Six1 is required for the early organogenesis of mammalian kidney. *Development* 130, 3085–3094. <https://doi.org/10.1242/dev.00536>
- Yang, H.-C., Zuo, Y., Fogo, A.B., 2010. Models of chronic kidney disease. *Drug Discov Today Dis Models* 7, 13–19. <https://doi.org/10.1016/j.ddmod.2010.08.002>
- Yang, X., Dormann, D., Münsterberg, A.E., Weijer, C.J., 2002. Cell Movement Patterns during Gastrulation in the Chick Are Controlled by Positive and Negative Chemotaxis Mediated by FGF4 and FGF8. *Developmental Cell* 3, 425–437. [https://doi.org/10.1016/S1534-5807\(02\)00256-3](https://doi.org/10.1016/S1534-5807(02)00256-3)
- Ying, Q.-L., Stavridis, M., Griffiths, D., Li, M., Smith, A., 2003. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat. Biotechnol.* 21, 183–186. <https://doi.org/10.1038/nbt780>

Appendix

Table 1. List of marker genes used for immunofluorescence characterization

Organoid structure/ cell type	Representative marker genes
Podocytes	NPHS1, PODXL, WT1
Proximal tubules	LTL, LRP2, CDH6
Loop of Henle	UMOD, JAG1
Distal tubules	CDH1, DBA, CALB1
Vascular endothelial cells	CD31, CD34, CD144
Stromal cells	MEIS1, FOXD1
Bowman's capsule-like cells	PDGFR β
Basement membrane	LMN1
Vascular progenitors	KDR
Nephron progenitors	SIX1, SIX2, SALL1, CITED1, PAX8, LHX1
Intermediate mesoderm	WT1, HOXD11
Primitive streak-like cells	T, MIXL1

Table 2. List of primers used in this study

Primer	Forward	Reverse
BMP2	ACTACCAGAAACGAGTGGGAA	GCATCTGTTCTCGGAAAACCT
BMP4	ATGATTCTGTAACCGAATGC	CCCCGTCTCAGGTATCAAACCT
BMP7	GGAACGCTTCGACAATGAGAC	GCAGGAAGAGATCCGATTCCC

CD31	AACAGTGTTGACATGAAGAGCC	TGTAAACAGCACGTCATCCTT
CD34	TGCTGAGTTTGCTGCCTTCTG	TGTTCCCTGGGTAGGTAAGTCTG G
EYA1	TTCAAGAACCGCCATCTGGCATC	GTGGATTGTGCTGTACTCTGCTG
FOXA2	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA
FOXF1	AATGCGCAAGTCTCCCTTTA	TTCGTGGCTCCTTTGACTTT
FOXH1	AAAGTAGCCATGGGTCATCG	GAGGGCTTCCACAATTAGCA
GAPDH	AGCAATGCCTCCTGCACCACCAA C	CCGGAGGGGCCATCCACAGTCT
GATA4	TCCCTCTTCCCTCCTCAAAT	TCAGCGTGTAAGGCATCTG
GATA6	CTCAGTTCCTACGCTTCGCAT	GTCGAGGTCAGTGAACAGCA
LHX1	CTTCTTCCGGTGTTTCGGTA	TCATGCAGGTGAAGCAGTTC
NPHS1	TCACCGTGAATGTCCTGTTCC	AGTGTGGCTAAGGGATTACC
NPHS2	AAGACAAGCCAAAGTGCGGATG	TGGAGGTATCGAAGCTGAACGG
OSR1	CCTTCCTTCAGGCAGTGAAC	CGGCACTTTGGAGAAAGAAG
PAX2	ACTCCATCAATGGGATCCTG	CCACACCACTCTGGGAATCT
PODXL	GAGCAGTCAAAGCCACCTTC	TGGTCCCCTAGCTTCATGTC
SOX17	CGCACGGAATTTGAACAGTA	GGATCAGGGACCTGTCACAC
TBX6	CATCGTGTCAAGCTCACCAACAG C	TTGTGCATGGAGTGCAGGATCAG G
VEGFA	AGGGCAGAATCATCACGAAGTG G	GTCTCGATTGGATGGCAGTAGC
WT1	GCGGAGCCCAATACAGAATA	GATGCCGACCGTACAAGAGT

Table 3. List of antibodies used in this study

Reagent or resource	Source	Identifier
AQP1 (Aquaporin-1)	Santa Cruz	Cat. #SC-25287; RRID:AB_626694
AQP2 (Aquaporin-2)	Santa Cruz	Cat. #SC-515770
CALB1 (Calbindin-1)	Sigma	Cat. #C9848; RRID:AB_476894
CDH1 (E-Cadherin)	BD Biosciences	Cat. #610182; RRID:AB_397581
CDH6 (K-Cadherin)	Gift from Professor Gregory Dressler, University of Michigan	N/A
CD31 (PECAM1)	Abcam	Cat. #ab76533; RRID:AB_1523298
CD31-PE (PECAM1)	BD Biosciences	Cat. #BD 555446; RRID:AB_395839
FOXD1	Abcam	Cat. #ab129324; RRID:AB_11150609
CK8 (Cytokeratin-8)	Developmental Studies Hybridoma Bank	Cat. #TROMA-I-S
HOXD11	Proteintech	Cat. #18734-1-AP
JAG1 (Jagged-1)	R&D Systems	Cat. #AF1277; RRID:AB_416699
KDR (VEGFR2/CD309)	Biolegend	Cat. #359916; RRID:AB_2565928
MEIS1	Active Motif	Cat. #A25/39795; RRID:AB_2750570
LHX1	Developmental Studies Hybridoma Bank	Cat. #4F2-C; RRID:AB_531784
LMN1 (Laminin)	Developmental Studies Hybridoma Bank	Cat. #2E8; RRID:AB_528343
LRP2 (Megalin)	R&D Systems	Cat. #NB110-96417
Lotus Tetragonolobus Lectin (LTL), Fluorescein labelled	Vector Laboratories	Cat. #FL-1321; RRID:AB_2336559
MIXL1	Proteintech	Cat. #22772-1-AP
NPHS1 (Nephrin)	Progen	Cat. #GP-N2; RRID:AB_1542487
OCT4	Santa Cruz	Cat. #sc-9081; RRID:AB_2167703

PAX2	Biolegend	Cat. #PRB-276P; RRID:AB_291611
PAX8	Proteintech	Cat. #10336-1-AP; RRID:AB_2236705
1PDGFR β	R&D Systems	Cat. #AF385; RRID:AB_355339
PODXL (Podocalyxin)	R&D Systems	Cat. #AF1658; RRID:AB_354920
SALL1	R&D Systems	Cat. #PP-K9814-00; RRID:AB_2183228
SIX1	Cell Signalling Technology	Cat. #D4A8K; RRID:AB_2753209
SIX2	Proteintech	Cat. #11562-1-AP; RRID:AB_2189084
SOX2	Millipore	Cat. #AB5603; RRID:AB_2286686
T (Brachyury)	Developmental Studies Hybridoma Bank	Cat. #PCRP-T-1A2; RRID:AB_2722364
WT1	Santa-Cruz Biotechnology	Cat. #SC-7385; RRID: AB_628448
WT1	Abcam	Cat. #ab89901; RRID:AB_2273027
Chicken anti-Rabbit IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A21443; RRID:AB_2535861
Donkey anti-Rabbit IgG, Alexa Fluor 488	Thermo Fisher Scientific	Cat. #A21206; RRID:AB_2535792
Goat anti-Rabbit IgG, Alexa Fluor 405	Thermo Fisher Scientific	Cat. #A31556; RRID:AB_221605
Goat anti-Rabbit IgG, Alexa Fluor 594	Thermo Fisher Scientific	Cat. #R37117; RRID:AB_2556545
Donkey anti-Mouse IgG, Alexa Fluor 488	Thermo Fisher Scientific	Cat. #A21202; RRID:AB_141607
Donkey anti-Mouse IgG, Alexa Fluor 594	Thermo Fisher Scientific	Cat. #A21203; RRID:AB_141633
Donkey anti-Mouse IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A31571; RRID:AB_162542
Goat anti-Mouse IgG, Alexa Fluor 633	Thermo Fisher Scientific	Cat. #A21053; RRID:AB_1500753
Goat anti-Mouse IgG, Alexa Fluor 405	Thermo Fisher Scientific	Cat. #A31553; RRID:AB_221604
Chicken anti-Goat IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A21469; RRID:AB_10374877

Chicken anti-Goat IgG, Alexa Fluor 488	Thermo Fisher Scientific	Cat. #A21467; RRID:AB_141893
Donkey anti-Goat IgG, Alexa Fluor 594	Thermo Fisher Scientific	Cat. #A11058; RRID:AB_142540
Donkey anti-Goat IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A21447; RRID:AB_141844
Goat anti-Guinea Pig IgG , Alexa Fluor 488	Thermo Fisher Scientific	Cat. #A11073; RRID:AB_142018
Goat anti-Guinea Pig IgG , Alexa Fluor 594	Thermo Fisher Scientific	Cat. #A11076; RRID:AB_141930
Chicken anti-Rat IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A21472; RRID:AB_1500700
Donkey anti-Rat IgG, Alexa Fluor 594	Thermo Fisher Scientific	Cat. #A21209; RRID:AB_2535795

Table 4. List of experimental models (cell lines and mouse strains) used in this study

Reagent or resource	Source	Identifier
Human embryonic stem cell H1	WiCell	Cat. #WA01; RRID:CVCL_9771
Human embryonic stem cell line H1 expressing Firefly Luciferase (H1-Luciferase)	From Professor Juan Carlos Izpisua Belmonte, Salk Institute	Cat. #WA01; RRID:CVCL_9771
Human embryonic stem cell H9	WiCell	Cat. #WA09; RRID:CVCL_9773
Human induced pluripotent stem cell line BJ-iPSC	From Professor Juan Carlos Izpisua Belmonte, Salk Institute	-
NOD.Cg- <i>Prkdc</i> ^{scid} <i>Il2rg</i> ^{tm1Wjl} /SzJ (NSG)	InVivos, Singapore	RRID:IMSR_JAX:005557
BALB/cJInv (CD-1)	InVivos, Singapore	RRID:IMSR_JAX:000651

Table 5. List of chemicals, peptides and recombinant proteins used in this study

Reagent or resource	Source	Identifier
Activin A	Stemgent	Cat. #03-0001
Axitinib	MedChemExpress (MCE)	Cat. #HY-10065
Cediranib	MedChemExpress (MCE)	Cat. #HY-10205
CHIR99021	Sigma-Aldrich	Cat. #SML1046
DAPI (6-diamidino-2-phenylindole dihydrochloride)	Sigma-Aldrich	Cat. #D9542
Dextran, Tetramethylrhodamine; 70,000 MW, Lysine Fixable	Thermo Fisher Scientific	Cat. #D1818
Dextran, Tetramethylrhodamine; 2,000,000 MW, Lysine Fixable	Thermo Fisher Scientific	Cat. #D7139
Growth Factor Reduced Matrigel	Corning	Cat. #354230
Luciferin	Perkin Elmer	Cat. #122799
Recombinant human Activin A	Stemgent, Inc.	Cat. #03-0001
Recombinant human fibroblast growth factor 2 (FGF2)	Joint Protein Central	Cat. #L16F02001
Recombinant human fibroblast growth factor 9 (FGF9)	PeptoTech	Cat. #100-23
Recombinant human Noggin	Joint Protein Central	Cat. #AA28-232
Recombinant human Transforming Growth Factor- β (TGF- β)	Sigma-Aldrich	Cat. #H8541
Tivozanib	MedChemExpress	Cat. #HY-10977
Y-27632 dihydrochloride	Sigma-Aldrich	Cat. #72308

Table 6. List of commercial assays used in this study

Reagent or resource	Source	Identifier
Chromium Single Cell A Chip Kit	10x Genomics	Cat. #PN-1000009
Chromium i7 Multiplex Kit	10x Genomics	Cat. #PN-120262
Direct-zol™ RNA Miniprep	Zymo Research	Cat. #R2050
DuoSet ELISA Ancillary Reagent Kit 2	R&D Systems	Cat. #DY008
Human VEGF DuoSet ELISA	R&D Systems	Cat. #DY293B
iScript™ cDNA Synthesis Kit	Bio-Rad	Cat. #1708841
iQ™ SYBR® Green Supermix	Bio-Rad	Cat. #1725125
TRIzol™ Reagent	Invitrogen	Cat. #15596018
Chromium Single Cell 3' Library & Gel Bead Kit v2	10x Genomics	Cat. #PN-120237