Human kidney organoids: progress and remaining challenges

Ryuichi Nishinakamura

Institute of Molecular Embryology and Genetics, Kumamoto University, Japan

Abstract

Kidney organoids are regarded as important tools with which to study the development of the normal and diseased human kidney. Since the first reports of human pluripotent stem cell (PSC)-derived kidney organoids 5 years ago, kidney organoids have been successfully used to model glomerular and tubular diseases. In parallel, advances in single-cell RNA sequencing have led to identification of a variety of cell types in the organoids, and have shown these to be similar to, but more immature than human kidney cells *in vivo*. Protocols for the in vitro expansion of stem cell-derived nephron progenitor cells (NPCs), as well as those for the selective induction of specific lineages, especially glomerular podocytes, have also been reported. While most of the current organoids are based on induction of nephron progenitors, the induction protocol for ureteric buds (collecting duct precursors) has also been developed, and generation of more complex kidney structures is coming into sight. Maturation of organoids is a major challenge, and more detailed analysis of developing kidney at a single cell level is needed. Eventually, organotypic kidney structures equipped with nephrons, collecting ducts, ureters, stroma, and vascular flow, are required to generate transplantable kidneys; such attempts are in progress.

[H1] Introduction

Organoids are self-organizing 3D aggregations of cells that represent the structure and function of organs. They can be derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Since the first reports of kidney organoids derived from human induced pluripotent stem cells (iPSCs) 5 years ago, many protocols for the induction of kidney organoids from iPSCs have been described, which differ in terms of their length of culture and growth factors used. The development of protocols for the generation of organoids has been driven by advances in our understanding of the processes and signals underlying kidney development. The availability of technologies such as single-cell RNA-sequencing (scRNA-seq) have provided

insights into the relevance of kidney organoids to the process of kidney development *in vivo*, and has potential to identify further approaches with which to enhance organoid maturation.

Kidney organoids have potential to advance the field of nephrology by providing a tool for the study of human kidney development and disease, by providing a tool for *in vitro* drug screening, and ultimately, for regenerative therapy; however, major barriers remain to the use of organoids for any of these purposes. In this Review I discuss progress in the development of kidney organoids and describes remaining challenges to the use of these cultures for the study of kidney physiology and disease. I describe the main components of the most commonly used protocols for generating kidney organoids, highlighting the main conceptual differences between them. I then describe how the field has advanced over the past 5 years, in terms of insights gained from sc RNA-seq, approaches to expand the nephron progenitor cell **[G]** (NPC) population and achieve higher-order structure, and how organoids have been used to model kidney disease. Finally, the Review discusses limitations of current organoid protocols, with an emphasis on issues relating to their immaturity, lack of vascularisation, and ureter formation.

[H1] Kidney development

Kidney organoids are an aggregation of cells that differentiate and self-assemble in response to environmental cues similar to those that are present in the developing kidney. An understanding of kidney development is therefore essential to understand the principles underlying kidney organoid induction.

The embryonic kidney is divided into the metanephric mesenchyme (MM) and ureteric bud (UB). NPCs in the MM produce the glomerular podocytes, Bowman's capsule, and renal tubules[1–4], whereas the UB produces the collecting ducts and ureter. At approximately embryonic day (E) 10.5 in mice, UB outgrowth from the posterior nephric duct epithelium occurs with invasion of the UB into the MM, as a result of MM-secreted glial cell line-derived neurotrophic factor (GDNF) acting on its receptor, RET, in the UB epithelia (Figure 1)[5–9]. In turn, the UB secretes WNT9B, which stimulates some NPCs within the MM to differentiate into nephron components [10]. By contrast, other NPCs remain undifferentiated, at least in part through the action of FGF9 derived from UBs and NPCs and FGF20 derived from NPCs[11].

Undifferentiated NPCs express a subset of transcription factors, such as SIX2, PAX2, and SALL1, which are essential for progenitor maintenance and differentiation[12–15]. The MM also contains another type of precursor cell — the stromal progenitor cell. Stromal progenitor cells give rise to interstitial cells, which fill spaces between nephron epithelia, and glomerular mesangial cells; moreover, stromal cells regulate the development of NPCs and the UB. For example, both cortical stromal cells and NPCs in the MM, secrete GDNF to stimulate UB branching[16]. Cortical stromal cells also express *Aldh1a2*, which encodes retinal dehydrogenase 2— an enzyme that produces retinoic acid and upregulates expression of RET in the UB, thereby contributing UB branching[17]. The protocadherin FAT4 is also expressed in cortical stromal cells and acts on the cadherin-related protein DCHS1 on NPCs to restrict their excessive expansion[18,19]. Medullary stromal cells are involved in the control of osmolality, which is critical for urine concentration, and their development requires WNT7B signalling from the UB[20]. Thus, NPCs and stromal progenitor cells within the MM, together with cells in the UB are the essential precursor components of the metanephric kidney; moreover, interactions between these cell types are critical for appropriate kidney development.

In the developing mouse embryo, both the MM and UB are reportedly derived from the intermediate mesoderm (IM), which expresses the transcription factor OSR1[21]. However, our group showed that the anterior and posterior IM are distinct populations[22,23]. The anterior IM of mice is established at E8.5 and subsequently differentiates into the UB lineage. By contrast, nascent mesoderm at the posterior end of E8.5 mouse embryos (the posterior nascent mesoderm) differentiates into the posterior IM at E9.5, and subsequently forms NPCs (Figure 2). Thus, the origins of the UB and MM are distinct, both spatially and temporally. It would be reasonable to hypothesize that this finding is likely to hold true in humans, although cell lineage trace experiments cannot be performed by using human embryos.

[H1] Kidney organoid induction protocols

Based on the finding that the UB and MM have distinct origins, we established a multi-step protocol to derive MM from mouse ESCs and human iPSCs cultured as three-dimensional (3D) spheres[22]. This protocol, which we term the Taguchi protocol, requires prolonged treatment with a high concentration of the Wnt agonist, CHIR99021 (CHIR), to promote development of the posterior nascent mesoderm

(Figure 2). We then apply a combination of retinoic acid, activin, BMP4, and an intermediate concentration of CHIR to induce the posterior nascent mesoderm to form posterior IM; subsequent treatment with FGF9 and a low concentration of CHIR generates NPCs that could give rise to podocytes, cells of the Bowman's capsule, and tubule epithelial cells[22].

The generation of kidney cells in 3D spheres from human ESCs was also reported by Takasato et al. in 2014[24]. In this study, the researchers used a two-dimensional (2D) induction step followed by 3D culture of the aggregated progenitor cells; however, the initial protocol was not based on the concept on posterior IM induction. In 2015, Takasato et al.[25] published a revised protocol that used CHIR treatment of varying duration to induce anterior or posterior specification of the IM. With optimal duration of CHIR treatment, this revised protocol, which we refer to as the Takasato protocol, generated human iPSC-derived kidney organoids that contained nephron progenitor-derived podocytes, Bowman's capsules, and renal tubules, as well as UB-like cells, stromal cells, and endothelial cells.

In a third protocol, Morizane et al.[26] also used CHIR treatment to specify the posterior IM lineage and induce human ESCs and iPSCs to form podocytes, Bowman's capsules, and renal tubules. This protocol, which we refer to as the Morizane protocol, like the Takasato protocol, also involved induction of 2D progenitor cultures, followed by 3D culture, but the duration of the entire process was shorter than that of the Takasato protocol. Morizane et al.[26] also reported that their protocol enabled highly efficient induction of NPCs, although this claim remains to be formally examined through a direct, parallel comparison of the different protocols using an iPSC clone expressing an NPC-specific reporter. Importantly, the kidney organoids induced by the Takasato and Morizane protocols exhibited some inherent renal tubule function, including absorptive capacity for dextran and sensitivity to nephrotoxic substances[25,26].

Although the above three protocols, as well as other published protocols[27,28], use CHIR to activate WNT, the protocols are otherwise distinct (Table 1, as well as other reviews[29,30]). For example, the duration of the CHIR treatment ranges from 1.5 days to 6 days. CHIR is critical for posteriorization (that is, development of the posterior nascent mesoderm and posterior IM); thus, a short duration of CHIR exposure might lead to insufficient posteriorization and eventual induction of the mesonephros, instead of the metanephros. The Takasato protocol has only one simple

step of FGF9 treatment after the CHIR step to induce NPCs, whereas the Morizane protocol has two steps following CHIR treatment: activin treatment followed by FGF9. The Taguchi protocol also has two steps post-CHIR treatment, but uses factors in addition to activin and FGF9 as described above. The complexity of the Taguchi protocol reflects the fact that this protocol was originally established to mimic gene expression levels in developing mouse embryonic kidneys. We found that the addition of each factor leads to increased expression levels of key genes such as *Six2, Pax2, Wt1*, and posteriorly expressed *Hox* genes, which suggests that induction efficiency or NPCs quality is increased in response to these factors. Thus different protocols are likely to produce nephrons of different quality and with different ratios of cell types; suitable protocols should be chosen in accordance with the purpose of each particular experiment.

[H2] Reproducibility and variability issues

Of note, however, considerable between-experiment variations have been observed in organoids, particularly in genes associated with their maturation, even in instances where a single protocol has been used with a single iPSC line [31]. Nephron patterning and cell proportions are also likely to fluctuate between experiments. Underlying reasons for these differences might include between-batch differences in reagents, fluctuations in the pluripotency state of the progenitor cells at the induction of differentiation (that is, between-passage variations), variability in the robustness of particular differentiation protocols, and variations in the specific handling techniques of individual researchers. Although clonal differences in the iPSCs used to generate organoid cultures are reportedly smaller than inter-experimental differences[31], we speculate that endogenous bone morphogenetic protein (BMP) signalling might vary from clone to clone. Although we do not have any direct evidence to support this hypothesis, the Morizane protocol uses the BMP inhibitor, noggin, depending on the ESC/iPSC clone[26]. In addition, we have found that optimisation of activin and/or BMP signalling in the initial induction steps prior to administration of CHIR in the Taguchi protocol can adjust for clonal variation, suggesting that variations in endogenous BMP signalling may contribute to inter-clonal variability [32]. Of note, poor inter-laboratory reproducibility has been reported for iPSC-derived neurons[33], and is likely to also be applicable to kidney organoids. Factors that affect each step of each protocol should therefore be identified to enable the organoids to be reproducibly

generated by researchers from different laboratories, as well as for the industrialised production of kidney organoids.

[H1] Opportunities and challenges

Kidney organoids have potential immediate applications, for example, as models to mimic and study *in vivo* kidney development, and to study human kidney disease. However, beyond issues relating to reproducibility and clonal variation as described above, many other challenges need to be addressed. These include approaches to increase the scalability of organoid cultures, approaches to generate specific cell types and approaches to improve vascularization of kidney organoids.

[H2] Modelling kidney development

sc RNA-seq enables detailed analysis of the expression of genes of individual cells**.** This technology is particularly useful for the study of complex organs that contain many cell types, such as the kidney[34]. A study that used sc RNA-seq to compare the gene expression of cells between organoids generated using the Takasato and Morizane protocols showed that organoids generated using the Morizane protocol contained more differentiated cells (including podocytes) than those generated using Ithe Takasato organoids [35]. However, these findings should be interpreted cautiously because inter-experimental and inter-clonal, and inter-laboratory variations might affect the outcome, as described above. I expect it is appropriate to assume that both organoid protocols generate a diverse range of kidney cells. The study also showed that both protocols generated off-target non-renal cells, including neural and muscle cells. Pseudotime analysis of sc RNA-seq data revealed that inhibition of signalling between brain-derived neurotrophic factor (BDNF) and its receptor, neurotrophic tyrosine kinase receptor, type 2 (NTRK2), significantly reduced the number of neural cells in kidney organoids, and resulted in more efficient induction of renal cells[35].

Importantly, scRNA-seq and transcriptional profiling studies have also demonstrated that renal cells within organoids are much more immature than those of the adult kidney. Indeed current kidney organoids seem to be similar to cell types present during the first or second trimester of gestation *in vivo*, indicating that further work is needed to generate mature organoids [25,36]. The availability of sc RNA-seq data for human embryonic kidneys provide insights into the gene expression profiles of different cell types during the developmental process[37–40], and will serve as important

references for the analysis of kidney organoid maturity. These studies are also likely to aid the identification of further signalling factors that drive renal cell development with the expectation that such findings could be applied to the development of protocols that generate more mature organoids. Indeed, little is currently known regarding the factors that govern kidney maturation during gestation and after birth, even in mice as most studies have focused on the early phases of kidney morphogenesis. Although sc RNA-seq is expected to facilitate the study of renal development, it is important to consider the limitations of this technique[41]. Many sc RNA-seq methods are available, each with their own advantages and disadvantages. One major disadvantage of sc RNA-seq is that the tissue dissociation process can cause cell stress and as a consequence, cells can change their gene expression patterns in response to the dissociation process. One approach to mitigate the effects of such dissociation artefacts is to sequence the nuclear RNA of single cells. The validity of single nuclear RNA sequencing has been demonstrated for adult kidney tissue but is yet to be applied to organoids [42]. The quality of data obtained through sc RNA-seq depends on many factors, such as the cell survival rate upon cell dissociation, the number of cells analysed, the number of genes detected per cell, and the thresholds set to minimize technical noise, and exhibit considerable variations between studies. Differences in these parameters can confound study results, and thus further improvements are needed with respect to approaches for both data acquisition and bioinformatics analysis.

[H2] Modelling human disease

One key area of interest in organoid research is in their use as models of disease, either through the introduction of disease-specific mutations or through the use of patient-derived iPSCs. We have shown that glomerular podocyte abnormalities can be recapitulated in kidney organoids generated using the Taguchi protocol from iPSCs derived from a patient with Finnish-type congenital nephrotic syndrome[43]. This disease is caused by mutations in *NPHS1*, which encodes NEPHRIN, a major component of the slit diaphragm in glomerular podocytes. The slit diaphragm functions as a molecular sieve that prevents leakage of serum proteins into the urine; defects in the slit diaphragm lead to severe proteinuria (that is, nephrotic syndrome). We showed that kidney organoids derived from healthy human iPSCs form slit diaphragm precursors (NEPHRIN+ rod-like structures) on the basolateral domains of

the podocytes *in vitro*. Following transplantation of the organoids into immunodeficient mice, they formed slit diaphragms on the basal domains of the podocytes adjacent to vasculature. However, patient-derived kidney organoids failed to form even slit diaphragm precursors as mutant NEPHRIN remained in the cytoplasm and was not transported to the cell surface (Figure 3, Table 1). Furthermore, genetic correction of the point mutation by gene editing corrected these abnormalities, clearly demonstrating that the point mutation in *NPHS1* was disease-causing. Another study used the Takasato protocol to generate kidney organoids from a patient with a different mutation of *NPHS1*[44]. Like our patient-derived organoids, these organoids also showed reduced levels of NEPHRIN but in contrast to our observations, also showed reduced expression of another slit diaphragm component, PODOCIN. Although the reason for the reduction in PODOCIN expression is unclear, these findings suggest the presence of allele-dependent differences in the consequences of these NEPHRIN mutations. They also show that patient-derived organoids can be used to recapitulate processes involved in glomerular disease abnormalities, and provide insights into disease mechanisms.

The Takasato protocol has also been used to establish organoids using iPSCs from a patient with nephronophthisis caused by mutations in intraflagellar transport protein 140[45]. Patient-derived organoids exhibited shortened primary cilia and impaired apicobasal polarity in renal tubule epithelia, consistent with the i*n vivo* consequences of this mutation, demonstrating the ability of kidney organoids to model renal tubule-related diseases as well as glomerular diseases. Importantly, correction of the gene defect through gene editing rescued these phenotypes (Figure 3, Table 1).

An alternative to the use of patient-derived iPSCs is to introduce disease-causing mutations through gene editing technology such as CRISPR/Cas9. Use of CRISPR/Cas9 to generate human iPSC-derived kidney organoids containing mutations in the *PODXL*, which encodes the podocyte protein PODOCALYXIN, demonstrated this protein is essential for the assembly of microvilli on the apical membranes of podocytes, as well as for the formation of intercellular spaces between lateral membranes of the podocytes[27,46]. These findings are consistent with observations in *Podxl*-knockout mice, demonstrating that podocalyxin has a conserved role in podocyte development [46]**.** Another study used gene editing to introduce mutations in the *PKD1* and *PKD2*, which encode the autosomal dominant polycystic kidney disease (ADPKD)-associated proteins, polycystin (PC1) and PC2,

respectively, in human iPSC-derived organoids. Renal tubules within organoids containing mutations in PC2 developed cysts of up to 1 cm in diameter [47] (Figure 3, Table 1). Detachment of the organoids from the adhesive plates promoted the cyst enlargement, suggesting that the microenvironment (or surrounding stroma) contributes to cyst formation. Organoids with *PKD1* mutations also formed cysts, but their size were not compared to that of *PKD2* mutant cysts. Of note, however, the kidney organoids used in that study had homozygous *PKD2* or *PKD1* mutations, whereas patients with ADPKD typically have heterozygous mutations**,** suggesting that these organoids may not represent true models of the disease. In addition, cysts in the organoids were detected proximal tubules, whereas cysts in patients with ADPKD are primarily present in the distal tubules and collecting ducts. Thus, reproduction of cysts from patient-derived iPSCs is needed to precisely model this disease. According to the researchers who developed the ADPKD organoid models[47], kidney organoids generated from patient-derived iPSCs exhibited marked line-to-line variations in their ability to form kidney organoids and cysts. Whether these differences relate to inadequacies in the organoid induction protocol or to the requirement of a 'second hit' in addition to the *PKD1* and *PKD2* mutations for cyst development is unknown[48].

In addition to their use as a model to study disease processes, patient-derived kidney organoids also have potential as a tool for the screening of potential therapeutic agents. The effective use of organoids for this purpose, however, requires their large-scale production for high throughput screening. For this purpose, a high-throughput semi-automated culture system has been developed for the purpose of optimising and expanding organoid induction protocols[49]. However, the organoids generated using this system were attached to the plate surface and were flatter than those produced via conventional suspension culture or those produced at the air-liquid interface; this difference may affect the precise nephron structure within the organoids, as well as the maturity of the induced nephrons. Careful experimental design and comparisons are therefore needed to ensure that high throughput systems generate organoids of high quality in a reproducible manner.

As mentioned earlier, currently available protocols are only able to produce immature kidney organoids. Therefore, only diseases that manifest abnormalities during early stages of embryogenesis can be reproduced by organoid technology. The development of more mature organoids is needed to enable the modelling and study of late-onset diseases.

[H2] Expansion of induced NPCs

The processes of NPC propagation and differentiation continue during development. The balance between propagation and differentiation is a critical determinant of nephron number and might affect kidney function in adult life[50]. In humans, all NPC differentiation occurs before birth, whereas in mice this process stops shortly after birth.

The in vitro expansion of NPCs has been challenging as these cells stop proliferating and lose their ability to differentiate in standard culture conditions. Most kidney organoid induction protocols generate NPCs within 9-14 days of culture. The ability to expand and freeze iPSC-derived NPCs would enable researchers to bypass the complicated induction process, and would provide more researchers with access to organoid technology. Furthermore, expansion of NPCs would be beneficial in settings in which large numbers of NPCs are required, such as regenerative cell therapy, disease modelling, and drug screening. Several groups, including ours, have reported protocols for the expansion of NPCs; all of these protocols involve administration of fibroblast growth factor (FGF), WNT, and BMP signalling factors, consistent with a role for these factors in NPC propagation in the developing kidney in vivo. For instance, FGF9 and FGF20 maintain NPCs whereas WNT9B and Smad-dependent BMP signalling induces NPC differentiation[10,11,51–53]. One study used FGF9, BMP7 and a WNT agonist to expand human ESC-derived NPCs[54]; however, this protocol led to the formation of renal tubule-like structures alone, without glomerular podocytes. Our group used leukaemia inhibitory factor (LIF), in addition to the reagents described above, to expand human iPSC-derived NPCs[55]. Although the *in vivo* role of LIF in nephrogenesis remains obscure, this protocol led to the successful generation of podocytes with Bowman's capsule as well as renal tubules. However, the expansion period was limited (1–2 weeks), and NPCs expanded beyond this period lost the ability to form nephrons. A separate study used similar reagents, including LIF**,** to propagate human embryo-derived NPCs for up to 7 months[56]. The researchers also claimed that human iPSC-derived NPCs were at least partially responsive to the same protocol. However, no studies have quantitatively assessed the proportion of NPCs among the expanded cells, nor have they succeeded in establishing methods for freezing human iPSC-derived nephron progenitors. Whether NPCs stop expanding at a certain age or stage, or change their characteristics during expansion in culture is

also unknown. The gene expression of mouse NPCs changes according to the developmental stage[57]; the changes that occur during human nephron development will likely be elucidated by sc RNA-seq analysis of human embryonic kidneys. Such information will be useful to identify whether progenitor 'ageing' occurs *in vitro*.

[H2] Generating specific nephron cell types

Most currently available protocols enable the transition of iPSCs to NPCs; however, the subsequent step, involving the transition of NPCs to one of multiple nephron cell types (particularly glomerular podocytes), is an interesting area of investigation, not only for purposes of understanding the developmental processes of lineage specification but also for the practical use of induced cells for drug screening and potentially reparative therapy.

Immortalised podocyte cell lines have long been used for *in vitro* studies of podocyte function; however, cells cultured in this manner do not retain the original characteristics of podocytes such as foot processes and slit diaphragms, and unlike podocytes *in vivo*, they express very low levels of slit diaphragm-related proteins such as NEPHRIN and PODOCIN [58–60]. Although several studies have reported methods for the induction of podocyte-like cells from human iPSCs[61–63], upregulation of podocyte-associated genes has not uniformly been observed during cell differentiation, with some studies reporting even lower expression of *NPHS1* (which encodes NEPHRIN) in the differentiated cells than in undifferentiated iPSCs [61,63]. We have shown that transient treatment of NPCs with WNT followed by inhibition of tumour growth factor beta (TGFβ) results in the highly efficient induction of podocytes from mouse embryos and human iPSCs[64]. The resultant podocytes exhibited gene expression signatures that were comparable to those of adult human podocytes; moreover, they expressed abundant slit diaphragm-associated proteins, such as NEPHRIN and PODOCIN and showed functional responsiveness to drug-induced injury. These iPSC-derived podocytes therefore offer a new resource for disease modelling and nephrotoxicity testing. Our protocol is consistent with a model of nephron progenitor recruitment and nephron patterning, which proposes that nephron progenitors exposed to UB-derived WNT9B for a short period of time will differentiate into podocytes [38]. Whether TGFβ signalling is involved in podocyte specification *in vivo* is, however, unknown. Of note, NEPHRIN remained located on

the lateral domains, in addition to the basal domains, of our induced podocytes, which is indicative that the slit diaphragms in our system are immature. Gene expression analysis also revealed low expression of the genes encoding collagen type IV alpha 3 and alpha 4 chains (*COL4A3* and *COL4A4*, respectively) in the induced podocytes, compared with levels in human adult podocytes [64]. These findings suggest the immature nature of a basement membrane on which podocytes normally sit and seem to be a consistent limitation of *in vitro* kidney organoids in general. For instance, low expression of *COL4A* isoforms was also observed in a comprehensive analysis of extracellular matrix in glomeruli that had been sieved to remove the other lineages of cells from organoids induced using the Takasato protocol [44].

In contrast to our in vitro findings, transplantation of iPSC-derived NPCs into mice resulted in maturation of the SD, accompanied by vascular integration from the host into the transplanted glomeruli [65]. Although formation of mesangial cells could not be confirmed owing to the insufficient availability of mesangium-specific markers, these findings suggest that interactions between podocytes, endothelial cells, and possibly mesangial cells, are probably important for podocyte maturation. Detailed sc RNA-seq analysis of human developing renal corpuscles will be helpful for the identification of candidate molecules that are important for the maturation of podocytes and other kidney components. Physical stretching evoked by vascularisation may also contribute to this maturation process, as discussed later.

[H2] Higher-order kidney organoid structure

Most of the above-described protocols generate kidney organoids with NPC-derived lineages, such as podocytes, Bowman's capsules, and renal tubules; however, the nephrons containing these lineages are separately positioned within the organoids and their organization does not mimic that of mammalian kidneys, in which nephrons are connected by collecting ducts that converge into the ureter. As mentioned earlier, the collecting ducts and the ureter are derived from the UB. Although the Takasato protocol[25] was initially reported to generate both MM-derived cells and UB-derived cells, sc RNA-seq analysis of these organoids showed that the UB-like cells do not express genes typical of UBs, such as *RET*, *WNT9b*, and *WNT11*, suggesting these cells resemble NPC-derived distal renal tubules rather than collecting ducts[35].

Our understanding of the distinct origins of MM and UB lineages during mouse development[22](Figure 2), has enabled us to establish a multistep method to

induce UBs from mouse ESCs and human iPSCs[32]. By following their respective developmental pathways, we first induce specification of the anterior IM, followed by induction of Wolffian duct precursors, and finally induction of UBs characterized by their typical gene signature (Figure 2). Interestingly, the UB induction protocol differs from the NPC induction protocol from the very first steps, suggesting that these two lineages might segregate at very early stages of development *in vivo* (for example, during the early epiblast and nascent mesoderm (primitive streak) stages). The window in which to administer CHIR to induce UB specification is very narrow (maximal efficiency was observed at 36 hours, and was dramatically reduced at 24 or 48 hours), in sharp contrast to the prolonged CHIR treatment (6 days) used to induce NPCs (Table 1). In addition, despite some overlap in the steps taken to induce the specification, commitment, and maturation of UB and MM lineages, the signalling processes required for the differentiation of these two lineages are clearly distinct (Figure 2). Finally, we applied previously published assembly methods for the dissociation and reaggregation of mouse embryonic kidney cells, [66,67], by combining mouse ESC-derived UBs and NPCs with mouse embryo-derived stromal cells to generate kidney organoids that showed robust UB branching, along with differentiated nephrons located at the periphery of the UB tips (Figure 4). Thus, we have demonstrated that this type of 'higher-order' kidney structure can be achieved by the differential induction of each lineage, followed by their combination with stromal cells in culture. We also successfully adapted this protocol to generate human UBs from human iPSCs; however, we do not yet have a suitable protocol for the induction of stromal cells from human iPSCs [35]. Moreover, these UB organoids were immature and further work is therefore needed to generate more mature systems for disease modelling, for example, of *PKD* mutations in the collecting ducts.

We have further shown that the transcription factor paired box gene 2 (*PAX2*), which is a causative gene for renal coloboma syndrome, is dispensable for the induction and differentiation of NPCs[68], but required for the process of mesenchymal-to-epithelial transition during UB induction [32]. The latter finding is consistent with the *Pax2*-knockout mouse phenotype: impaired cell integrity (tight and adherent junctions) of Wolffian duct epithelia [69], whereas the former is not as *Pax2*-knockout mice have impaired NPC maintenance and differentiation[70]. The generation of lineage-specific knockout mice might aid understanding of the role of factors such as PAX2 in lineage specification. At present, it is difficult to determine whether the

discrepancies observed between human kidney organoids and knockout mice are caused by species differences or by incomplete *in vitro* modelling of the *in vivo* status.

[H2] Stromal cells in the organoids

In our protocol, mouse embryo-derived stromal cells were essential for the generation of higher-order kidney structures from mouse ESCs[32]. Simply combining mouse ESC-derived or human iPSC-derived nephron progenitors and UBs resulted in poor branching. Although embryo-derived stromal cells can be obtained fairly easily from mice, they are difficult to routinely obtain from human embryos. In our preliminary experiments, the addition of mesenchymal stem cells or stroma-like cells to the kidney organoids was also insufficient to induce branching. Thus, the induction of a bona fide stromal cell lineage from human iPSCs is likely needed to generate a similar higher-order structure for human kidney reconstruction (Figure 4). This proposal is consistent with the accumulating body of evidence showing that stromal cells have important roles in kidney organogenesis[16–20]. Most stromal cells in the kidney are derived from Forkhead box D1 (FOXD1)-positive stromal progenitors that are located at the periphery of the developing kidney[71,72]. The Takasato protocol induces the development of stromal cells, some of which express *FOXD1*, simultaneously to the development of NPCs[25]. The stromal cells in organoids generated using the Morizane protocol proliferate in response to IL-1β, possibly mimicking the kidney fibrosis [73]. The importance of stromal cells for the development of other renal cells, suggests that we should test the ability of these stroma-like cells to support the generation of higher-order structures in human kidney organoids, by aggregating with iPSC-derived NPCs and UBs. Concomitantly, studies should examine the similarities and differences between organoid stromal cells and those found *in vivo*. sc RNA-seq analyses have shown that stromal cells found **in the mouse and human kidney** *in vivo* are not homogeneous[37,74]. Thus, the subpopulations of stromal cells that contribute to the formation of the complex kidney structures should be identified, in order to establish protocols for the induction of such subpopulations in vitro.

[H2] Securing the urinary exit route

Organoids could potentially be used to replace renal function in patients with end stage renal diseases. A key barrier to the use of kidney organoids for regenerative therapy is the need to generate a urinary exit tract following transplantation of the

organoids. None of the available kidney organoid protocols are able to generate a ureter that elongates outside the organoids. If, following transplantation, blood flow from the host is supplied to kidney organoids and the glomeruli begin to produce urine, the absence of a ureter would eventually cause hydronephrosis. Kidney organoids thus require a ureter as a one-way exit, which should be connected to the host's ureter or urinary bladder to allow urine to flow without obstruction. This would also promote maturation of the organoids *in vivo*. However, ureters generated within kidney organoids might not be sufficiently large to be sutured to host tissues, and technical improvements therefore are needed to resolve these limitations. For example, one study achieved a draining system by transplanting a rat embryonic metanephros together with the cloaca, as precursor tissue of the urinary bladder, into host rats[75], Several weeks after transplantation, the researchers removed the left native kidney and connected the host ureter to the newly formed cloaca-grown bladder of the graft, allowing the graft to grow without hydronephrosis. However, induction of a urinary bladder is needed to apply this system to kidney organoids, a process that requires a separate protocol since the bladder is derived from endoderm and is formed in a developmental pathway that differs from that of the mesoderm-derived kidney.

[H2] Vascularisation of kidney organoids

Glomeruli in the kidney filter blood to generate urine; thus, an adequate vasculature is essential for kidney function. Although some vascular endothelial cells are present within kidney organoids[25], most glomeruli within organoids remain avascular *in vitro*. We have previously reported that human iPSC-derived glomeruli become efficiently vascularised when NPC-type organoids (which do not possess UBs) are transplanted under the kidney capsules of immunodeficient mice[65]. The reason why organoids are so poorly vascularized in vitro but are readily vascularized following transplantation *in vivo* remains unknown; however, mechanical flow within the host vasculature might have a role. The importance of blood flow for adequate kidney vascularisation is illustrated by a study showing that chemically induced heartbeat arrest in zebrafish impairs integration of the vasculature into glomeruli[76]. Support for a role of vascular flow in inducing vascularization is further provided by a study showing that culture of kidney organoids under conditions of flow on a microfluidic device induces the formation of vascular networks inside the organoids[77]. Nephron epithelia, including podocytes and renal tubules, cultured in this system expressed higher levels of lineage

markers than nephron epithelia cultured under conventional static conditions, probably as a result of fluidic shear stress and upregulation of VEGF expression in the organoids. The researchers demonstrated that the vasculature could be perfused ; however, the extent to which fluid flows through glomeruli and renal tubules within these organoids and degree to which the cells in these devices resemble human fetal or adult kidney cells *in vivo* is unclear.

In our transplantation system, we found that most endothelial cells within the glomeruli of our transplanted organoids were derived from the host animals, rather than from the graft[65,78]. By contrast, two other reports detected partial integration of human iPSC-derived endothelial cells upon transplantation[43, 79]. Further studies are therefore needed to determine the relative contributions of donor and host-derived vasculatures within transplanted kidney organoids, whether endothelial cells need to be induced from the human iPSCs, and whether endothelial cells must be integrated into kidney organoids before they are transplanted. If an external source of endothelial cells is required, it is important to determine whether these need to be kidney-specific or whether general endothelial cells are sufficient. Such issues must be addressed to generate functional kidney organoids.

One additional issue is that the vessels that invade kidney organoids using current transplantation methods[65] are much smaller than renal arteries observed *in vivo*, which carry 20%–25% of the cardiac output. Therefore, it is necessary to generate larger arteries, both inside and outside of the kidney organoids, which will permit the necessary volume of blood flow once transplanted. However, little is currently known regarding the mechanisms of extra- and intra-renal artery development. At E 11.5 in mice, the kidney is located on the ventral sides of the common iliac artery. A day later, short stalks bud out from the aorta and branch into the kidney, but the kidney also connected with common iliac artery[80]. By E15.5, the stems of the arteries are unified into a single renal artery. The blood vessels inside the developing kidney are reported to form mainly by angiogenesis **[G]** rather than by vasculogenesis **[G]** [81,82]. At E11.5, the UB invades into the MM at which time vessels surround the UB and spread into the kidney within the following 1-2 days. Most of these blood vessels seem to connect to extrinsic vessels, suggesting that an angiogenesis-mediated process is involved. If this is the case, a strategy of connecting kidney organoids with extrinsic blood vessels would be more beneficial than trying to accelerate vasculogenesis in the organoids; however, further research is needed to

elucidate the process of renal vessel formation and to recapitulate the complex architecture of the renal vasculature.

[H2] Reconstructing human kidneys in animals

Several attempts have been made to generate organs in animals for use in transplantation. One strategy involves the generation of interspecies chimeras by blastocyst complementation (Figure 5). This technique involves injection of iPSCs into the blastocysts that have been manipulated to lack a particular organ, so that the iPSCs contribute dominantly to the developing organ *in vivo*. As mentioned earlier, the transcription factor SALL1 is expressed by NPCs; deletion of *Sall1* in mice leads to kidney agenesis or hypoplasia[14]. One study demonstrated that injection of undifferentiated mouse ESCs into *Sall1*-deficient rat blastocysts led to the successful generation of mouse ESC-derived nephron epithelia in newborn rat kidneys[83]. UBs, stroma, and vasculature in the kidneys were a mixture of mouse and rat cells, because *Sall1* is cell-autonomously required for the formation of NPCs, but not for the other lineages. This strategy might be useful for the generation of human kidneys for transplantation in large animals, such as pigs. However, at least three aspects must be considered for such an approach to be feasible. First, residual pig cells, especially endothelial cells, will cause hyperacute rejection following transplantation; thus, in addition to NPCs, all UBs, stromal cells, and endothelial cells must be replaced with human cells. Deletion of *Sall1* or *Six2* in host pigs is not sufficient to avoid immune-mediated rejection, because depletion of these factors will only deplete NPC-derived lineages. All other renal lineages will need to be depleted in the host animals, while ensuring that non-renal organs remain intact. Second, although rat–mouse chimeras have been reported in which a mouse organ has been grown in a rat host [83,84], it is currently unknown whether it is possible to generate chimeras between humans and pigs. One study that injected human iPSCs into wild-type pig blastocysts and implanted the blastocysts into a pig uterus, reported the integration of human cells into the pig embryos[85]. However, the efficiency and level of interspecies chimerism were low. Furthermore, many embryos showed retarded growth, suggesting that the contribution of human iPSCs may have interfered with normal pig development. Third, it is currently not possible to regulate the proportions of chimeric cells within the host animals. Following injection into the blastocyst, human cells may distribute

to the kidney, but may also distribute to other off-target organs, including the brain and germ cells with unknown consequences.

Importantly, the issue of off-target effects could be avoided by injecting donor cells at the kidney-forming stage (Figure 5), rather than at the blastocyst stage. In an initial step towards such an approach, one study injected mouse NPCs into ex vivo embryonic kidneys from transgenic mice expressing the diphtheria toxin receptor in Six2-positive NPCs; administration of diphtheria eliminated host-derived NPCs without affecting the donor cell population [86]. The resultant tissues were transplanted into other mice together with the cloaca, leading to the development of donor-mouse nephrons. The researchers also injected rat NPCs into mouse embryonic kidneys ex vivo and observed their differentiation into glomeruli and renal tubules following depletion of host-derived NPCs, although the researchers did not transplant these organs into another host or examine functions of the resultant chimeric kidneys. The next step would be to inject human iPSC-derived NPCs into the developing kidneys of rats and pigs. However, since other host-derived cells (for example, those derived from the UBs, as well as stromal cells and endothelial cells) will not be eliminated even if host NPCs are successfully eliminated, [87] potential interspecies interactions between donor-derived NPCs and host-derived cells must be assessed. If interactions do occur, it might be possible to attempt the generation of more mature human–pig chimeric kidney tissues. Realistically, safe human kidney transplantation will likely require complete elimination of host-derived tissues. Such elimination might eventually be possible given technological advances in gene editing technologies such as CRISPR-Cas9. Nevertheless, it remains unclear whether the simple injection of mixed donor cells of many lineages to a vacant niche will be sufficient to generate an entire complex kidney structure in host animals.

[H1] Conclusions

Advances in iPSC-derived kidney organoids have developed rapidly over the past 5 years. Kidney organoids, which to date have mainly comprised cells of the MM lineage, have been successfully used to model glomerular and tubular diseases. A protocol for the UB organoids has also been developed. However, major hurdles remain to the use of these systems as experimental models and in transplantation. Notably sc RNA-seq and transcriptional profiling studies have shown that organoids represent a very immature kidney system. Current protocols for the induction of

kidney organoids also do not generate the full complement of renal cells, particularly with regard to heterogeneous stromal cell populations, and higher-order kidney structures with vasculature are also yet to be established. To overcome these hurdles, better understanding of gene expression profiles of human and mouse embryonic kidneys at a single cell level at different developmental stages are needed. Better understand of the molecular mechanisms of organ development and maturation can then be applied to drive the development of more mature organs containing a complete complement of renal cell types. Such efforts, combined with other emerging techniques such as gene editing and interspecies chimera generation, will accelerate scientific advances towards the generation of transplantable organs, as opposed to ‘organoids, in the future.

References

1. Grobstein, C. Inductive interaction in the development of the mouse metanephros. *J. Exp. Zool.* **130**, 319–339 (1955).
2. Auerbach, R. & Grobstein, C. Inductive interaction of embryonic tissues after dissociation and reaggregation. *Exp. Cell Res.* **15**, 384–397 (1958).
3. Kobayashi, A. *et al.* Six2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development. *Cell Stem Cell* **3**, 169–181 (2008).
4. Osafune, K., Takasato, M., Kispert, A., Asashima, M. & Nishinakamura, R. Identification of multipotent progenitors in the embryonic mouse kidney by a novel colony-forming assay. *Development* **133**, 151–161 (2006).
5. Pichel, J. G. *et al.* Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73–76 (1996).
6. Moore, M. W. *et al.* Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76–79 (1996).
7. Sanchez, M. P. *et al.* Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382**, 70–73 (1996).
8. Schuchardt, A., D’Agati, V., Larsson-Blomberg, L., Costantini, F. & Pachnis, V. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367**, 380–383 (1994).

9. Costantini, F. & Kopan, R. Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development. *Dev Cell* **18**, 698–712 (2010).
10. Carroll, T. J., Park, J.-S., Hayashi, S., Majumdar, A. & McMahon, A. P. 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 (2005).
11. Barak, H., Huh, S., Chen, S. & Jeanpierre, C. FGF9 and FGF20 maintain the stemness of nephron progenitors in mice and man. *Dev Cell* **22**, 1191–1207 (2012).
12. Self, M. *et al.* Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney. *EMBO J* **25**, 5214–5228 (2006).
13. Torres, M., Gómez-Pardo, E., Dressler, G. R. & Gruss, P. Pax-2 controls multiple steps of urogenital development. *Development* **121**, 4057–4065 (1995).
14. Nishinakamura, R. *et al.* Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development. *Development* **128**, 3105–3015 (2001).
15. Kanda, S. *et al.* Sall1 Maintains Nephron Progenitors and Nascent Nephrons by Acting as Both an Activator and a Repressor. *J. Am. Soc. Nephrol.* **25**, 2584–2595 (2014).
16. Magella, B. *et al.* Cross-platform single cell analysis of kidney development shows stromal cells express Gdnf. *Dev. Biol.* **434**, 36–47 (2017).
17. Rosselot, C. *et al.* Non-cell-autonomous retinoid signaling is crucial for renal development. *Development* **137**, 283–292 (2010).
18. Bagherie-Lachidan, M. *et al.* Stromal Fat4 acts non-autonomously with Dachsous1/2 to restrict the nephron progenitor pool. *Development* **142**, 2564–2573 (2015).
19. Mao, Y., Francis-West, P. & Irvine, K. D. A Fat4-Dchs1 signal between stromal and cap mesenchyme cells influences nephrogenesis and ureteric bud branching. *Development* **142**, 2574–2585 (2015).
20. Yu, J. *et al.* A Wnt7b-dependent pathway regulates the orientation of epithelial cell division and establishes the cortico-medullary axis of the mammalian kidney. *Development* **136**, 161–171 (2009).
21. Mugford, J. W., Sipilä, P., McMahon, J. a & McMahon, A. P. Osr1 expression demarcates a multi-potent population of intermediate mesoderm that undergoes

progressive restriction to an Osr1-dependent nephron progenitor compartment within the mammalian kidney. *Dev Biol* **324**, 88–98 (2008).

22. Taguchi, A. *et al.* 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 (2014).
23. Taguchi, A. & Nishinakamura, R. Nephron reconstitution from pluripotent stem cells. *Kidney Int* **87**, 894–900 (2015).
24. Takasato, M. *et al.* Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney. *Nat Cell Biol* **16**, 118–126 (2014).
25. Takasato, M. *et al.* Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature* **526**, 564–568 (2015).
26. Morizane, R. *et al.* Nephron organoids derived from human pluripotent stem cells model kidney development and injury. *Nat. Biotechnol.* **33**, 1193–1200 (2015).
27. Freedman, B. S. *et al.* Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. *Nat Commun* **6**, 8715 (2015).
28. Przepiorski, A. *et al.* A Simple Bioreactor-Based Method to Generate Kidney Organoids from Pluripotent Stem Cells. *Stem Cell Reports* **11**, 1–15 (2018).
29. Morizane, R. & Bonventre, J. V. Kidney Organoids: A Translational Journey. *Trends Mol. Med.* **23**, 246–263 (2017).
30. Little, M. H., Kumar, S. V. & Forbes, T. Recapitulating kidney development: Progress and challenges. *Semin. Cell Dev. Biol.* in press (2018). doi:10.1016/j.semcdb.2018.08.015
31. Phipson, B. *et al.* Evaluation of variability in human kidney organoids. *Nat. Methods* **16**, 79–87 (2019).
32. Taguchi, A. & Nishinakamura, R. Higher-Order Kidney Organogenesis from Pluripotent Stem Cells. *Cell Stem Cell* **21**, 730–746 (2017).
33. Volpato, V. *et al.* Reproducibility of Molecular Phenotypes after Long-Term Differentiation to Human iPSC-Derived Neurons: A Multi-Site Omics Study. *Stem Cell Reports* **11**, 897–911 (2018).
34. Potter, S. S. Single-cell RNA sequencing for the study of development, physiology and disease. *Nat. Rev. Nephrol.* **14**, 479–492 (2018).

35. Wu, H. *et al.* Comparative Analysis and Refinement of Human PSC-Derived Kidney Organoid Differentiation with Single-Cell Transcriptomics. *Cell Stem Cell* **23**, 869-881.e8 (2018).
36. Combes, A. N., Zappia, L., Er, P. X., Oshlack, A. & Little, M. H. Single-cell analysis reveals congruence between kidney organoids and human fetal kidney. *Genome Med.* **11**, 3 (2019).
37. Lindström, N. O. *et al.* 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 (2018).
38. Lindström, N. O. *et al.* 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 (2018).
39. Wang, P. *et al.* Dissecting the Global Dynamic Molecular Profiles of Human Fetal Kidney Development by Single-Cell RNA Sequencing. *Cell Rep.* **24**, 3554-3567.e3 (2018).
40. Menon, R. *et al.* Single-cell analysis of progenitor cell dynamics and lineage specification in the human fetal kidney. *Development* **145**, dev164038 (2018).
41. Miyoshi, T., Hiratsuka, K., Garcia Saiz, E. & Morizane, R. Kidney Organoids in Translational Medicine: Disease Modeling and Regenerative Medicine. *Dev. Dyn.* 1–12 (2019). doi:10.1002/dvdy.22
42. Wu, H., Kirita, Y., Donnelly, E. L. & Humphreys, B. D. Advantages of Single-Nucleus over Single-Cell RNA Sequencing of Adult Kidney: Rare Cell Types and Novel Cell States Revealed in Fibrosis. *J. Am. Soc. Nephrol.* ASN.2018090912 (2018). doi:10.1681/ASN.2018090912
43. Tanigawa, S. *et al.* Organoids from Nephrotic Disease-Derived iPSCs Identify Impaired NEPHRIN Localization and Slit Diaphragm Formation in Kidney Podocytes. *Stem Cell Reports* **11**, 727–740 (2018).
44. Hale, L. J. *et al.* 3D organoid-derived human glomeruli for personalised podocyte disease modelling and drug screening. *Nat. Commun.* **9**, 5167 (2018).
45. Forbes, T. A. *et al.* Patient-iPSC-Derived Kidney Organoids Show Functional Validation of a Ciliopathic Renal Phenotype and Reveal Underlying Pathogenetic Mechanisms. *Am. J. Hum. Genet.* **102**, 1–16 (2018).
46. Kim, Y. K. *et al.* Gene-edited Human Kidney Organoids Reveal Mechanisms of Disease in Podocyte Development. *Stem Cells* **35**, 2366–2378 (2017).

47. Cruz, N. M. *et al.* Organoid cystogenesis reveals a critical role of microenvironment in human polycystic kidney disease. *Nat. Mater.* **16**, 1112–1119 (2017).
48. Pei, Y. A 'two-hit' model of cystogenesis in autosomal dominant polycystic kidney disease? *Trends Mol. Med.* **7**, 151–156 (2001).
49. Czerniecki, S. M. *et al.* High-Throughput Screening Enhances Kidney Organoid Differentiation from Human Pluripotent Stem Cells and Enables Automated Multidimensional Phenotyping. *Cell Stem Cell* **22**, 929-940.e4 (2018).
50. Lackland, D. T., Bendall, H. E., Osmond, C., Egan, B. M. & Barker, D. J. P. Low Birth Weights Contribute to the High Rates of Early-Onset Chronic Renal Failure in the Southeastern United States. *Arch. Intern. Med.* **160**, 1472 (2000).
51. Karner, C. M. *et al.* Canonical Wnt9b signaling balances progenitor cell expansion and differentiation during kidney development. *Development* **138**, 1247–1257 (2011).
52. Blank, U., Brown, A., Adams, D. C., Karolak, M. J. & Oxburgh, L. BMP7 promotes proliferation of nephron progenitor cells via a JNK-dependent mechanism. *Development* **136**, 3557–3566 (2009).
53. Brown, A. *et al.* Role for compartmentalization in nephron progenitor differentiation. *Proc Natl Acad Sci USA* **110**, 4641–4645 (2013).
54. Brown, A. C., Muthukrishnan, S. D. & Oxburgh, L. A synthetic niche for nephron progenitor cells. *Dev Cell* **34**, 229–241 (2015).
55. Tanigawa, S., Taguchi, A., Sharma, N., Perantoni, A. O. & Nishinakamura, R. Selective In Vitro Propagation of Nephron Progenitors Derived from Embryos and Pluripotent Stem Cells. *Cell Rep* **15**, 801–813 (2016).
56. Li, Z. *et al.* 3D Culture Supports Long-Term Expansion of Mouse and Human Nephrogenic Progenitors. *Cell Stem Cell* **19**, 516–529 (2016).
57. Chen, S. *et al.* Intrinsic age-dependent changes and cell-cell contacts regulate nephron progenitor lifespan. *Dev Cell* **35**, 49–62 (2015).
58. Mundel, P. *et al.* Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines. *Exp Cell Res* **236**, 248–258 (1997).

59. Saleem, M. A. *et al.* A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J. Am. Soc. Nephrol.* **13**, 630–8 (2002).
60. Chittiprol, S., Chen, P., Petrovic-Djergovic, D., Eichler, T. & Ransom, R. F. Marker expression, behaviors, and responses vary in different lines of conditionally immortalized cultured podocytes. *Am J Physiol Ren. Physiol* **301**, F660–F671 (2011).
61. Song, B. *et al.* The Directed Differentiation of Human iPS Cells into Kidney Podocytes. *PLoS One* **7**, e46453 (2012).
62. Ciampi, O. *et al.* Generation of functional podocytes from human induced pluripotent stem cells. *Stem Cell Res.* **17**, 130–139 (2016).
63. Musah, S. *et al.* Mature induced-pluripotent-stem-cell-derived human podocytes reconstitute kidney glomerular-capillary-wall function on a chip. *Nat. Biomed. Eng.* **1**, 0069 (2017).
64. Yoshimura, Y. *et al.* Manipulation of Nephron-Patterning Signals Enables Selective Induction of Podocytes from Human Pluripotent Stem Cells. *J. Am. Soc. Nephrol.* **30**, 304–321 (2019).
65. Sharmin, S. *et al.* Human Induced Pluripotent Stem Cell-Derived Podocytes Mature into Vascularized Glomeruli upon Experimental Transplantation. *J. Am. Soc. Nephrol.* **27**, 1778–1791 (2016).
66. Unbekandt, M. & Davies, J. A. Dissociation of embryonic kidneys followed by reaggregation allows the formation of renal tissues. *Kidney Int.* **77**, 407–416 (2010).
67. Ganeva, V., Unbekandt, M. & Davies, J. a. An improved kidney dissociation and reaggregation culture system results in nephrons arranged organotypically around a single collecting duct system. *Organogenesis* **7**, 83–87 (2011).
68. Kaku, Y. *et al.* PAX2 is dispensable for in vitro nephron formation from human induced pluripotent stem cells. *Sci Rep* **7**, 4554 (2017).
69. Soofi, A., Levitan, I. & Dressler, G. R. Two novel EGFP insertion alleles reveal unique aspects of Pax2 function in embryonic and adult kidneys. *Dev Biol* **365**, 241–250 (2012).
70. Naiman, N. *et al.* Repression of Interstitial Identity in Nephron Progenitor Cells by Pax2 Establishes the Nephron-Interstitium Boundary during Kidney Development. *Dev. Cell* **41**, 349-365.e3 (2017).

71. Humphreys, B. D. *et al.* Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. *Am J Pathol* **176**, 85–97 (2010).
72. Kobayashi, A. *et al.* Identification of a Multipotent Self-Renewing Stromal Progenitor Population during Mammalian Kidney Organogenesis. *Stem Cell Reports* **3**, 650–662 (2014).
73. Lemos, D. R. *et al.* Interleukin-1 β Activates a MYC-Dependent Metabolic Switch in Kidney Stromal Cells Necessary for Progressive Tubulointerstitial Fibrosis. *J. Am. Soc. Nephrol.* **29**, 1690–1705 (2018).
74. Combes, A. N. *et al.* Single cell analysis of the developing mouse kidney provides deeper insight into marker gene expression and ligand-receptor crosstalk. *Development* in press (2019). doi:10.1242/dev.178673
75. Yokote, S. *et al.* Urine excretion strategy for stem cell-generated embryonic kidneys. *Proc. Natl. Acad. Sci. USA* **112**, 12980–12985 (2015).
76. Serluca, F. C., Drummond, I. A. & Fishman, M. C. Endothelial Signaling in Kidney Morphogenesis. *Curr. Biol.* **12**, 492–497 (2002).
77. Homan, K. A. *et al.* Flow-enhanced vascularization and maturation of kidney organoids in vitro. *Nat. Methods* (2019). doi:10.1038/s41592-019-0325-y
78. van den Berg, C. W. *et al.* 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 (2018).
79. Bantounas, I. *et al.* Generation of Functioning Nephrons by Implanting Human Pluripotent Stem Cell-Derived Kidney Progenitors. *Stem Cell Reports* **10**, 766–779 (2018).
80. Murakami, Y. *et al.* Reconstitution of the embryonic kidney identifies a donor cell contribution to the renal vasculature upon transplantation. *Sci. Rep.* **9**, 1172 (2019).
81. Munro, D. A. D., Hohenstein, P. & Davies, J. A. Cycles of vascular plexus formation within the nephrogenic zone of the developing mouse kidney. *Sci. Rep.* **7**, 3273 (2017).
82. Munro, D. A. D. & Davies, J. A. Vascularizing the Kidney in the Embryo and Organoid: Questioning Assumptions about Renal Vasculogenesis. *J. Am. Soc. Nephrol.* **29**, 1593–1595 (2018).
83. Goto, T. *et al.* Generation of pluripotent stem cell-derived mouse kidneys in Sall1-targeted anephric rats. *Nat. Commun.* **10**, 451 (2019).

84. Yamaguchi, T. *et al.* Interspecies organogenesis generates autologous functional islets. *Nature* **542**, 191–196 (2017).
85. Wu, J. *et al.* Interspecies Chimerism with Mammalian Pluripotent Stem Cells. *Cell* **168**, 473–486 (2017).
86. Yamanaka, S. *et al.* Generation of interspecies limited chimeric nephrons using a conditional nephron progenitor cell replacement system. *Nat. Commun.* **8**, 1719 (2017).
87. Howden, S. E., Vanslambrouck, J. M., Wilson, S. B., Tan, K. S. & Little, M. H. Reporter-based fate mapping in human kidney organoids confirms nephron lineage relationships and reveals synchronous nephron formation. *EMBO Rep.* e47483 (2019). doi:10.15252/embr.201847483

Acknowledgements

I thank Atsuhiro Taguchi, Shunsuke Tanigawa**,** and all the other members of Nishinakamura laboratory for their contributions to the establishment of kidney organoid protocols and for helpful discussions.

Competing interests

The author declares no competing interests.

Key points

- Kidney organoids are useful for modelling early onset diseases that affect glomeruli and renal tubules

- Better understanding of the gene expression changes that occur at a single cell level during human embryonic kidney is necessary to guide further maturation

of kidney organoids; technologies such as single-cell RNA-sequencing represent powerful tools for this purpose

- The induction of branching ureteric buds can be achieved using a protocol that differs from that used for the induction of nephron progenitor cells

- Notable challenges remain to the use of organoids for regenerative medicine, including approaches fort the generation of higher-order structures, organoid maturation, vascularisation, and single-ureter formation

Figure 1 | Interactions between the nephron progenitor, ureteric bud and stromal lineages during kidney development.
The left panels correspond to embryonic day (E)11.5 kidneys in mice, while the right panel to E15.5. $SIX2^+$ nephron progenitor cells (NPCs) in the metanephric mesenchyme (MM) secrete glial cell line-derived neurotrophic factor (GDNF), which acts on its receptor RET in the ureteric bud (UB) epithelia to trigger UB invasion and branching. The UB secrets WNT9B, which stimulates some NPCs to differentiate into cells of nascent nephrons, including glomerular podocytes, epithelial cells of the Bowman's capsule, and renal tubules (not shown accurately in the right panel). Other NPCs remain undifferentiated by the action of FGF9 derived from UBs and NPCs and FGF20 from NPCs. The MM also contains the $FOXD1^+$ stromal progenitor cells. Like NPCs, cortical stromal cells also secrete GDNF to stimulate UB branching. They also produce retinoic acid (RA), which upregulates expression of RET in the UBs. FAT4, which is expressed by cortical stromal cells acts on DCHS1 on NPCs to restrict their excessive expansion. Development of the medullary stromal cells requires WNT7B signalling from the UBs.

Figure 2 | Distinct developmental paths of nephron progenitor cells and the ureteric bud during mouse kidney development *in vivo* and *in vitro*. The origins of nephron progenitor cells (NPCs) and cells of the ureteric bud (UB) are both spatially

and temporally distinct. NPCs are derived from the posterior nascent mesoderm (formed at E8.5) and posterior intermediate mesoderm (formed at E9.5) *in vivo*, whereas cells of the UB lineage are derived from anterior intermediate mesoderm (formed at E8.5)[22]. The signalling steps required for the induction, specification and commitment of these two lineages as determined by findings from *in vitro* experiments are shown[32]. Some steps seem to overlap between the two lineages (shown in the same colours) but the two pathways are clearly distinct. Our protocol for the induction of UB differed from the NPC induction protocol from the initial steps, suggesting that these two lineages may segregate at very early stages of development *in vivo*. +, ++ and +++: strength of Wnt signalling

Figure 3 |Disease modelling using human induced pluripotent stem cells. Glomerular podocyte abnormalities can be recapitulated in kidney organoids either through the use of patient-derived induced pluripotent stem cells (iPSCs) to generate nephron progeitor cells (NPCs) or through the use of gene editing to introduce mutations into the organoids. a | Kidney organoids have been generated from induced pluripotent stem cells iPSCs from a patient with Finnish-type congenital nephrotic syndrome[43], a disease caused by mutations in NPHS, which encodes the slit diaphragm (SD) protein NEPHRIN. These patient-derived kidney organoids failed to form SD precursors and demonstrated retention of mutant NEPHRIN within the cytoplasm of podocytes. Genetic correction of the point mutation by gene editing restored NEPHRIN localization on the basolateral domains of the podocytes. b | Kidney organoids established from iPSCs from a patient with nephronophthisis caused by mutations in intraflagellar transport protein 140 exhibited renal tubule epithelia with shortened primary cilia and impaired apicobasal polarity[45]. Use of gene editing to correct the mutation rescued these phenotypes. c | Use of CRISPR/Cas9 technology has been used to generate iPSC-derived kidney organoids containing mutations in the genes encoding polycystin (PC) 1, or polycystin (PC) 2, as a model of autosomal dominant polycystic kidney disease. Proximal tubule cells within organoids lacking functional PC2 formed large cysts [47].

Figure 4 | An approach to generate higher order structure of the kidney *in vitro*. Most available protocols for the generation of kidney organoids generate cells of the nephron progenitor cell (NPC) lineage (that is, podocytes, cells of the Bowman's

capsule and renal tubule epithelia) but do not adequately generate cells of the ureteric bud (UB) lineage (that is, collecting ducts and ureters). a | We have established a protocol that enables successful generation of NPCs and UBs from mouse embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs). By combining NPCs and UBs generated from mouse ESCs with mouse embryo-derived stromal cells, we can generate kidney organoids that show robust UB branching and differentiated nephrons located at the periphery Thus differential induction of each lineage, followed by combined culture, is an effective strategy to construct a higher-order structure of the kidney within an organoid. A drawback of this strategy is that it requires mouse embryo-derived stromal cells [32].We do not yet have a suitable protocol for the induction of stromal cells from human iPSCs, which would be needed to generate a similar higher-order structure of the human kidney. In addition, the formation of a single ureter and integration of the vasculature is still required in order to generate a functional artificial kidney. b | Section of a mouse kidney organoid generated using this protocol and stained with SIX2 (magenta, to identify NPCs), CYTOKERATIN 8 (red, to identify the UB), and E-CADHERIN (green, to identify renal tubules and UB).

Figure 5 | Strategies for the reconstruction of kidneys in large animals. a | Blastocyst complementation involves injection of pluripotent stem cells (PSCs) into blastocysts that have been manipulated to lack a kidney, so that PSCs contribute dominantly to the developing kidney *in vivo*. However, it should be noted that residual pig cells will cause hyperacute rejection; thus, all nephron progenitor cells (NPCs), cells of the ureteric bud (UB) lineage, stromal cells, and endothelial cells must be replaced with human cells. It is also unknown whether the generation of interspecies human–pig chimeras is possible. Finally, the induction of human PSCs into blastocysts might result in the distribution of human cells not only to the kidney but also to other organs, with potential off-target effects. b | The problem of off-target effects can be minimized by injecting donor cells directly into the developing kidney, rather than the blastocyst. However, injection of human induced (i)PSC-derived NPCs would result in the generation of chimeric kidneys containing some pig-derived tissues (for example, UBs, stromal cells, and endothelial cells (ECs)). Whether injection of a population of mixed donor cells comprising many lineages (that is,

NPCs, UBs, stromal cells, and ECs) to a completely vacant niche could result in the construction of the entire complex kidney in animals is also unclear.

Table 1 | Methods for the induction of kidney organoids

Step		Protocol					
		Taguchi 2014[22]	Morizane 2015[26]	Freedman 2015[27]	Takasato 2015[25]		Taguchi 2017[32]
Duration of time in culture until formation of NPCs		13-14 days	9 days	n.d.	10 days		12.5 days[a]
Pre-CHIR step		(+) Activin low	(-)	(+)	(-)		(+) Activin high +BMP
Duration of CHIR step		6 days	4 days (+/-noggin)	1.5 days	4 days		1.5 days
Duration between CHIR step and formation of NPCs		2 steps	2 steps	n.d.	1 step		5 steps[b]
Cell types in the organoids	Glomerulus and/or renal tubule	(+)	(+)	(+)	(+)		(-)
	stroma	n.d.	(+)	(+)	(+)		n.d.
	endothelial cell	n.d.	(+)	(+)	(+)		n.d.
	ureteric bud	(-)	(-)	(-)	(+/-)		(+)
Disease modelling or knockout		NEPHRIN PAX2		PODXL PC1, /PC2	NEPHRIN IFT140		PAX2

[a] Duration of time in culture until formation of the ureteric bud. [b] Duration of time between CHIR step and formation of the UB.

n.d.: not determined; PODXL, PODOCALYXIN; NPCs, nephron progenitor cells.

(+), (-) and (+/-) : presence or absence of steps or structures.

Glossary

Induced pluripotent stem cells
(iPSCs)which is generated by forced expression of several transcription factors in somatic cells and can differentiate into a variety of cell types.

Nephron progenitor
A population in the embryonic kidney that can differentiate into glomerular podocyte, Bowman's capsule, renal tubule, and loop of Henle.

Metanephric mesenchyme
A population of cells accumulated around the ureteric bud tips. It contains nephron progenitors and stromal progenitors.

Ureteric bud: a population of cells in the embryonic kidney that undergoes extensive branching and differentiates into collecting ducts and ureters.

Bowman's capsule
An epithelial sac surrounding the glomerulus. A structure consisting of Bowman's capsule and a glomerulus is referred to as a renal corpuscle.

Mesonephros
The embryonic kidney that develops earlier and more anteriorly than the metanephros. After forming Wolffian ducts, most parts of mesonephros degenerate during development.

Metanephros
The embryonic kidney that appears last and develops into the permanent kidney.

Wolffian duct (mesonephric duct)

The epithelial duct of the mesonephros that elongates in an anterior-to-posterior direction. A portion close to the posterior end sprouts to form the ureteric bud.

Primitive streak
An elongated furrow formed along the axis of gastrulation-stage embryos. Mesodermal and endodermal cells are generated from the primitive streak.

Renal coloboma syndrome
A condition that presents kidney and eye abnormalities. It is mainly caused by PAX2 mutations.

Angiogenesis
The branching of existing vessels

vasculogenesis
The *de novo* formation of vessels from mesodermal precursors