Generate vascularized kidney organoids for studying kidney development

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**GENERATE VASCULARIZED KIDNEY ORGANOIDS FOR
STUDYING KIDNEY DEVELOPMENT**

ZHANG TIAN

SCHOOL OF BIOLOGICAL SCIENCES

2020

GENERATE VASCULARIZED KIDNEY ORGANOIDS FOR STUDYING KIDNEY DEVELOPMENT

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SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Master of Science

2020

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25/07/2019 Zhang Tian

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Generate Vascularized Kidney Organoids for Studying Kidney Development

ABSTRACT

Human pluripotent stem cells (hPSCs)-derived organoids recapitulate organ architecture and development processes. Here we employ a hPSCs-derived three-dimensional (3D) kidney organoid platform to study human kidney development in vitro, which cannot be realized otherwise due to ethical considerations. Using this platform, we demonstrate that a subpopulation of nephron progenitor cells (NPCs) display similar property of vascular progenitors, which eventually differentiate to renal endothelial cells in both in vitro differentiation and in vivo development. Based on this platform, we further develop an approach to modulate the differentiation of nephron compartments via manipulating metabolic pathway. Overall, hPSCs-derived kidney organoids offer new avenues for studying renal lineage specification, as well as for disease modeling in an organ-specific manner.

INTRODUCTION

Kidney

The kidneys are a pair of bean-shaped organs, located on both sides of the spine, at the back of the abdominal cavity. The primary function of kidney is waste management via filtration and urine excretion to maintain blood homeostasis including acid-base balance, hormone secretion, osmolarity, and blood pressure regulation. Compromised kidney function disrupts the homeostasis of body fluid, which eventually causes systemic damage to multiple organs.

Kidneys perform their critical roles via millions of function unit, nephron, which is composed of renal corpuscle as the filtration apparatus, and tubulars to process and modify the primary filtrate, before disposal into urethra (Boron, 2004). As shown in Figure 1, renal corpuscle is comprised of glomerulus that wraps around glomerular capillary tufts. Glomerulus contains two layers of specialized epithelial cells, namely visceral epithelial cells/podocytes (inner layer) and parietal epithelial cells (outer layer). The filtration barrier consists one layer of inter-digited podocytes, one layer of glomerular basement membrane, and one layer of fenestrated endothelium. The primary filtrate is generated within Bowman's capsule, before entering renal tubules, including proximal convoluted tubule, loop of Helen, distal convoluted tubule, and connecting tubule. The renal tubules are surrounded by tiny blood vessels (peri-tubular vessels) that reabsorb essential substances back to the bloodstream, such as glucose, amino acids, and potassium (Clapp, 2009).

Consistent with its function in maintaining physiological homeostasis, kidney is a highly vascularized organ. Kidney vascular network is established in a manner that it can properly associate with the relevant renal compartments, and precisely accomplish regionally specialized functions. The glomerular capillaries are constituted with fenestrated endothelial cells, which enable the filtration of fluid, solutes, and small proteins (<68 kDa), but not blood cells, platelets,

and large proteins (>68 kDa) (Marshall & Bangert, 2008). The endothelial cells in close proximity to renal tubules are mainly responsible for reabsorption of protein, glucose, salts, and others (Clapp, 2009).

Figure 1. Schematic of adult human kidneys

Left panel represents whole human kidney. The middle one represents nephron structures with surrounding vasculature. Right panel focused on the detail structures of glomerular capillary.

Mammalian Kidney Development

Mammalian kidney development proceeds through three successive phases, pronephros, mesonephros, and metanephros, reminiscent of the evolution of this organ. In mammals, the definitive adult kidney is entirely derived from metanephros, which are the last of three excretory organs generated from intermediate mesoderm (IM) during gestation (Little & McMahon, 2012). *Osr1*⁺ IM cells give rise to multiple cellular components of metanephric kidney including nephric duct, nephron, interstitial cells, and endothelial cells (Mugford, Sipila, McMahon, & McMahon, 2008). Kidney is developed via the reciprocal interaction between ureteric bud (UB) and metanephric mesenchyme (MM), both of which are originated from distinctive regions of IM. Genetic mapping of mouse kidney development showed that a portion of *Osr1*⁺ IM cells

differentiate into nephric duct (*Pax2+Lhx1+Gata3+*), from which UB grows out. The initial UB branching was induced by the signals derived from the nearby *Hoxd11*⁺ MM that wraps around UB tips. As development proceeds, UB continues to grow and branch, establishing the collecting system (Lauri, 1987). Meanwhile, the induced *Six2*⁺ cap mesenchyme (CM) undergoes a mesenchyme-to-epithelial transition (MET) to form renal vesicles (RV). The main epithelial segments of nephron are developed from the RV, from glomerulus, proximal tubule, loop of Henle, to distal-connecting tubules (Boyle et al., 2008; Kobayashi et al., 2008). Within MM, *Foxd1*⁺ cells generate interstitial cells including pericytes, and mesangial cells within the glomerulus (Hatini, Huh, Herzlinger, Soares, & Lai, 1996; Humphreys et al., 2010). Another MM subpopulation that expresses endothelial marker *Flk1* may also play roles in supporting UB growth (Abrahamson DR, 2003). These *Flk1*⁺ MM cells may also participate in renal vasculogenesis, which will be discussed later (Gao X, 2005; Mugford et al., 2008).

Cap mesenchyme (CM) located the closest to ureteric tips is considered as the precursor of most nephron epithelial cells. The densely packed and histologically distinct CM is a multipotent and self-renewal progenitor population (Little & McMahon, 2012). *Six2* is a critical regulator of nephron progenitors, because removal of *Six2* grievously disrupts nephrogenesis (Self et al., 2006). Within the *Six2*⁺ CM compartment, *Wnt4*⁺ nephron progenitor cells (NPCs) are more committed to nephrogenesis (Kobayashi et al., 2008), while *Cited1*⁺ cells represent more naive NPCs (Mugford et al., 2008). CM undergoes MET and further morphogenesis following the primary induction signal Wnt9 secreted by the nearby ureteric tips. Two Wnt9b downstream target genes *Wnt4* and *Fgf8* further regulate the formation of RVs (Carroll TJ, 2005). The expression of marker genes that indicate proximal-distal pattern formation is readily observed upon the formation of RVs, which will sequentially develop into common shape body, S-shape body, and ultimately mature nephron.

Kidney Vascular Network

Kidney vascular network presents a unique and complex 3D architecture to realize its critical function. The development of kidney vascular network initiates at the early stages of kidney morphogenesis. Vascular development and nephrogenesis are two processes that closely correlated with each other (Gomez, Norwood, & Tufro-McReddie, 1997). Angiogenesis refers to the outgrowth of capillaries from existing blood vessels (Risau et al., 1988); while vasculogenesis is the *de novo* formation of endothelial network from vascular endothelial precursors (Risau & Ekblom, 1986; Risau et al., 1988). Both angiogenesis and vasculogenesis are believed to contribute to the formation of kidney vascular network (Sariola, 1991). The development of vascular network is regulated by various factors including genetic regulators, vascular growth factors, extracellular matrix, and mechanical forces.

However, the embryonic origin of renal vasculature remains unresolved, though classic xeno-transplantation experiments pointed out that both extra- and intra-renal sources contribute to the formation of renal vasculature. More specifically, invasion of host blood vessels into avascular metanephric kidneys upon xeno-transplantation into neonatal mice suggested the possibility of extra-renal vascular origin during the kidney development (Sariola, 1991). On the other hand, upon transplantation into adult mouse kidney or anterior eye chamber, avascular metanephric kidney established vasculature that were entirely originated from the implants, indicating an intra-renal origin (Hu et al., 2016). As mentioned, within in MM, there is a subset of cells express endothelial markers, which may later contribute to the renal vascularization (Little & McMahon, 2012).

Human Pluripotent Stem Cells (hPSCs)

Our understanding of embryonic development is largely based on studies using different model organisms, such as *Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio*, and *Mus*

musculus. The divergence between human and model organisms may lead to misinterpretation of human development. However, it is impractical and unethical to use human embryos for studying peri- and post-implantation development. In 1998, the successful isolation and *in vitro* culture of human embryonic stem cells (ESCs) from blastocysts open a new avenue for understanding human development (Thomson et al., 1998). Moreover, the generation of induced pluripotent stem cells (iPSCs) from somatic cells with four Yamanaka factors (*c-Myc*, *SOX2*, *KLF4*, *OCT4*) has revolutionized the field of regenerative medicine (Takahashi et al., 2007). These human pluripotent stem cells (PSCs) have the capability of unlimited self-renew and differentiation, providing unprecedented opportunities to understand human development and disease pathogenesis *in vitro*.

Embryonic stem cells (ESCs) are pluripotent cells derived from inner cell mass of blastocyst, which can divide indefinitely and maintain the capacity to differentiate into all derivatives of the three primary germ layers. Induced pluripotent stem cells (iPSCs) can be converted from somatic cells through nuclear reprogramming to acquire the properties of ESCs (Yamanaka, 2010). iPSC is highly like ESCs in terms of transcription profile (Takahashi and Yamanaka, 2006) and epigenetic profile (Bock et al., 2011).

The production of iPSCs enable disease modeling and drug screening to be carried out in a patient-specific manner. As pluripotent cells are obtained from individual patient, it is possible to uncover pathogenesis of inherited diseases. Also tissue or organ structures derived from iPSCs can be used as unlimited replacement for rare biopsy.

The immaturity of iPSC-derived tissue and organ structures limits their clinical application. Current differentiation protocols were only able to generate fetal stage tissue and organ

structures from iPSCs (Rossi et al., 2019). Immature organoids might not be able to fully recapitulate organ structures and disease phenotypes.

hPSCs-derived 3D Organoids

hPSCs represent an unlimited resource for generating all cell types inside our body by direct differentiation *in vitro*. Traditional *in vitro* differentiation approaches employ a two-dimensional (2D) culture format, which could not faithfully recapitulate tissue architecture and cell-cell/cell-matrix contact (D'Amour, 2006; Osakada et al., 2009). Hence, 3D embryonic bodies have been adapted for directed differentiation to replace traditional 2D monolayer culture (Lian et al., 2012; Zhang, Wernig, Duncan, Brüstle, & A.Thomson, 2001). However, differentiation factors cannot be evenly distributed throughout 3D embryonic bodies, leading to non-consistent differentiation outcomes (Wieses, Kania, Rolletschek, Blyszczuk, & Wobus, 2006). In order to establish *in vitro* platforms that could faithfully recapitulate organogenesis in terms of spatiotemporal patterning and structural sophistication, various types of 3D organoids have been generated for modeling organ/tissue biology (Clevers, 2016).

Over the last decade, organoid platforms have been established for a multitude of organ and tissue structures. 3D organoid cultures have the capacity to resemble most types of organs, including brain (Lancaster et al., 2013), liver (Takebe et al., 2013), lung (Wong et al., 2012), intestine (Spence et al., 2011), blood vessels (Wimmer et al., 2019), and more. Besides organoids recapitulating organ structures, tumor organoid (Clevers and Tuveson, 2019) and embryonic organoid models (Rivron et al., 2018; Harrison et al., 2017; Shao et al., 2017; Baillie-Johnson et al., 2015) have been developed for developmental studies and disease modeling. Organoids can also be classified according to the starting cell types: human pluripotent stem cells (hPSCs), adult stem cells (ASCs), and cancer cells (Rossi et al., 2018).

Organoids provide reliable and accessible model to study development and organogenesis. Compared to animal models, organoids have greater experimental accessibility. Divergences between human and other animals also limit developmental studies using animal models. To overcome this challenge, validations using human embryonic and fetal tissues are preferred, but restricted by extremely limited tissue sources and related ethical concerns. Moreover, organoids together with reprogramming and gene editing techniques can be used for disease modeling and precision medicine. Compared to disease modeling with cell lines, organoids enable the understanding of disease mechanism at organ level.

3D Kidney Organoids

hPSCs have been successfully steered into 3D kidney organoids that are reminiscent of human fetal kidney regarding both cell identity and structural organization, providing a novel avenue for studying development and diseases. Differentiation protocols for generating ureteric bud (UB) progenitor-like cells (Xia et al., 2013) and metanephric mesenchyme (Taguchi et al., 2014) from hPSCs were established independently. Although the UB progenitor differentiation protocol is chemically defined, the realization of UB progenitor functionality is dependent upon the formation of 3D chimeric organoids with mouse embryonic day 12.5 (E12.5) kidney cells. Nishinakamura's group established the protocol for differentiating mouse and human PSCs into MM, which could further differentiate into metanephric kidney structures upon induction by spinal cord or Wnt4-expressing cells (Taguchi et al., 2014). However, the differentiation protocol is not fully chemically defined and there is no presence of *de novo* vasculature.

In 2015, two chemically defined differentiation protocols were reported independently by Melissa's and Boventre's groups (Morizane et al., 2015; Takasato et al., 2015). Both studies demonstrated high differentiation efficiency of approximately 70-80% of NPCs. These hPSC-derived 3D kidney organoids contained renal epithelial structures, interstitial cells, and

endothelial cells. The continuous and organized arrangement of the nephron-like structures resembled the segmental pattern of developing nephrons *in vivo*. The study by Melissa and colleagues also indicated the presence of UB-like structures within their kidney organoids. Transcriptomic profiling showed that hPSCs-derived kidney organoids have the highest congruence with the first trimester human kidney.

Two recent studies demonstrate the contribution of physical parameters of microenvironment to *in vitro* kidney organoids generation. One group reported that soft hydrogel could expedite the derivation of kidney organoids (Garreta et al., 2019). The other study has shown that fluid flow could enhance vascularization and maturation of kidney organoids *in vitro* (Homan et al., 2019). According to the published study, kidney organoids cultured under flow developed perfusable vascular. Also, tubular structures undergo maturation and became polarized, ciliated compartment. Moreover, developed foot processes and penetrated vascular were found within glomerular. Overall, flow culture enhanced the maturation of kidney organoids (Homan et al., 2019).

3D kidney organoids that are reminiscent of human kidney structures represent an ideal *in vitro* platform for studying development and modeling disease. Numerous studies have used kidney organoids for investigating polycystic kidney disease (Cruz et al., 2017; Freedman et al., 2015). Alongside gene-editing techniques, organoids enable us to study the contribution of specific genetic lesions to their respective disease phenotypes. Moreover, lineage tracing within kidney organoids has been established to study renal development (Howden, Vanslambrouck, Wilson, Tan, & Little, 2019). However, this lineage tracing study only provides a proof-of-concept, without being able to attend some controversial questions, such as the origin of renal vascular network.

Glutamine metabolism in kidney

The kidneys play critical roles in the metabolism of proteins, glucose, lipids, carbohydrates, and others. Among these, kidney participates in glucose homeostasis via metabolizing glucose for energy production, gluconeogenesis, and glucose reabsorption. Kidney gluconeogenesis contribute to approximately 20-25% of the whole-body glucose production (DeFronzo, Norton, & Abdul-Ghani, 2017). Proximal tubule with appropriate enzymes is responsible for kidney gluconeogenesis (Mather and Pollock, 2011).

Glutamine metabolism is a pathway closely related to glucose metabolism. Glutamine is a glucose precursor in kidney as it can contribute to the carbon pool for glucose. Glutamine-related metabolic pathways are summarized in Figure 2: glutamine is converted into glutamate, which could either enter gluconeogenic pathway via α -ketoglutarate or form glutathione (GSH) that is an antioxidant (Stumvoll, Perriello, Meyer, & Gerich, 1999). It was shown that glutamine metabolism may be implicated in a variety of metabolic diseases such as diabetes and cancer. In type II diabetic patients, the increased conversion from glutamine to glucose was reported (Stumvoll et al., 1999).

Stumvoll et al: Glutamine in human carbohydrate metabolism

Figure 2. Glutamine Metabolism in the Kidney

Main pathways and key enzymes are listed: (1) glutamate dehydrogenase (2) glutamate oxaloacetate transaminase (3) glutamine synthetase (4) glutaminase (5) glutamate semialdehyde dehydrogenase.

Altered metabolic pathways could affect kidney development, as well as its response to disease pathology (Rhee et al., 2018). Many metabolites serve as cofactors or substrates for chromatin-modifying enzymes, which are critical for chromatin remodeling and histone modification, such as α -ketoglutarate. Hence nutrients including glucose and glutamine are intricately involved in regulating cell identity via spatiotemporal epigenetic modulation of lineage-specific modifiers (Wang, Long, Chang, Zhao, & Lu, 2018). Kidney organoid model offers an amenable platform for investigating the possible roles of metabolites in regulating kidney development and diseases.

The capacity to resemble organ architecture and organogenesis enable hPSCs-derived kidney organoids to be used as *in vitro* model for human development study. Using kidney organoids, we aimed to investigate the lineages interactions during organ development, and metabolism pathways involved. Here we have identified a non-conventional nephron progenitor cells (NPCs)-derived endothelial population with *in vitro* organoid system. We further verified the presence of this unique population in the second trimester human fetal kidneys. Employing our kidney organoid platform, we demonstrate the possibility to modulate the specification of nephron components via interfering with metabolic pathways. And this novel correlation between glutamine metabolism with kidney development warrants further investigation, as it may be relevant not only to human kidney developmental, but also to kidney disease pathogenesis.

RESULTS

Differentiation of hPSCs into Vascularized 3D Kidney Organoids

In this protocol, hPSCs were directed to differentiate in monolayer culture to control cell fates more precisely than in embryonic bodies. Within the monolayer culture system, inductive factors could be accessed by hPSCs in a more uniform fashion to achieve a high efficiency. At the first stage of differentiation, posterior primitive streak cells were induced by 4 days of CHIR99021 treatment. During the second stage, these posterior primitive streak cells were driven towards nephrogenic intermediate mesoderm with 3 days culture without any growth factor (Figure 3A). Then NPCs were efficiently generated from intermediate mesoderm by co-treatment with FGF9 and CHIR99021 (Figure 3B). These NPCs further differentiate and self-assemble to establish pre-tubular aggregate (PTA)-like structures (Figure 3C). In the presence of FGF9, some of the PTA-like cell clusters downregulated NPCs marker and acquired LHX1 and JAG1 expression, indicating the development into renal vesicle (RV)-like structures (Figure 3D). These RV-like clusters underwent further morphogenesis that is reminiscent of nephron formation *in vivo* (Figure 3E). We observed the mutually exclusive expression of NPCs marker SIX2 and epithelial marker JAG1, which suggested the downregulation of progenitor markers during nephrogenesis. This was important as retention of progenitor markers in maturing nephron structures could lead to the formation of Wilm's tumors.

Figure 3. Differentiate hPSCs into 3D kidney organoids

- A. Schematic of the differentiation process.
- B. FACS analysis of NPCs population (labelled by SIX1 and SIX2) from day 10 to day 14.
- C. Representative bright field images of the differentiation culture from day 0 to day 20.
Scale bars, 100µm.
- D. Immunofluorescence analysis for markers of NPC (SIX2) and RV (LHX1 and JAG1).
Scale bars, 10 µm (upper panel); 20µm (lower panel).
- E. Immunofluorescence analysis for markers of S-shape body (JAG1). Scale bars, 20 µm.

By day 24, multiple nephron structures were present in kidney organoids, including both tubules (LTL⁺CDH1⁺) and podocytes (PODXL⁺SYNPO⁺WT1⁺). (Figure 4A, B). Furthermore, endothelial cells (CD31⁺CD34⁺) surround and disperse throughout the whole organoid to form a highly elaborate vascular network (Figure 4C). Transmission electron microscopy (TEM) analysis of day 24 kidney organoids revealed the presence of podocyte-like cells with developing cellular processes, as well as tubular epithelium displaying a brush border (Figure 4D).

Figure 4. Vascularized kidney organoids recapitulate renal structures

- A. Representative bright-field image of 3D kidney organoids (left panel: day 16 kidney organoid in liquid culture; right panel: day 24 kidney organoid in liquid-air interface culture). Scale bars, 200 μ m.
- B. Immunofluorescence analysis for markers of tubular structure (LTL, CDH1) and podocyte (PODXL, SYNPO, WT1) in day 24 kidney organoids. Scale bars, 20 μ m.
- C. Whole-mount immunofluorescence analysis of 3D kidney organoid (Day 24) for vascular (CD31 and CD34) and podocyte (NPHS1) compartments. Scale bars, 200 μ m.
- D. Representative TEMs of tubular-like structure (left panel) and podocyte-like structure (right panel) within day 24 organoids.

Transcriptomic Profiling of the Differentiation Process

Next, we performed bulk RNA sequencing of kidney organoids at day 0, day 4, day 7, day 10, day 24. We first observed the peak of IM and NPC markers on day 7 and 10 of differentiation, respectively. As differentiation proceeds, we observed a temporal loss of NPC gene expression but an increase in multiple nephron segment markers, including the podocytes, proximal and distal tubules (Figure 5, Table 1). We also observed upregulation of endothelial markers (*KDR*, *TEK*, *FLT1*, *CDH5*, *CD34*) during differentiation, and *KDR* expression was robustly detected in the NPC stage, which might imply the temporal correlation between renal vasculogenesis and nephrogenesis (Figure 5, Table 1)

Figure 5. Transcriptomic profiling of the differentiation process

Log2 fold changes of representative genes of different renal lineages (normalized to day 0)

Table 1. Representative markers used in transcriptomic profiling

	Cell Types	Gene
1	NPC	OSR1
2	NPC	SIX1
3	NPC	SIX2
4	NPC	CITED1
5	NPC	EYA1
6	NPC	SALL1
7	NPC	GDNF
8	NPC	ETV4
9	NPC	COL2A1
10	NPC	HES1
11	NPC	CRABP2
12	NPC	LEF1
13	PTA	LHX1
14	PTA	WNT4
15	PTA	CCND1
16	Renal Vesical	JAG1
17	Renal Vesical	SFRP2
18	Renal Vesical	DLL1
19	S-shaped Body Medial/Distal	POU3F3
20	S-shaped Body Medial/Distal	SIM2
21	S-shaped Body Medial/Distal	SOX9
22	S-shaped Body Medial/Distal	IRX2
23	S-shaped Body Medial/Distal	IRX3
24	S-shaped Body Proximal	CDH6
25	S-shaped Body Proximal	HNF1A
26	S-shaped Body Proximal	AMN
27	S-shaped Body Podocyte	FOXC2
28	S-shaped Body Podocyte	MAFB
29	S-shaped Body Podocyte	OLFM3
30	S-shaped Body Podocyte	WT1
31	Connecting Tubule	CDH1
32	Distal Tubule/Loop of Helen	PAPPA2
33	Distal Tubule/Loop of Helen	CLCN5
34	Distal Tubule/Loop of Helen	SLC12A1
35	Podocyte	PTPRO
36	Podocyte	NPHS2
37	Podocyte	NPHS1
38	Podocyte	PODXL
39	Podocyte	TGFBR3
40	Podocyte	CLIC5
41	Podocyte	CITED2
42	Interstitial Cells	DES
43	Interstitial Cells	COL3A1
44	Interstitial Cells	COL1A1
45	Interstitial Cells	SERPINE2
46	Interstitial Cells	FGF7
47	Mesengial Cells	ACTA2
48	Mesengial Cells	TPM2
49	Mesengial Cells	PDGFRB
50	Mesengial Cells	MCAM
51	Mesengial Cells	CSPG4
52	Mesengial Cells	CD248
53	Endothelial Cells	KDR
54	Endothelial Cells	TEK
55	Endothelial Cells	FLT1
56	Endothelial Cells	CDH5
57	Endothelial Cells	CD34

***In Vitro* Functional Validation of hpSC-derived Kidney Organoids**

To validate the physiologically relevant features of hPSC-derived kidney organoids, *in vitro* dextran uptake assay was employed. After 24 hours incubation with dextran of various molecule weights, 70 kDa dextran, but not 2000 kDa dextran, was identified within proximal tubule epithelial cells (LTL⁺) (Figure 6).

Figure 6. *In vitro* functional assay of kidney organoids

Immunofluorescence analysis of kidney organoids following dextran uptake assay. Organoids were incubated with fluorescence-labelled dextran of various molecular weights. Detection of dextran within proximal tubule (LTL) was shown. Scale bars: 20 μm .

Use FACS Analysis to Examine the Endothelial Population during Differentiation

We further performed fluorescence-activated cell sorting (FACS) analysis to investigate the lineage relationship between NPC and endothelial cell. On day 11, we detected a subpopulation of NPCs co-expressing endothelial progenitor marker KDR, but not mature endothelial marker CD31 (Figure 7A). From day 12 to day 14, the percentage of NPC⁺KDR⁺CD31⁻ subpopulation decreased, while more NPC⁺KDR⁺ cells acquired CD31. The results suggest the presence of a NPC subpopulation that possesses endothelial progenitor signature (NPC⁺KDR⁺CD31⁻), which can further differentiate into mature endothelial population (Figure 7A, 6B).

Shown population to NPC

B

Figure 7. Time course FACS analysis of NPC and endothelial markers

- A. FACS analysis for the dynamic expression of NPC (SIX1, SIX2, CITED1, and SALL1) and endothelial markers (CD31 and KDR) during day 11-14 of differentiation.
- B. Line chart and doughnut shown the percentages of NPC+KDR+CD31⁻ and NPC+KDR+CD31⁺ subpopulations from day 11 to day 14.

Validate the Non-conventional Renal Vascular Origin Using Human Samples

The in vitro kidney organoid differentiation platform enabled the identification of a non-conventional renal vascular origin. Thus we continued to validate the vascular origin using the second trimester human fetus kidney. Human fetus kidney dissociated into single cells were stained with individual NPC markers (SIX1, SIX2, CITED1, or SALL1) and endothelial marker CD31. In total, five human kidneys from week 16 to week 21 of gestation were examined, and this NPC⁺CD31⁺ population was successfully identified within four kidneys. All cells went through twice of single cell gating to avoid false double positive population due to doublets. Within all samples, NPCs populations individually labeled with SIX1, SIX2, CITED1, or SALL1 were identified (Figure 8). Moreover, NPC⁺CD31⁺ population have been identified within human fetus kidneys (Figure 8). We observed relatively lower expression of NPC markers in the NPC⁺CD31⁺ population, possibly owing to the undergoing maturation of endothelial lineage (Figure 8).

Week 16 Sample 1

Week 17 Sample 3

Figure 8. FACS analysis of the non-conventional endothelial origin in human samples

Human fetal kidneys were co-stained with one NPC marker (SIX1, SIX2, CITED1, or SALL1) and endothelial markers (CD31). Subpopulations co-expressed both NPC markers and endothelial markers were found.

To further validate the existence of this population at single cell resolution, we explored the feasibility of mining a published single cell RNA sequencing analysis database. The published single cell transcriptomic (scRNA-seq) dataset (GEO: GSM3143601) of week 16 human fetal kidney cortex was employed for re-analysis. As shown in Figure 9A, NPC clusters and endothelial cluster were identified by the expression of lineage-specific markers. Among all 6,602 cells that were available for re-analysis, the endothelial cluster included 223 cells. Reads of endothelial markers KDR and CD34 were detected in all endothelial cells in this database. In order to find cells with both NPC and endothelial signatures, we searched for cells that are positive for NPC marker reads within the endothelial cluster. As shown in Figure 9B, cells expressing both endothelial markers and NPCs markers were present in the published human fetus kidney scRNA-seq dataset.

Figure 9. Single cell transcriptomic analysis of human fetus kidneys (re-analysis of published dataset)

B. Table showing the number and percentages of NPC⁺ endothelial cells.

Modulate the Proportion of Different Renal Components via Regulating Metabolism

In the absence of glutamine, neither podocyte nor vascular network was present in day 21 kidney organoids (Figure 10A). This result strongly suggest that podocyte differentiation and the establishment of vascular network were interrupted due to the altered glutamine metabolism pathway. To investigate the effects of glutamine on established podocyte structures, we performed glutamine deprivation at a later differentiation time point (from day 9 onward). On day 21, we observed less podocyte structures in non-glutamine treated organoids, while no podocyte could be identified in day 24 organoids (Figure 10B). These results implied that the absence of glutamine during differentiation can also cause damage to established podocyte structures.

Figure 10. Effects of metabolites in modulating the proportion of different renal components.

- A. Whole-mount immunofluorescence analysis of day 21 kidney organoid for proximal tubule (LTL), podocyte (PODXL), and vascular (CD34) compartments (upper panel: without glutamine from day 0; lower panel: with glutamine). Scale bars, 100 μ m.

- B. Whole-mount immunofluorescence analysis of kidney organoid cultured without glutamine after NPCs stage for proximal tubule (LTL) and podocyte (NPHS1). Scale bars, 100 μm .

DISCUSSION

Kidney organoids provide a unique opportunity to study human kidney development *in vitro*. Here, we have identified a non-conventional renal endothelial origin, and confirmed the existence of this NPC-derived endothelial subpopulation in human fetus kidneys by employing single cell analysis. The synergism of organoid biology and single cell technologies provide a more detailed understanding of the development of kidney vasculature. As both angiogenesis and vasculogenesis were involved in the formation of renal vascular network (Sariola, 1991), vasculogenesis was mainly believed to be differentiated from preexisting blood vessels (Saxen, 1987). These resident vessels may invade or migrate into nephron structures during the early stages of organogenesis (Kazimierczak, 1971; Potter, 1965; Sariola, 1991). Our studies have suggested that NPCs could also be one type of endothelial precursors, which at least partially contribute to the renal vasculature. The NPC-derived endothelial population may have kidney-specific signature linked with organ-specific functions, which warrants further investigation.

Our studies have shown that with highly efficient differentiation protocol, kidney organoid differentiation platform could be used to uncover novel aspects of human kidney development. Furthermore, this kidney organoid platform enables us to study the effects of metabolic pathways on kidney development and physiology. We have found out that glutamine-related metabolic pathways may be linked to nephrogenesis in a segment-specific manner. The damage on the established podocyte structures could be caused by the lack of antioxidant agent glutathione that plays crucial roles in compensating the reactive oxidative stress caused by glycolysis. We will be able to dissect the complex interaction between metabolism and developmental pathways using organoids.

FUTURE WORK

Origin of Renal Vascular Network

It has been agreed that renal vascular network may have multiple origins. To fully recapitulate the formation of renal vascular network, lineage tracing system based on Cre-recombination could be employed. In addition, as this differentiation platform enables us to generate renal-specific endothelial cells from hPSCs, studies of vascular development and diseases in an organ-specific manner could be conducted in the future.

Renal Metabolism

As glutamine pathways are closely related glucose metabolism, glutamine metabolism may be involved in diabetic nephropathy. Therefore, studies could be performed to dissect the roles of glutamine metabolism during kidney development as well as in pathological conditions.

CONCLUSION

Here we have generated vascularized 3D kidney organoids for *in vitro* studies of kidney development. The organoids recapitulate both the developmental timeline and the complex 3D structures of human kidneys. Using this organoid platform, we have validated the presence of NPC-derived vascular progenitors, which could partially contribute to the renal vascular network. Similar population has been found in human fetal kidney samples. Moreover, we were able to modulate the development of kidney compartments through the manipulation of metabolic pathways. Specifically, the number of podocytes within kidney organoids could be adjusted due to the modulation of glutamine pathways. These studies have provided evidence that organoid platform is a valuable *in vitro* tool for developmental and disease studies.

METHODS

Human pluripotent stem cell (hPSCs) culture

All experiments presented except *in vitro* dextran uptake assay used human embryonic stem cell H9 (Wicell). ARPKD patient-derived iPSC was used for *in vitro* dextran uptake assay, which was a gift from Professor Juan Carlos Izpisua Belmonte, Salk Institute. hPSCs were cultured on growth-factor-reduced Matrigel-coated (Corning) plates with TeSR. For maintenance, hPSCs were treated with Dispase (Life Technology) for dissociation when confluency reached about 70-80%. Then cells were broken into small clumps by pipetting and passaged at a ratio of 1:5-1:10.

Induction of kidney organoids from human pluripotent stem cells (hPSCs)

hPSCs were dissociated into single cells with Accutase (STEMCELL Technologies) and seeded on Matrigel-coated plates 3-4 days before differentiation. Seeding density was about 15,000 to 24,000 cells /cm². Basic differentiation media used was Advanced RPMI 1640 (Life Technology) with 1X GlutaMAX (Life Technology) and 1X NEAA (Life Technology). Cells were treated with 10 µM CHIR99021 (Sigma) for 4 days, then followed by 3 days culture in basic medium without any growth factor. From day 7 to day 8, cells were treated with 50ng/ul FGF9 (Peprotech) and 3uM CHIR99021, followed by culture in basic medium with FGF9 (50ng/ul) until day 20. From day 20, differentiated cells would be cultured in basic differentiation medium.

Generating three-dimensional (3D) kidney organoids

From day 10 to day 12, differentiating cells were dissociated into single cells using Accutase and aggregated using round bottom ultra-low attachment 96-well plated (Corning) (25,000-100,000 cells/well). Cells were cultured with basic differentiation medium supplemented with FGF9 and 10 µM Y-27632 (Sigma). 3D kidney organoids were then transferred onto the chambers of transwell (Corning) for liquid-air culture 5 to 7 days after aggregation.

FACS analysis

Samples were dissociated into single cells with Accutase, followed by passing through a 40 µm filter to remove cell clumps. Samples were blocked with blocking buffer (2% FBS and 2% BSA in 1X PBS) for 20 mins on ice. Cells were then stained with surface markers for 20 mins on ice. After washing with washing buffer (1% FBS and 1% BSA in 1XPBS), cells were fixed with 2% paraformaldehyde for 10 mins followed by permeabilization with 0.3% Triton-X in 1X PBS. After washing, cells were staining with other primary antibodies, followed by secondary staining. Then cells were washed and resuspended in PBS before analysis. Stained samples were analysis using LSRFortessa X-20. The FACS data were processed with Flowjo version 10, and BD FACSDIVA™ software.

Immunofluorescence staining

Samples were washed with PBS and fixed in 4% paraformaldehyde for 10 mins. Samples were permeabilized with 0.3% Triton-X in 1X PBS and blocked with 2% FBS and 2% BSA in 1X PBS. Samples were stained with primary antibodies overnight at 4 degree. After three times of washing, samples were stained with secondary antibodies for 2-3 hours at room temperature. Samples were mounted with Fluoromount-G® Mounting Medium (SouthernBiotech) and analysed with ZEN Lite.

***In vitro* Dextran uptake assay**

Organoids were incubated with basic differentiation medium supplemented with 100 µg/mL dextran for 4 hours and washed with fresh medium. Kidney organoids were then cultured in fresh cell culture medium without dextran for another 24 hours. Samples were fixed and used for immunofluorescence analysis.

Transmission Electron Microscopy

Day 24 organoids were fixed in 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer for 15 mins and washed for 5 times. 2% osmium tetroxide solution containing 2% potassium ferrocyanide was used for staining (2 h), followed by dehydration with ethanol, acetone, and resin (Polysciences Inc, PA, USA) before curing at 60 °C for two days. Blocks were sectioned using Leica EM UC7 ultramicrotome (Leica Microsystems, Wetzlar, DE) at 500 µm for semi-thin sections and 130 µm for ultra-thin sections. Imaging was performed on either the JEOL JEM1010 or JEOL JEM1200.

Single Cell Analysis of Human Kidney Cortex

Single cell sequencing dataset of 16 weeks human kidney was published by the Semrau group (GEO: GSM3143601). Single cell transcriptomic information of 6,602 cells were available for re-analysis. The dataset could be achieved from

<https://home.physics.leidenuniv.nl/~semrau/humanfetalkidneyatlas/>

RNA Extraction for RNA-seq

Samples were harvested in 1 mL TRIzol (Themofisher) for RNA extraction. Samples were mixed with 200 µL chloroform and centrifuged for 15 mins at 4 degree. The upper layer of sample was transferred and mixed with 500 µL isopropanol, followed by 2-3 hours incubation at -20 degree. Direct-zol™ RNA Miniprep (Zymo Research) was used for the rest parts of RNA extraction.

RNA-seq data processing

Sequenced reads were aligned to GRCh37 with STAR (version 2.5.2a), and transcripts were assembled with RSEM (version 1.3.0). Quartile normalize fragments per kilobase per million reads (FPKM) expression matrix of genes were obtained with Cuffnorm (version 2.2.1).

Heatmap of expression of nephron stage markers and segment markers was generated with R

package pheatmap version 1.0.10 (<https://cran.r-project.org/web/packages/pheatmap/index.html>).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CDH1 (E-Cadherin)	BD Biosciences	Cat. #610182; RRID:AB_397581
KDR (VEGFR2/CD309)	Biolegend	Cat. # 359916; RRID:AB_2565928
MEIS1	Active Motif	Cat. #A25/39795; RRID:AB_2750570
Lotus Tetragonolobus Lectin (LTL), Fluorescein labelled	Vector Laboratories	Cat. #FL-1321; RRID:AB_2336559
NPHS1 (Nephrin)	Progen	Cat. #GP-N2
PODXL (Podocalyxin)	R&D Systems	Cat. #AF1658
SALL1	R&D Systems	Cat. # PP-K9814-00
SIX1	Cell Signalling Technology	Cat. #D4A8K
SIX2	Proteintech	Cat. # 11562-1-AP
WT1	Abcam	Cat. #ab89901
Chicken anti-Rabbit IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A21443
Donkey anti-Rabbit IgG, Alexa Fluor 488	Thermo Fisher Scientific	Cat. #A21206
Goat anti-Rabbit IgG, Alexa Fluor 405	Thermo Fisher Scientific	Cat. #A31556
Goat anti-Rabbit IgG, Alexa Fluor 594	Thermo Fisher Scientific	Cat. #R37117

Donkey anti-Mouse IgG, Alexa Fluor 488	Thermo Fisher Scientific	Cat. #A21202
Donkey anti-Mouse IgG, Alexa Fluor 594	Thermo Fisher Scientific	Cat. #A21203
Donkey anti-Mouse IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A31571
Goat anti-Mouse IgG, Alexa Fluor 633	Thermo Fisher Scientific	Cat. #A21053
Goat anti-Mouse IgG, Alexa Fluor 405	Thermo Fisher Scientific	Cat. #A31553
Chicken anti-Goat IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A21469
Chicken anti-Goat IgG, Alexa Fluor 488	Thermo Fisher Scientific	Cat. #A21467
Donkey anti-Goat IgG, Alexa Fluor 594	Thermo Fisher Scientific	Cat. #A11058
Donkey anti-Goat IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A21447
Goat anti-Guinea Pig IgG , Alexa Fluor 488	Thermo Fisher Scientific	Cat. #A11073
Goat anti-Guinea Pig IgG , Alexa Fluor 594	Thermo Fisher Scientific	Cat. #A11076
Chicken anti-Rat IgG, Alexa Fluor 647	Thermo Fisher Scientific	Cat. #A21472

Donkey anti-Rat IgG, Alexa Fluor 594	Thermo Fisher Scientific	Cat. #A21209
Biological Samples		
Human fetal kidney samples	Human fetal kidney samples from donors 16-23 weeks of age were obtained from Kandang Kerbau Women's and Children's Hospital (KKH) with written consent from donors. SingHealth and National Health Care Group Research Ethics Committees Singapore specifically approved this study (CIRB Ref: 2012/064/B)	
Chemicals, Peptides, and Recombinant Proteins		
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
Recombinant human fibroblast growth factor 2 (FGF2)	Joint Protein Central	Cat. #L16F02001
Recombinant human fibroblast growth factor 9 (FGF9)	PeproTech	Cat. # 100-23
Recombinant human Transforming Growth Factor- β (TGF- β)	Sigma-Aldrich	Cat. # H8541
Y-27632 dihydrochloride	Sigma-Aldrich	Cat. #72308
Critical Commercial Assays		
Direct-zol™ RNA Miniprep	Zymo Research	Cat. #R2050
TRIzol™ Reagent	Invitrogen	Cat. #15596018
Experimental Models: Cell Lines		
Human embryonic stem cell H9	WiCell	Cat. #WA09
ARPKD patient-derived and gene corrected iPSCs	From Professor Juan Carlos Izpisua Belmonte, Salk Institute	
Software and Algorithms		

pheatmap v1.0.10		https://cran.r-project.org/web/packages/pheatmap/index.html
R 3.5.1	R project	https://www.r-project.org
Seurat v2.2.0	(Butler, Hoffman, Smibert, Papalexi, & Satija, 2018)	http://satijalab.org/seurat/
STAR v2.5.2a	(Dobin et al., 2013)	https://github.com/alexdobin/STAR
ZEN Lite	Carl Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite.html
BD FACSDIVA™ SOFTWARE	BD FACS	http://www.bdbiosciences.com/en-us/instruments/research-instruments/research-software/flow-cytometry-acquisition/facsdiva-software
Flowjo Version 10	FACS Analysis	https://www.flowjo.com/
Other		
10X Phosphate Buffered Saline	1st BASE Biochemicals	Cat. #BUF-2040
Accutase®	STEMCELL Technologies Inc.	Cat. #07920
Advanced RPMI 1640	Thermo Fisher Scientific	Cat. #12633020

Bovine Serum Albumin (BSA)	Biowest	Cat. #P6154
Dispase	Invitrogen	Cat. #17105-041
DMEM/F-12	Thermo Fisher Scientific	Cat. #12400024
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific	Cat. #10270106
Glutaraldehyde	Electron Microscopy Services	Cat. #16020
Fluoromount-G® Mounting Medium	SouthernBiotech	Cat. #0100-01
GlutaMAX Supplement	Thermo Fisher Scientific	Cat. #35050-061
Lead nitrate	Nacalai Tesque	Cat. #20230-12
MEM	Thermo Fisher Scientific	Cat. #31985070
Non-Essential Amino Acids	Thermo Fisher Scientific	Cat. #11140-050
Normocin	Invivogen	Cat. #ant-nr-1
Nuclease-Free Water	VWR	Cat. #E476-1L
Osmium tetroxide	Electron Microscopy Services	Cat. #19152
Paraformaldehyde	Sigma-Aldrich	Cat. #P6148
Phosphate buffer pH 7.0	Electron Microscopy Services	Cat. #19340-70
Poly/Bed 812 – BDMA embedding kit	Polysciences Inc.	Cat. #21844-1
Potassium ferrocyanide	Electron Microscopy Services	Cat. #20150
Sodium hydroxide	Sigma Aldrich	Cat. #367176-500g
Triton-X100	Bio-Rad	Cat. #161-0407
Uranyl acetate	Electron Microscopy Services	Cat. #2240

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