Current Transplantation Reports (2023) 10:29–39
https://doi.org/10.1007/s40472-023-00397-2

Engraftment of Kidney Organoids In Vivo

Benjamin S. Freedman[1,2] · Benjamin Dekel[3]

Accepted: 5 April 2023 / Published online: 25 April 2023

Abstract

Purpose of Review Kidney organoids are heterocellular structures grown in vitro that resemble nephrons. Organoids contain diverse cell types, including podocytes, proximal tubules, and distal tubules in contiguous segments, patterned along a proximal-to-distal axis. Human organoids are being explored for their potential as regenerative grafts, as an alternative to allograft transplants and hemodialysis. Earlier work, analyzing grafts of developing human kidney tissue and whole human embryonic kidney rudiments, serves as a baseline for organoid implantation experiments.

Recent Findings When transplanted into immunodeficient mice beneath the kidney capsule, kidney organoid xenografts can form vascularized, glomerulus-like structures, which exhibit a degree of filtration function. However, the absence of an appropriate collecting duct outlet and the presence of abundant stromal-like cells limits the functionality of such grafts and raises safety concerns. Recently, ureteric-like organoids have also been generated, which extend projections that resemble collecting ducts.

Summary Combining nephron-like and ureteric-like organoids, along with renal stromal cells, may provide a path towards more functional grafts.

Keywords Autologous · iPS cells · Pluripotent stem cells · Mini-organ · Organ-on-chip

Introduction

Renal replacement therapy, which is the only known therapy for end-stage renal disease, constitutes either dialysis or kidney transplant [1]. Both of these therapies are decades old and have substantial side effects. Dialysis patients have higher mortality rates and lower quality of life, compared to the general population [2]. Kidney transplant is considered the gold standard for therapy and is generally associated with better outcomes. However, donor organs are in short supply, thus patients typically spend years on a waiting list before one becomes available. The scarcity of these organs also dictates that not all patients who need a transplant will be eligible to receive one. The Advancing American Kidney Health Initiative, launched in 2019, sets as a goal to double the number of kidney transplants available by 2030. Beyond supply limitations, it is important to recognize even successful transplants require immunosuppressive medications that can have substantial side effects, including an increased risk of infection or tumorigenesis. The COVID-19 pandemic illustrates this, as many transplant recipients remain relatively confined at home for fear of contracting severe illness or losing their precious graft. Ultimately, even "perfect match" kidney transplants are eventually rejected by the body and need to be replaced.

As an alternative to allograft transplants and hemodialysis, renal organoids are being explored for their potential as regenerative therapeutics for the kidneys. Such organoids could be produced on-demand, which would enable a greater number of patients to receive grafts, and in a more timely manner. An organoid graft could conceivably be derived from the patient's own cells, which would eliminate the need for immunosuppressive medication, and would be a permanent graft that does not become rejected over time.

✉ Benjamin S. Freedman
benof@uw.edu

1 Division of Nephrology, Kidney Research Institute, and Institute for Stem Cell and Regenerative Medicine, Departments of Medicine, Pathology (Adjunct), and Bioengineering (Adjunct), University of Washington School of Medicine, Seattle, WA, USA

2 Plurexa LLC, Seattle, WA, USA

3 Division of Pediatric Nephrology and the Pediatric Stem Cell Research Institute, Sagol Center for Regenerative Medicine, Sheba Medical Center, School of Medicine, Tel Aviv University, Tel Aviv-Yafo, Israel

However, there are a range of concerns regarding this therapeutic approach, which must be addressed in a comprehensive manner before it can be tested clinically. There are also a wide range of options that need to be explored, in order to best prepare the technology for success.

Definitions of Kidney Organoids

Kidney organoids are heterocellular structures grown in vitro that resemble nephrons. For the purposes of this review, we will focus on kidney organoids that have been derived from pluripotent stem cells, which includes both embryonic stem cells and induced pluripotent stem (iPS) cells. The hallmark of these differentiated kidney organoids is the presence of cells resembling podocytes, proximal tubules, and distal tubules in contiguous segments patterned along a proximal-to-distal axis (Fig. 1) [3, 4, 5, 6]. Both mouse and human kidney organoids have been described and appear to be relatively similar in their general patterning and composition [5].

A consensus definition of what constitutes a kidney organoid is not yet established in the field. In most cases, identification of organoids is based upon the presence of gene expression markers or labels, such as nephrin in podocytes, lotus lectin in proximal tubules, and E-cadherin in distal tubules, in morphologies and patterns suggestive of nephron lineage [3, 4, 5, 6]. Single-cell RNA-sequencing may also be utilized, and the structures may be compared to actual tissue from kidneys in vivo to further bolster the identification. Whether these descriptive measures are sufficient, and how to quantify them, remain open questions. "Organoids" are not the same as "tissues" and differences exist between cell types in organoids and their counterparts in vivo, which can be demonstrated in side-to-side comparisons [7, 8].

Functional criteria for kidney organoids are not yet well defined and physiological activities in these structures remain rather primitive, compared to actual kidneys. Organoids lack perfusion but are capable of accumulating a variety of compounds, which can be visualized by live fluorescence. Organoid tubules are also capable of swelling in response to secretagogue stimulation. Such assays are providing new ways to study renal physiology [9•]. How exactly these behaviors in cell culture correlate with actual nephron functions in vivo, however, is not yet clear. Essential functions such as glomerular filtration and perfusion of filtrate into nephron tubules are absent in kidney organoids in vitro. Recent work has therefore investigated whether implantation of organoids into immunodeficient host animals might provide a context in which to elicit these important functionalities.

Renal Subcapsular Implantation of Kidney Organoids

Several different groups have succeeded in implanting organoids beneath the kidney capsule of immunodeficient mice [10, 11, 12, 13]. A consistent finding amongst these groups is the emergence of more sophisticated, glomerulus-like structures within the grafts (Table 1) [12, 13]. In these experiments, capillaries arise within aggregates of peripheral podocyte-like cells, producing bouquet-like structures that resemble capillary loop stage glomeruli in developing human kidneys. This is a striking finding that has no consistent parallel in kidney organoid cultures in vitro, where interactions between the organoid podocytes and endothelial cells, which arise as an off-target population in pluripotent stem cell-derived organoid cultures, are only occasionally observed and lack the specificity observed in grafts in vivo [3, 8].

The bulk of the endothelium within kidney organoid subcapsular grafts appears to derive from the host mouse kidney microvascular endothelium, which extends into the graft from the underlying renal cortex. This raises a number of questions regarding how this process works. For instance,

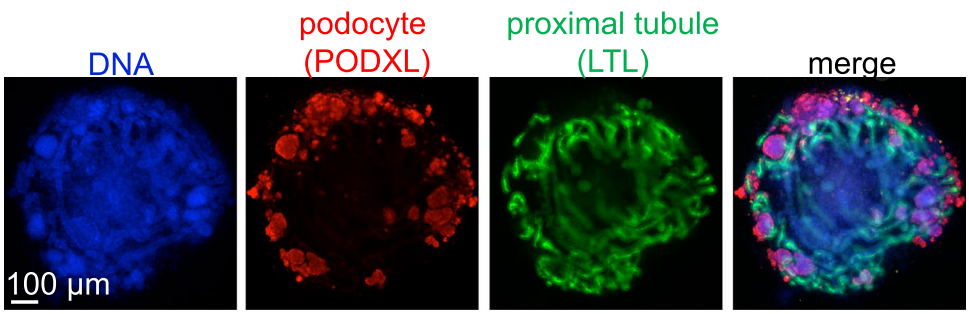

Fig. 1 Nephron-like structures grown in vitro. Wide-field immunofluorescence micrographs of nephron markers in a human kidney organoid derived from pluripotent stem cells. Clusters of podocytes form at the periphery. Elongated proximal tubules forming beneath them. The core contains distal tubular structures. From Freedman et al., *Nature Communications,* 2015

Table 1 Selected studies of kidney organoid transplantation in mice

First/last author	Freedman	Sharmin	van den Berg	Nam	Gupta	Bantounas	Tanigawa
Year	2015	2015	2018	2019	2019	2020	2022
Host mice	NOD-SCID	NOD-SCID/JAK3null	NOD-SCID	NOD-SCID	NOD-SCID	SCID/*beige*	NOD-SCID
Host age	Neonate	Adult	Adult	Adult	Adult	Adult	Adult
Implanted cell origin	Human PSC	Human PSC + EC + MSC	Human PSC	Human PSC	Human PSC	Human PSC	Mouse PSC
Diff. Protocol	Freedman	Taguchi	Takasato	Freedman	Morizane	Takasato	Taguchi
Graft site	Cortex	Capsule	Capsule	Capsule	Capsule	Subcutaneous	Capsule
Days in vivo	21	20	7–28	7–42	21	84	14
Complex epithelia	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Prox. tubules (LTL^+)	Yes	Yes	Yes	Yes	Yes	ND	Yes
Glomeruli	ND	Yes	Yes	Yes	Yes	ND	Yes
Stroma	ND	Yes	Yes	Yes	Yes	Yes	Yes
Cartilage	ND	ND	Yes	Yes	ND	Yes	ND
Functional asssay	ND	ND	i.v. dextran	ND	i.v. dextran, μ-CT	i.v. dextran	ND

do the endothelial cells that contribute to glomerulus-like structures derive from glomerular endothelial cells, or peritubular endothelial cells? If the latter, which would appear to be more likely given the mouse kidney cortical geometry, then this would indicate an innate ability of the kidney microvasculature to adapt to different segments of the nephron. What are the cues that attract endothelial cells into the podocytes specifically, to form glomerulus-like structures? Are there also peritubular-like structures within these grafts with specific interactions? None of these questions has yet been answered, thus this remains a fertile area of research.

The extent to which these glomerulus-like structures contain functional glomerular basement membranes requires significant study. The intimate interactions between podocytes and endothelial cells in these grafts, in which thin layers of podocytes and endothelial cells form on opposite sides of a single basement membrane, generally resembles the architecture of a glomerular basement membrane in vivo. At the ultrastructural level, it is not yet clear whether the basement membranes that form in these structures are truly reminiscent of the glomerular basement membrane. Glomerulus-like structures derived from a patient with congenital nephrotic syndrome associated with a missense mutation in *NPHS1* showed mislocalization of nephrin protein, and ultrastructural deficiencies in slit-diaphragm like structures as assessed by transmission electron microscopy [14]. Another study, in which grafts were produced subcutaneously rather than beneath the kidney capsule, observed some structures with more mature, trilaminar glomerular basement membranes, while other structures showed membranes with two lamina rarae suggesting an immature state [15].

Red blood cells have been observed inside the blood vessels of glomerulus-like structures by electron microscopy, indicating they are connected to the blood supply, and suggestive of filtration [10, 13]. A series of studies has demonstrated the tendency of lower molecular weight dextrans to pass through glomerulus-like structures, while larger molecular weight dextrans are retained. These filtration events can be visualized using an abdominal window, which was installed in the mice such that kidneys can be monitored (Fig. 2A) [12]. Systemically injected fluorescent dextran molecules rapidly appeared inside the branched capillaries of glomerulus-like structures, indicating these were connected to the host blood supply (Fig. 2B). Electron microscopy suggested maturation of both podocytes and tubular structures after transplantation [12]. Smaller dextrans (10 kDa) penetrated compartments of the organoid graft that larger dextrans (70 kDa) could not [16]. These experiments suggest that glomerulus-like structures in organoid grafts in vivo can form functional filtration slits. As leakage of endothelial vessel structures is also a possible interpretation, further work is required to determine the precise role of podocytes in size-selective dextran accumulation [17].

Despite the evidence for filtration, the ability of the podocytes within grafts to form tertiary foot processes, and the underlying endothelial cells to form fenestrae, is not yet clear. These questions require dedicated electron microscopy and possibly newer methods such as super-resolution microscopy to address. It will be important to understand how these structures compare at a quantitative level to native glomeruli, both structurally and functionally. This requires side-by-side comparisons and the development of specialized assays and metrics to measure glomerular ultrastructural features. In the previous experiments studying dextran accumulation, the mouse kidney cortex neighboring the graft appeared superior to the graft itself in filtration and clearance characteristics.

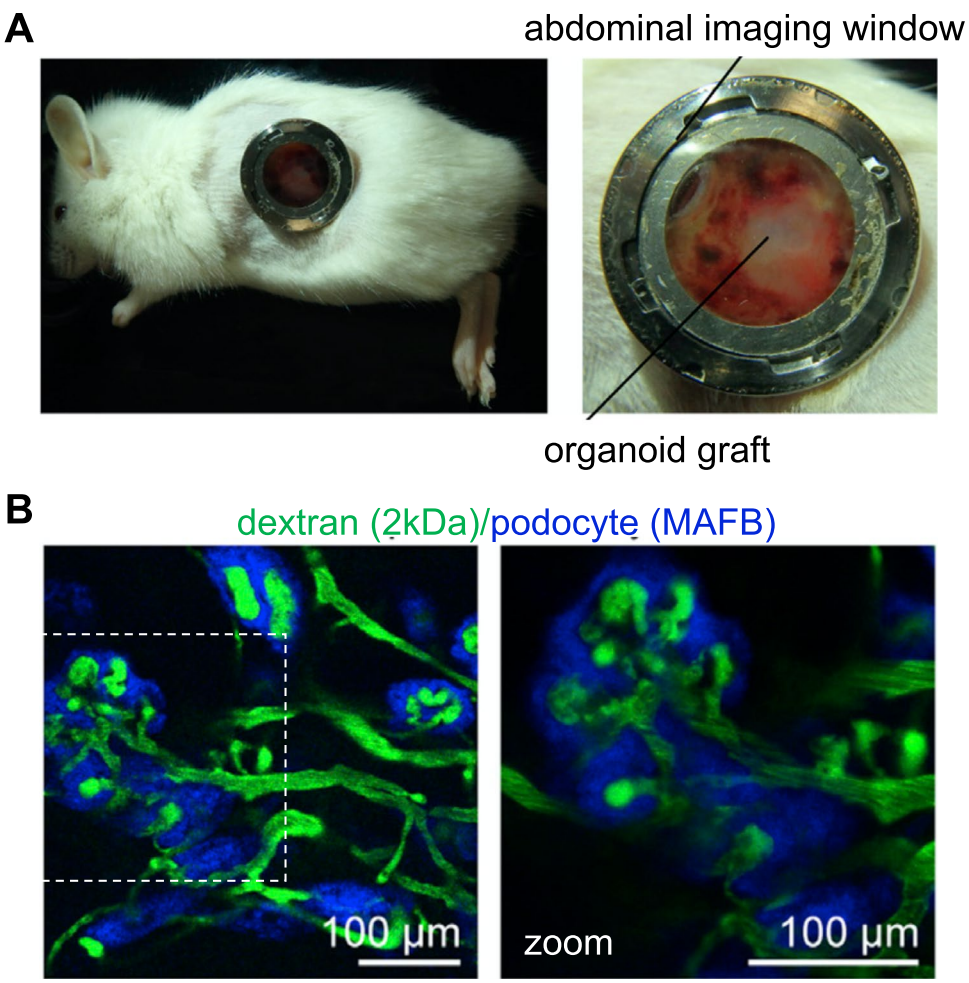

Fig. 2 Vascularized glomerulus-like structures in kidney organoid grafts. **A** Abdominal imaging window used to monitor organoid grafts. **B** Fluorescence images of living grafts seven days after implantation, following injection of a green fluorescent dextran intravenously. From van den Berg et al., *Stem Cell Reports,* 2018

Beyond podocytes and endothelial cells, there are several other prominent cell types in native glomeruli, whose presence in glomerulus-like structures in grafts is less well characterized. Glomerulus-like structures are occasionally surrounded by a thin layer of $CLDN1^{+}PAX8^{+}$ epithelium that resembles parietal epithelial cells (Fig. 3) [10, 13]. Similar parietal epithelial-like cells have also been observed to intermediate between proximal tubules and podocytes in organoids in vitro, but such structures appear collapsed, with little space between podocytes and the surrounding "capsule" [8, 18]. In contrast, in organoid grafts in vivo, an open lumen resembling a Bowman's space is often observed between parietal epithelial cells and podocytes [10, 13]. Other cell types within glomeruli, such as mesangial cells and cells of renin lineage, remain poorly studied in kidney organoid grafts. Proximal tubular structures connected with glomerulus-like structures have also been paid relatively little attention. It is unclear whether these become perfused with filtrate from upstream glomerulus-like structures, and whether a peritubulular vasculature arises alongside them within the grafts, through which the tubules might perform reabsorptive and secretory functions.

Innovation of Ureteric Organoids

The human kidney organoids described above, sometimes referred to as "nephron organoids," lack a collecting duct and ureteric component. This has emerged as a significant limitation of these organoids. During kidney development, the ureteric bud and metanephric mesenchyme undergo iterative inductive interactions. These two stem cell pools sustain each other and promote a regulated balance of differentiation and self-renewal. Ultimately this produces the phenomenon known as branching morphogenesis wherein nephrons form from "T-buds" in an iterative, fractal architecture. In contrast, kidney organoids appear to be derivatives of metanephric mesenchyme in the absence of ureteric bud-derived epithelium. This presents two challenges. First, there is no obvious mechanism for nephron progenitor cells to undergo cycles of inductive self-renewal and differentiation, thus the absence of ureteric bud limits the process of nephrogenesis to a single round. Second, the nephron structures that arise lack collecting ducts, thus there is no ready outlet for urinary filtrate.

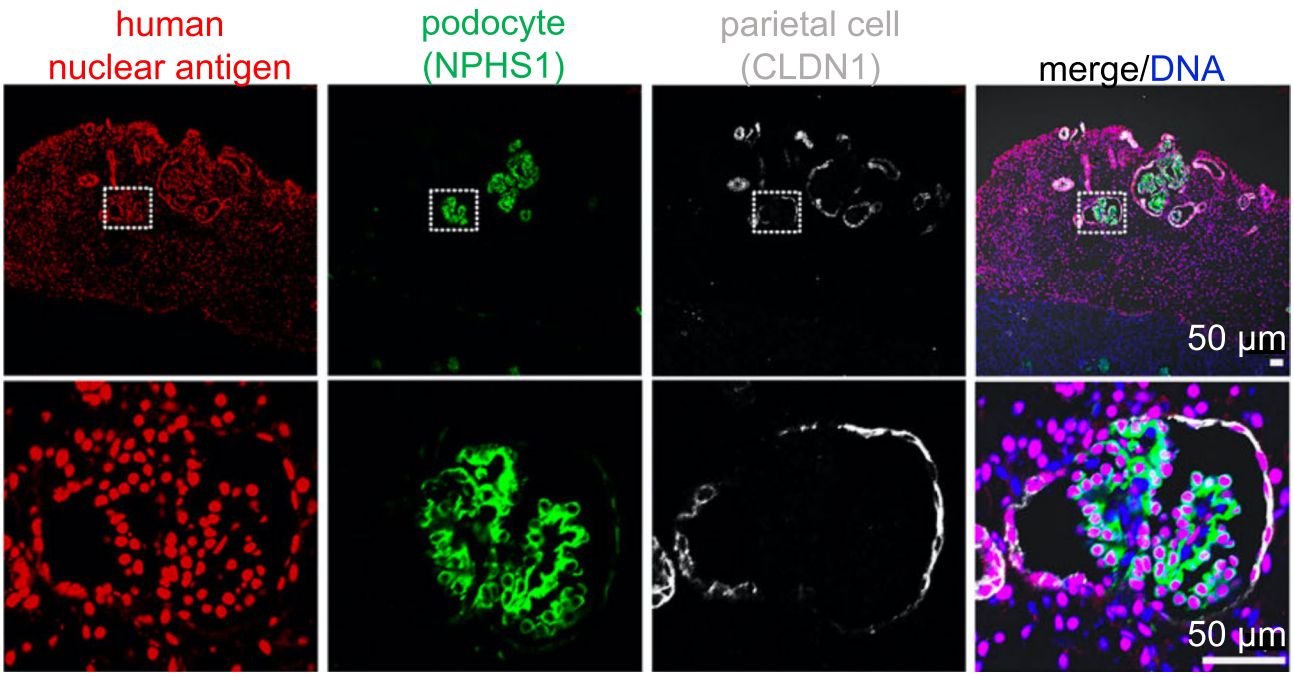

Fig. 3 Organoid grafts contain Bowman's capsule-like structures. Low-magnification (top) and zoomed (bottom) views of human organoid graft beneath the kidney capsule of an immunodeficient mouse host. HNA marks human cells, which are co-localized with markers of podocytes and parietal epithelial cells. From Nam et al., *Exp. Mol. Medicine,* 2019

In theory, introduction of a ureteric compartment into organoid cultures should solve both of these problems. Early claims of ureteric bud differentiation from pluripotent stem cells date back to 2012, but have been partially corrected in the literature [6, 19, 20, 21•, 22]. In recent years, new differentiation protocols have been introduced, describing structures that express markers consistent with ureteric identity, and show tubular extensions in a radial fashion, with RET^{+} tips [23, 24, 25•, 26•]. These ureteric bud-like structures show a propensity to connect up with nephron-like organoid tubules, producing connected segments. However, such structures still appear immature, lacking strong marker expression for principal cells (e.g. aquaporin-2) and intercalated cells. In mouse pluripotent stem cells, the addition of stromal progenitors (derived separately) enhances the growth and survival of ureteric bud-like structures, which when further combined with kidney organoids can produce improved organotypic structures resembling kidney cortex (Fig. 4) [27••].

There remain certain challenges to this approach. First, none of the ureteric bud structures appear to be fully functional in their ability to promote branching morphogenesis, even after implantation in vivo beneath the kidney capsule, thus the resultant structures remain limited in size. Second, the differentiation of the ureteric structures generally appears less mature than bona fide collecting ducts, at least in the absence of a supportive stroma. Third, despite successful connection between the nephron and ureteric structures, there is still no ready way to connect collecting ducts in organoid grafts to the native collecting system of the kidneys. In the absence of drainage, there is nowhere for the filtrate to go, which would result in a hydronephrosis phenotype.

To address the issue of drainage, alternatives to the renal capsule are being explored. In neonatal mice, kidney organoids have been injected directly into the mouse kidney cortex, which is relatively soft at this stage of development. Three weeks after implantation, human proximal tubular structures were identified in the mouse kidneys by immunofluorescence (Fig. 5). This experiment provides proof of principle that human kidney organoid tubules could survive and engraft in vivo within the cortex of the kidneys [3]. Similarly, mouse nephron progenitor cells implanted into neonatal mouse kidneys can produce chimeric nephrons [28]. Whether human organoids implanted at this stage might form functional connections with mouse collecting ducts is not yet known. Notably, the mouse continues to undergo nephrogenesis during the first three days of neonatal life, raising the possibility that human organoids might come into contact with ureteric bud. While this would remain a proof of concept experiment, it would help us understand the potential of human organoids to form fully functional nephrons.

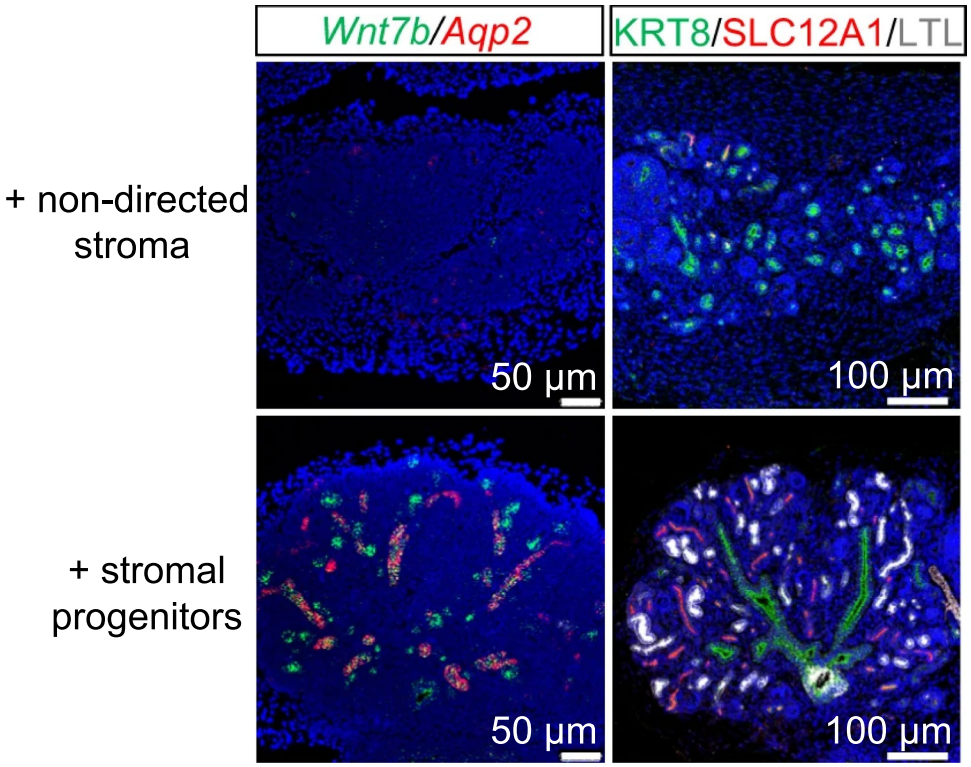

Fig. 4 Stromal progenitors and ureteric bud improve kidney organoid differentiation. Comparative immunofluorescence analysis of nephron markers in mouse kidney organoids differentiated with ureteric bud in vitro, in combination with either non-specific stroma (top) or induced stromal progenitors (bottom). From Tanigawa et al., *Nature Communications,* 2022

Metanephric Grafts—Lessons from History

Organoids resemble human fetal kidney tissues. Therefore, to consider transplantation of organoids, it is valuable to consider earlier experiments engrafting human fetal kidney/metanephric tissue and subsequently whole human and pig embryonic rudiments [29, 30]. Moreover, for the field to progress from organoids to artificial kidneys, it will be important to demonstrate therapeutic proof of concept. In this regard, we may learn lessons from the history of metanephric implants. Metanephroi are embryonic kidney primordia, which include ureteric bud and nephron progenitor cells in arrangements suited to generate mature kidney tissues. Metanephroi are generally better organized and more complex than organoids, thus the extent to which organoids are likely to succeed as grafts may be inferred from metanephroi.

Several papers demonstrate that metanephroi grow substantially after transplantation, during which time they undergo nephrogenesis. When implanted beneath the kidney capsule of immunodeficient mice, metanephroi from pigs or humans readily formed expanding tissue grafts, which in some cases were as large or larger than the native mouse kidneys [30, 31]. Interestingly, earlier metanephroi fare better than later ones in these experiments [30].

Importantly, in the metanephric kidney, epithelial ($Six2^+Cited1^+$), endothelial ($Flk1^+$/Scl/$Tal1^+$), and stromal ($Foxd1^+$) cellular lineages are within strict lineage boundaries and therefore, an in vitro differentiation protocol aimed strictly at enriching $Six2^+Cited1^+$ nephron epithelial progenitors will likely not include endothelial and stromal progenitors required to generate glomerular, endothelial, and mesangial cells [32]. Moreover, complicating the concomitant in vitro derivation of vascular progenitors along with epithelial or stromal counterparts is the question of whether a direct shared precursor exists between these different types of cells within the kidneys [33].

Accordingly, a human pluripotent stem cell–derived cell suspension enriched for nephron progenitors and depleted of endothelial progenitors will pattern into nephrons but will give rise to avascularized glomeruli in vitro, and no donor-derived glomerular vessels will be observed after organoid grafting. In contrast, when using graft material, which harbors all metanephric cell lineages, glomerulogenesis with donor-derived vessels can occur; for instance, grafts of early human and pig metanephric kidneys, prior to glomerular formation, which contain epithelial, stromal, and endothelial progenitors in their niches and that maintain proper progenitor cell interactions, can generate upon transplantation in some instances vascularized glomeruli with donor human capillaries that are connected to mouse vasculature altogether forming chimeric vessels [30, 32].

Thus, as with human organoid grafts, grafted metanephric tissues indeed became vascularized from the host animal, pointing towards an innate ability of differentiating nephron progenitors to recruit vasculature. Nevertheless,

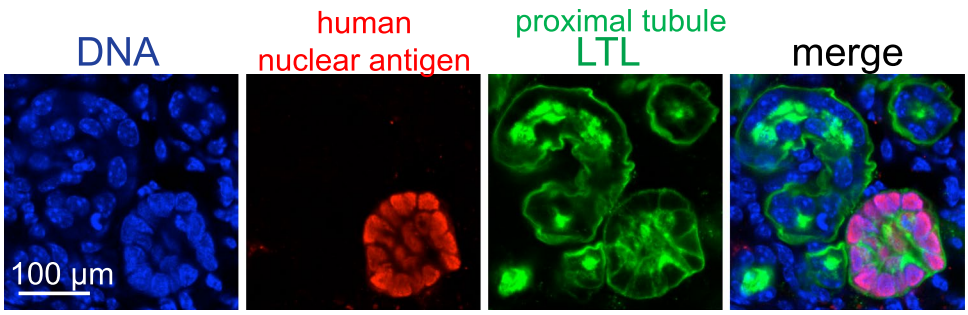

Fig. 5 Neonatal kidney organoid graft. Immunofluorescence of mouse kidney cortex (immunodeficient), 3 weeks after injection of the neonatal (P0) kidney with cells from kidney organoid cultures. From Freedman et al., *Nature Communications,* 2015

glomerulus-like structures within the metanephric grafts can harbor chimeric vessels while those in organoid grafts are host-derived. Means to increase donor-derived vessels and vascularization may be induced by adding to the cell composition vessel-forming cells (namely human mesenchymal stromal cells and endothelial colony-forming cells) as recently shown for adult kidney spheroids [34••, 35]. This is crucial when grafting into chronically injured kidney harboring deranged vasculature [36]. While the results were promising in terms of the capacity of new nephrons to form in adults after transplantation, research into metanephroi as a source of transplantable grafts has largely stalled. Evidence for renal function of transplanted metanephroi remains rather limited as only dilute urine was generated and creatinine clearance levels did not approach those of actual transplants [37].

It is unclear whether further studies in this field might have led to greater improvements and demonstration of potential for renal replacement, or whether the technology itself has fundamental limitations. Of note, kidney organoids remain inferior to metanephroi in their composition and potential for nephrogenesis, as organoids are cell cultures rather than natural organ rudiments. Nevertheless, organoids raise new possibilities for transplantation, which may provide more attractive alternatives to renal replacement therapy than metanephric allografts. Now that organoid exist, it may be worthwhile to revisit experiments involving metanephric implantation, as positive controls and to guide the general approach. In addition, in contrast to metanephroi at the time, we might not think of organoid transplantation as means to increase the nephron mass but rather as an autologous graft that generates paracrine effects that induces in turn kidney repair and regeneration.

Limitations of Kidney Organoids

Organoids derived from pluripotent stem cells are not fully mature or pure renal cultures. A variety of different protocols exist to produce kidney organoids from pluripotent stem cells. Empirical comparisons of these protocols by third party groups has strengthened our understanding of organoids and their limitations [38]. All such kidney organoid differentiation protocols appear to produce similar compositions of cells, although their geometries, relative heterocellularity, and precise gene expression patterns may differ due to culture conditions. Some cultures are adherent, with the organoids forming along a tissue culture plate, while in other cultures the organoids may form at an air–liquid interface or in a pellet. Different geometries may have different utilities, for instance, it is easier to follow organoids over time in adherent cultures. As was noted early on, kidney organoids contain cell types that are not of nephron origin, such as neurons. Such cells are natural "off target" products of pluripotent stem cell differentiation [3, 22, 39].

The cell types within kidney organoids are not fully complete or mature. Collecting ducts do not differentiate alongside proximal nephron lineages, being derivatives of a distinct stem cell pool during development [3, 21•, 40]. Organoid podocytes formed in vitro lack bona fide foot processes, and the endothelial cells within these cultures do not generally invade the podocytes to form glomeruli in vitro [3, 7, 12]. Proximal tubules lack well-differentiated brush borders and are deficient in strongly-expressed markers such as aquaporin-1 [3, 12, 39]. Organoid functionality in vitro is also limited, with no perfusion or filtration. As noted above, implantation of organoids can overcome some of these limitations. The extent to which fully mature and functional structures can be generated in such grafts remains an important question.

Variability of organoid cultures remains a substantial concern. There is variability in the composition of organoids from well to well, even within the same batch, and this variability increases between different batches [8, 41, 42]. Variables include the efficiency of differentiation (yield of organoids), the presence and relative proportions of different cell types within the organoids, and the geometry of the nephron compartments relative to one another. This necessitates rigorous quality control metrics and practices to demonstrate reproducibility, including repetition of experiments in multiple pluripotent stem cell lines (derived from different individuals or guide RNAs) and distinct batches of organoids (each batch starting with the plating of pluripotent cells) [9•]. One approach to reduce variability is to optimize

differentiation methods and reagents. Rather than source differentiation reagents from multiple vendors, which is a major source of variability between batches, it is now possible to order organoid differentiation kits, which have been vetted for this purpose, and tested in multiple stem cell lines [43]. This is anticipated to increase the reproducibility of kidney organoid technology. The culture conditions themselves are also being continually optimized. For instance, treating organoid cultures with a nephron progenitor cell expansion media during the time course of differentiation produces better organized nephron-like structures, which form a bouquet around a central "hub" and express stronger markers of proximal tubules [44••]. This more regular geometry, compared to the original protocol, may aid in assessing both descriptive and functional characteristics.

Within organoid grafts, variability remains a significant barrier to functional studies, as the tissues lack the regularity and reproducibility observed in natural kidney tissues. There is no established method to evaluate how reproducible grafts are from one animal to the next. The grafts, which comprise tissue masses a few millimeters in diameter, are commonly sampled in tissue slices, which are descriptive in nature and lack three-dimensional details. Robust immunofluorescence markers should be used to distinguish human from mouse cells in renal subcapsular grafts, to avoid confusing mouse nephrons with human ones, and clearly identify the relative host and donor contributions to chimeric structures. Useful markers include anti-human nuclear antigen (HNA) and anti-human podocalyxin (PODXL), for which surrounding mouse tissues provide negative controls. Human and mouse vasculature can be distinguished using anti-human and anti-mouse CD31, respectively. Low-magnification views of grafts can be useful to appreciate general features and compare to neighboring mouse tissue, while higher magnification provides details (above Fig. 3) [3, 10, 11, 12]. Micro-computed (μ-CT) tomography provides an alternative to tissue slices, in which a contrast agent is first injected, after which the anesthetized mouse is imaged on a μ-CT scanner for several minutes. This reveals focal differences in contrast within grafts, suggesting heterogeneity amongst areas of differential vascularization [11].

For grafts, optimization of the route of organoid administration, as well as the specific stage of differentiation (nephron progenitor versus mature organoid), may be critical to generating more functional tissues, as previously shown for metanephric grafts [30]. Implantation of organoids beneath the kidney capsule of the mouse requires substantial expertise. As an alternative, grafts may be implanted subcutaneously instead, which is easier to accomplish. When implanted subcutaneously, human pluripotent stem cell-derived cells at an intermediate stage of differentiation produced epithelial structures with features of glomeruli and tubules after 12 weeks [15]. However, nephron marker assessment was limited, and lacked neighboring kidney parenchyma as a quality control. As human pluripotent stem cells can produce a wide variety of structures after implantation in vivo, including epithelial structures that resemble glomeruli, deeper characterization of subcutaneous grafts is required to verify their potential to form glomerulus-like structures [45].

Organoids have also been grown in chick allantoic membranes, which have some capacity for vascularization. Glomerulus-like structures have been reported in these, although conclusive co-localization between podocytes and endothelial cells was lacking [46]. A further limitation of allantoic membranes is that they have a relatively limited window of implantation in culture of approximately five days, before which the allantoic membranes has not yet developed, and after which they may be subject to fouling [46, 47•]. As an alternative, the coelomic cavity of the chick embryo has also been explored as a potential site for human kidney organoid grafts, which allows for longer periods of implantation, and is compatible with organoid vascularization [47•]. In this setting, most of the vasculature appears to derive from the graft, rather than the host, and glomerulus-like structures were not observed [47•]. Neither subcutaneous nor chick embryonic implants have a ready route to connect back to native ureters or kidneys, which is a significant limitation for functional studies. In conclusion, while alternatives to the mouse renal capsule exist, to date such sites remain underdeveloped, and suffer from their own significant limitations.

Regenerative Therapeutics

The functionality of kidney organoids for regenerative medicine applications has yet to be demonstrated in a convincing way. Organoid grafts have not been shown to generate urine or perform renal functions such as uremic solute clearance, water and electrolyte reabsorption and secretion, modulation of blood pressure, erythropoiesis, and vitamin D production. Even for metanephroi, there is relatively little data to suggest functional engraftment via addition of nephron mass. Organoid grafts remain immature relative to neighboring renal tissues, both in nephron-like architecture and organization, as well as gene expression patterns [10]. As described above, there is currently a lack of connectivity with the endogenous renal collecting system. Tubular structures in grafts tend to widen into cysts several weeks after implantation [10].

There are also significant safety concerns associated with pluripotent stem cell-derived organoid grafts, which become rapidly overgrown in vivo by stromal cells (vimentin^{+}) and cartilage [10, 11, 12, 13, 15]. The emergence of cartilage in these grafts is reminiscent of "teratoma" tumors that arise from undifferentiated pluripotent stem cells after implantation in vivo [45]. Growths containing cartilage and bone can

also arise when very early metanephric tissues, taken from embryos, are implanted [30]. Abundant stroma also arises after implantation of primary mouse nephron progenitor cells beneath the kidney capsule, despite improved numbers of glomerulus-like structures compared to pluripotent stem cell-derived grafts [11]. Thus, the kidney capsule may a sub-optimal location for implanting kidney cell grafts, due to efficacy and safety limitations.

In conclusion, human organoid cultures can engraft into mice and form nephron-like structures, including vascularized glomerulus-like structures. This is an important step towards regenerative therapy. Whether such grafts can provide functional benefit and perform kidney functions is an important question. Combining metanephric organoids with ureteric organoids may provide a path to establishing more complete tissue grafts. Lessons can be learned from prior studies of metanephros transplantation, which have exhibited a substantial capacity of such grafts for growth and differentiation, but have not yet translated into a clinical protocol. Revisiting such studies may be useful as a positive control for work in human organoids, which resemble primitive metanephroi. The ability to grow new nephrons is now within our reach. Dedicated optimization of human nephrogenesis to achieve safety and efficacy milestones in vivo may yield the ultimate prize of regenerative medicine for the kidneys, with availability and immunocompatibility well beyond today's standard of care.

Funding This work was supported by a Regular Award from the United States-Israel Binational Science Foundation, NIH Awards U01DK127553, R01DK117914, UG3TR002158, UG3TR003288, and UC2DK126006, NASA Proposal #21-3DTMPS_2-0001, Department of Defense Award W81XWH2110007, and Cystinosis Research Foundation grant CRFF-2021–003.

Declarations

Conflicts of interest BSF is an inventor on patents and/or patent applications related to human kidney organoid differentiation (these include "Three-dimensional differentiation of epiblast spheroids into kidney tubular organoids modeling human microphysiology, toxicology, and morphogenesis" [Japan, USA, and Australia], licensed to STEMCELL Technologies; "High-throughput automation of organoids for identifying therapeutic strategies" [PTC patent application pending]; "Systems and methods for characterizing pathophysiology" [PTC patent application pending]). BSF has ownership interest in Plurexa LLC. BD is a co-founder, shareholder, and board member at KidneyCure. None of the preceding interests affected in any way the results of the paper or would be affected by them, but they are shared by way of transparency.

Human and Animal Rights All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

Informed Consent Not applicable. No identifying information is provided.

References

Papers of particular interest, published recently, have been highlighted as:
• Of importance
•• Of major importance

1. Jha V, Garcia-Garcia G, Iseki K, Li Z, Naicker S, Plattner B, Saran R, Wang AY, Yang CW. Chronic kidney disease: global dimension and perspectives. Lancet. 2013;382:260–72.
2. Robinson BM, Zhang J, Morgenstern H, Bradbury BD, Ng LJ, McCullough KP, Gillespie BW, Hakim R, Rayner H, Fort J, Akizawa T, Tentori F, Pisoni RL. Worldwide, mortality risk is high soon after initiation of hemodialysis. Kidney Int. 2014;85:158–65.
3. Freedman BS, Brooks CR, Lam AQ, Fu H, Morizane R, Agrawal V, Saad AF, Li MK, Hughes MR, Werff RV, Peters DT, Lu J, Baccei A, Siedlecki AM, Valerius MT, Musunuru K, McNagny KM, Steinman TI, Zhou J, Lerou PH, Bonventre JV. Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. Nat Commun. 2015;6:8715.
4. Morizane R, Lam AQ, Freedman BS, Kishi S, Valerius MT, Bonventre JV. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. Nat Biotechnol. 2015;33:1193–200.
5. Taguchi A, Kaku Y, Ohmori T, Sharmin S, Ogawa M, Sasaki H, Nishinakamura R. Redefining the in vivo origin of metanephric nephron progenitors enables generation of complex kidney structures from pluripotent stem cells. Cell Stem Cell. 2014;14:53–67.
6. Takasato M, Er PX, Chiu HS, Maier B, Baillie GJ, Ferguson C, Parton RG, Wolvetang EJ, Roost MS, de Chuva Sousa Lopes SM, Little MH. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. Nature. 2015;526:564–8.
7. Kim YK, Refaeli I, Brooks CR, Jing P, Gulieva RE, Hughes MR, Cruz NM, Liu Y, Churchill AJ, Wang Y, Fu H, Pippin JW, Lin LY, Shankland SJ, Vogl AW, McNagny KM, Freedman BS. Gene-edited human kidney organoids reveal mechanisms of disease in podocyte development. Stem Cells. 2017;35:2366–78.
8. Czerniecki SM, Cruz NM, Harder JL, Menon R, Annis J, Otto EA, Gulieva RE, Islas LV, Kim YK, Tran LM, Martins TJ, Pippin JW, Fu H, Kretzler M, Shankland SJ, Himmelfarb J, Moon RT, Paragas N, Freedman BS. High-throughput screening enhances kidney organoid differentiation from human pluripotent stem cells and enables automated multidimensional phenotyping. Cell Stem Cell. 2018;22:929-940 e924.
9.• Freedman BS. Physiology assays in human kidney organoids. Am J Physiol Renal Physiol. 2022;322:F625–38. **Review of the field including best practices for organoid quality control and figure preparation.**
10. Nam SA, Seo E, Kim JW, Kim HW, Kim HL, Kim K, Kim TM, Ju JH, Gomez IG, Uchimura K, Humphreys BD, Yang CW, Lee

JY, Kim J, Cho DW, Freedman BS, Kim YK. Graft immaturity and safety concerns in transplanted human kidney organoids. Exp Mol Med. 2019;51:1–13.
11. Kumar Gupta A, Sarkar P, Wertheim JA, Pan X, Carroll TJ, Oxburgh L. Asynchronous mixing of kidney progenitor cells potentiates nephrogenesis in organoids. Commun Biol. 2020;3:231.
12. van den Berg CW, Ritsma L, Avramut MC, Wiersma LE, van den Berg BM, Leuning DG, Lievers E, Koning M, Vanslambrouck JM, Koster AJ, Howden SE, Takasato M, Little MH, Rabelink TJ. Renal subcapsular transplantation of psc-derived kidney organoids induces neo-vasculogenesis and significant glomerular and tubular maturation in vivo. Stem Cell Rep. 2018;10:751–65.
13. Sharmin S, Taguchi A, Kaku Y, Yoshimura Y, Ohmori T, Sakuma T, Mukoyama M, Yamamoto T, Kurihara H, Nishinakamura R. Human Induced pluripotent stem cell-derived podocytes mature into vascularized glomeruli upon experimental transplantation. J Am Soc Nephrol. 2016;27:1778–91.
14. Tanigawa S, Islam M, Sharmin S, Naganuma H, Yoshimura Y, Haque F, Era T, Nakazato H, Nakanishi K, Sakuma T, Yamamoto T, Kurihara H, Taguchi A, Nishinakamura R. Organoids from nephrotic disease-derived iPSCs Identify impaired nephrin localization and slit diaphragm formation in kidney podocytes. Stem Cell Rep. 2018;11:727–40.
15. Bantounas I, Ranjzad P, Tengku F, Silajdzic E, Forster D, Asselin MC, Lewis P, Lennon R, Plagge A, Wang Q, Woolf AS, Kimber SJ. Generation of functioning nephrons by implanting human pluripotent stem cell-derived kidney progenitors. Stem Cell Rep. 2018;10:766–79.
16. van den Berg CW, Koudijs A, Ritsma L, Rabelink TJ. In vivo assessment of size-selective glomerular sieving in transplanted human induced pluripotent stem cell-derived kidney organoids. J Am Soc Nephrol. 2020;31:921–9.
17. Wang X, Phan DT, Sobrino A, George SC, Hughes CC, Lee AP. Engineering anastomosis between living capillary networks and endothelial cell-lined microfluidic channels. Lab Chip. 2016;16:282–90.
18. Kaku Y, Taguchi A, Tanigawa S, Haque F, Sakuma T, Yamamoto T, Nishinakamura R. PAX2 is dispensable for in vitro nephron formation from human induced pluripotent stem cells. Sci Rep. 2017;7:4554.
19. Xia Y, Nivet E, Sancho-Martinez I, Gallegos T, Suzuki K, Okamura D, Wu MZ, Dubova I, Esteban CR, Montserrat N, Campistol JM, Izpisua Belmonte JC. Directed differentiation of human pluripotent cells to ureteric bud kidney progenitor-like cells. Nat Cell Biol. 2013;15:1507–15.
20. Takasato M, Er PX, Chiu HS, Maier B, Baillie GJ, Ferguson C, Parton RG, Wolvetang EJ, Roost MS, Lopes SM, Little MH. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. Nature. 2015;526:564–8.
21.• Howden SE, Wilson SB, Groenewegen E, Starks L, Forbes TA, Tan KS, Vanslambrouck JM, Holloway EM, Chen YH, Jain S, Spence JR, Little MH. Plasticity of distal nephron epithelia from human kidney organoids enables the induction of ureteric tip and stalk. Cell Stem Cell. 2021;28:671-684 e676. **Clarifies potential of nephron organoids to generate ureteric-like progenitor cells.**
22. Wu H, Uchimura K, Donnelly EL, Kirita Y, Morris SA, Humphreys BD. Comparative analysis and refinement of human psc-derived kidney organoid differentiation with single-cell transcriptomics. Cell Stem Cell. 2018;23:869-881 e868.
23. Taguchi A, Nishinakamura R. Higher-order kidney organogenesis from pluripotent stem cells. Cell Stem Cell. 2017;21:730-746 e736.
24. Mae SI, Ryosaka M, Toyoda T, Matsuse K, Oshima Y, Tsujimoto H, Okumura S, Shibasaki A, Osafune K. Generation of branching ureteric bud tissues from human pluripotent stem cells. Biochem Biophys Res Commun. 2018;495:954–61.
25.• Uchimura K, Wu H, Yoshimura Y, Humphreys BD. Human pluripotent stem cell-derived kidney organoids with improved collecting duct maturation and injury modeling. Cell Rep. 2020;33:108514. **Combination of nephron-like and ureteric bud-like differentiation protocols suggests improved maturation.**
26.• Zeng Z, Huang B, Parvez RK, Li Y, Chen J, Vonk AC, Thornton ME, Patel T, Rutledge EA, Kim AD, Yu J, Grubbs BH, McMahon JA, Pastor-Soler NM, Hallows KR, McMahon AP, Li Z. Generation of patterned kidney organoids that recapitulate the adult kidney collecting duct system from expandable ureteric bud progenitors. Nat Commun. 2021;12:3641. **A new protocol for inducing ureteric bud-like progenitor cells from iPS cells.**
27.•• Tanigawa S, Tanaka E, Miike K, Ohmori T, Inoue D, Cai CL, Taguchi A, Kobayashi A, Nishinakamura R. Generation of the organotypic kidney structure by integrating pluripotent stem cell-derived renal stroma. Nat Commun. 2022;13:611. **Study in mouse iPS cells combines multiple progenitors to generates advanced kidney-like tissues with connected tubules and ducts.**
28. Li Z, Araoka T, Wu J, Liao HK, Li M, Lazo M, Zhou B, Sui Y, Wu MZ, Tamura I, Xia Y, Beyret E, Matsusaka T, Pastan I, Rodriguez Esteban C, Guillen I, Guillen P, Campistol JM, Izpisua Belmonte JC. 3D Culture supports long-term expansion of mouse and human nephrogenic progenitors. Cell Stem Cell. 2016;19:516–29.
29. Dekel B, Burakova T, Ben-Hur H, Marcus H, Oren R, Laufer J, Reisner Y. Engraftment of human kidney tissue in rat radiation chimera: II. Human fetal kidneys display reduced immunogenicity to adoptively transferred human peripheral blood mononuclear cells and exhibit rapid growth and development. Transplantation. 1997;64:1550–8.
30. Dekel B, Burakova T, Arditti FD, Reich-Zeliger S, Milstein O, Aviel-Ronen S, Rechavi G, Friedman N, Kaminski N, Passwell JH, Reisner Y. Human and porcine early kidney precursors as a new source for transplantation. Nat Med. 2003;9:53–60.
31. Dekel B, Amariglio N, Kaminski N, Schwartz A, Goshen E, Arditti FD, Tsarfaty I, Passwell, JH, Reisner Y, Rechavi G. Engraftment and differentiation of human metanephroi into functional mature nephrons after transplantation into mice is accompanied by a profile of gene expression similar to normal human kidney development. J Am Soc Nephrol. 2002;13(4):977–90. https://doi.org/10.1681/ASN.V134977
32. Dekel B. The Ever-Expanding Kidney Repair Shop. J Am Soc Nephrol. 2016;27:1579–81.
33. Murakami Y, Naganuma H, Tanigawa S, Fujimori T, Eto M, Nishinakamura R. Reconstitution of the embryonic kidney identifies a donor cell contribution to the renal vasculature upon transplantation. Sci Rep. 2019;9:1172.
34.•• Harari-Steinberg O, Omer D, Gnatek Y, Pleniceanu O, Goldberg S, Cohen-Zontag O, Pri-Chen S, Kanter I, Ben Haim N, Becker E, Ankawa R, Fuchs Y, Kalisky T, Dotan Z, Dekel B. Ex vivo expanded 3D Human kidney spheres engraft long term and repair chronic renal injury in mice. Cell Rep. 2020;30:852-869 e854. **Demonstration of potential to enhance endogenous repair using 3D culture of primary kidney epithelial cells.**
35. Pleniceanu O, Harari-Steinberg O, Omer D, Gnatek Y, Lachmi BE, Cohen-Zontag O, Manevitz-Mendelson E, Barzilai A, Yampolsky M, Fuchs Y, Rosenzweig B, Eisner A, Dotan Z, Fine LG, Dekel B, Greenberger S. Successful Introduction of human renovascular units into the mammalian kidney. J Am Soc Nephrol. 2020;31:2757–72.

36. Namestnikov M, Pleniceanu O, Dekel B. Mixing Cells for Vascularized Kidney Regeneration. Cells. 2021;10:1119.
37. Rogers SA, Hammerman MR. Prolongation of life in anephric rats following de novo renal organogenesis. Organogenesis. 2004;1:22–5.
38. Freedman BS. Better Being Single? Omics Improves Kidney Organoids. Nephron. 2019;141:128–32.
39. Subramanian A, Sidhom EH, Emani M, Vernon K, Sahakian N, Zhou Y, Kost-Alimova M, Slyper M, Waldman J, Dionne D, Nguyen LT, Weins A, Marshall JL, Rosenblatt-Rosen O, Regev A, Greka A. Single cell census of human kidney organoids shows reproducibility and diminished off-target cells after transplantation. Nat Commun. 2019;10:5462.
40. Yoshimura Y, Taguchi A, Nishinakamura R. Generation of a Three-Dimensional Kidney Structure from Pluripotent Stem Cells. Methods Mol Biol. 2017;1597:179–93.
41. Phipson B, Er PX, Combes AN, Forbes TA, Howden SE, Zappia L, Yen HJ, Lawlor KT, Hale LJ, Sun J, Wolvetang E, Takasato M, Oshlack A, Little MH. Evaluation of variability in human kidney organoids. Nat Methods. 2019;16:79–87.
42. Cruz NM, Freedman BS. Differentiation of human kidney organoids from pluripotent stem cells. Methods Cell Biol. 2019;153:133–50.
43. Freedman BS. A commercially available kit to generate human kidney organoids. (Re)Building a Kidney Consortium. 2021. https://www.rebuildingakidney.org/id/17-EBYM.
44.•• Vanslambrouck JM, Wilson SB, Tan KS, Groenewegen E, Rudraraju R, Neil J, Lawlor KT, Mah S, Scurr M, Howden SE, Subbarao K, Little MH. Enhanced metanephric specification to functional proximal tubule enables toxicity screening and infectious disease modelling in kidney organoids. Nat Commun. 2022;13:5943. **Use of an improved media formulation enables impressive geometric patterning of kidney organoids with proximal tubular characteristics.**
45. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145–7.
46. Garreta E, Prado P, Tarantino C, Oria R, Fanlo L, Marti E, Zalvidea D, Trepat X, Roca-Cusachs P, Gavalda-Navarro A, Cozzuto L, Campistol JM, Izpisua Belmonte JC, Hurtado Del Pozo C, Montserrat N. Fine tuning the extracellular environment accelerates the derivation of kidney organoids from human pluripotent stem cells. Nat Mater. 2019;18:397–405.
47.• Koning M, Dumas SJ, Avramut MC, Koning RI, Meta E, Lievers E, Wiersma LE, Borri M, Liang X, Xie L, Liu P, Chen F, Lin L, Luo Y, Mulder J, Spijker HS, Jaffredo T, van den Berg BM, Carmeliet P, van den Berg CW, Rabelink TJ. Vasculogenesis in kidney organoids upon transplantation. NPJ Regen Med. 2022;7:40. **Detailed analysis of organoid vascularization after transplantation into chick coelom.**