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REVIEW

From organoids to transplantable artificial kidneys

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SUMMARY

It is difficult to restore kidney function following chronic kidney damage. Although dialysis is currently used to treat patients with chronic kidney disease, it does not cure the disease, while severely restricting the patient's daily and social activities. Kidney transplantation is an alternative and curative therapy, but donor numbers remain limited. However, the generation of kidney organoids from human induced pluripotent stem cells represents an important recent advance in regenerative medicine. Kidney organoids are expected to be used for disease modeling and drug discovery, and may eventually be applicable for transplantation. In this review, we describe the current status of kidney organoids and discuss the hurdles that need to be overcome to generate transplantable artificial kidneys.

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artificial kidney, iPS cell, kidney organoid, nephron progenitor, transplantation, ureteric bud

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Introduction

The nephron, including the glomeruli and renal tubules, comprises the basic functional unit of the kidney. The human adult kidney contains approximately one million nephrons, and their correct three-dimensional alignment is required for effective kidney functioning. Glomeruli filter the blood to produce primitive urine, which flows into the renal tubules, and water, electrolytes, and sugar are reabsorbed as the urine flows through the proximal and distal renal tubules. The distal renal tubules are connected to the collecting ducts, which converge into a single ureter that thus acts as a one-way exit for the urine produced by the approximately one million nephrons.

Three embryonic kidney progenitors

In mammals, the kidney develops in the order of pronephros, mesonephros, and metanephros. The metanephros eventually functions as the adult kidney, while most parts of the pronephros and mesonephros regress during development. The embryonic kidney (metanephros) is formed from at least three populations of precursor cells: nephron progenitors that form nephrons, ureteric buds that form the urinary drainage tract, and stromal progenitors that differentiate into the renal interstitium. Nephron progenitors differentiate into glomeruli and renal tubules via mutual interactions between the nephron progenitors and ureteric buds, while the urinary bud branches repeatedly to form the collecting ducts and ureter (Fig. 1).

Generation of kidney organoids from induced pluripotent stem cells via nephron progenitors

We previously developed induction protocols for nephron progenitors [1] using mouse embryonic stem cells (ESCs, mESCs) and human induced pluripotent stem cells (iPSCs, hiPSCs). Our analysis of nephron progenitor development in mice indicated a different origin for nephron progenitors than expected.

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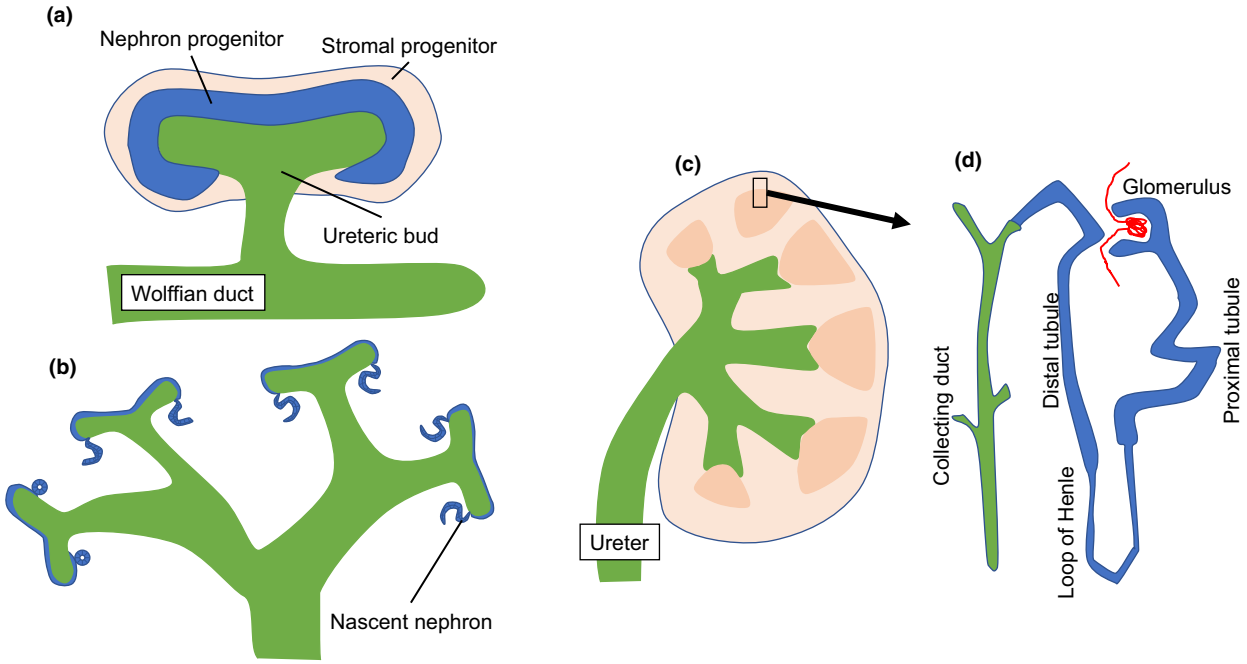

Figure 1 The adult kidney is derived from three precursors. (a) The ureteric bud (green) protrudes from the Wolffian duct and invades into the metanephric mesenchyme, containing nephron and stromal progenitors (blue and beige, respectively). (b) The ureteric bud repeatedly branches and induces nephron progenitors to differentiate into nephrons. (c) Adult kidney. Renal pelvis and ureter (green) are derived from the ureteric bud. (d) Higher magnification of the renal parenchyma (rectangle in panel c). Glomeruli and renal tubules are derived from nephron progenitors (blue). Collecting ducts are derived from the ureteric bud (green). These differentially originated tubules connect to form the urinary tracts.

Although all the kidney progenitors were considered to be derived from intermediate mesoderm, we found that nephron progenitors were derived from posterior intermediate mesoderm, while ureteric buds were derived from anterior intermediate mesoderm (Fig. 2). We further showed that the posterior intermediate mesoderm was derived from the posterior nascent mesoderm, located at the posterior end of early-stage embryos. Based on these findings, we successfully induced nephron progenitors that differentiated into kidney organoids containing glomeruli and renal tubules *in vitro*.

Several other groups have also reported protocols for inducing kidney organoids via nephron progenitors from human ESCs (hESCs) or iPSCs [2–4]. Takasato *et al.* [5,6] developed a relatively simple protocol, while the method of Morizane *et al.* [7] required a shorter culture time. Recently, Przepiorski *et al.* [8] reported a simple and cost-effective protocol using a bioreactor. These protocols share certain features but also have some distinct aspects. Although the similarities and differences between the nephron progenitors and nephrons induced by these different protocols need to be determined, the induced proximal renal tubules have been shown to carry out some functions, including reabsorption of low-molecular-weight dextran [6,8] and selective cell death in response to nephrotoxic drugs [6,7].

Disease modeling using kidney organoids

hiPSC-derived kidney organoids can also be applicable to disease modeling. Freedman *et al.* [9] partially reproduced polycystic kidney disease (PKD) using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome-editing system to delete the *PKD1* or *PKD2* genes, which are recognized as the causative genes of autosomal dominant PKD. These knockout kidney organoids showed cyst formation from renal tubules. Forbes *et al.* [10] generated iPSCs from a patient with nephronophthisis, which is caused by cilia abnormalities, and reproduced shortened cilia in the kidney organoids derived from the patient's iPSCs. We recently established iPSCs from a patient with congenital nephrotic syndrome who had a single amino acid mutation in the gene encoding NEPHRIN [11], which is a main component of the filtration apparatus in the glomerulus slit diaphragm. We induced kidney organoids from patient-derived iPSCs and found mislocalization of NEPHRIN and impaired slit diaphragm

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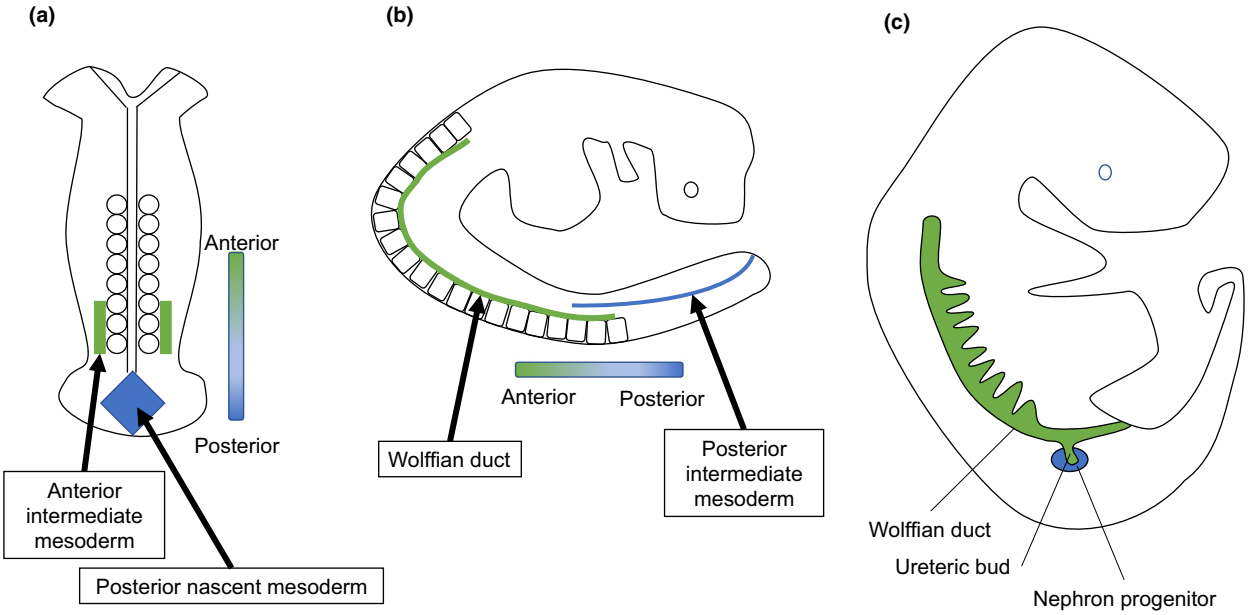

Figure 2 Scheme of kidney development in mice. The nephron progenitor and ureteric bud have spatially and temporally different origins. (a) Embryonic day (E) 8.5. Anterior intermediate mesoderm (green) appears before the posterior intermediate mesoderm. The precursor of the posterior intermediate mesoderm is detected as posterior nascent mesoderm (blue). (b) E9.5. Posterior intermediate mesoderm (blue) is formed from posterior nascent mesoderm. Wolffian ducts (green), formed from the anterior intermediate mesoderm, elongate caudally. (c) E11.5. The ureteric bud (green) protrudes toward the nephron progenitors (blue) and initiates interactions to form the metanephros.

formation. These abnormalities were resolved when the mutation was corrected by genome editing, confirming that this single amino acid mutation was responsible for causing the disease. These disease models may serve as useful platforms for drug discovery for the treatment of hereditary kidney diseases.

Expansion of nephron progenitors

Many researchers are unfamiliar with handling hiPSCs and it would therefore be useful to establish methods for expanding these cells and making frozen stocks of hiPSC-derived nephron progenitors that could then be distributed throughout the research community. Nephron progenitors cease propagation and are terminally differentiated within a few days after birth in mice and at approximately 36 weeks of gestation in humans [12,13]; however, several groups, including ours, have succeeded in expanding nephron progenitors *in vitro*, at least partially [14–16]. All the various protocols utilize Wnt, bone morphogenetic protein, and fibroblast growth factor signaling, based on the accumulated findings *in vivo*. Brown *et al.* [14] expanded hESC-derived nephron progenitors, but the expanded cells only formed renal tubule-like structures and no glomeruli. Our group [15] succeeded in propagating hiPSC-derived nephron progenitors that could differentiate into both glomeruli and renal tubules; however, the expansion period was limited to 1–2 weeks. Li *et al.* [16] reported propagation of human embryo-derived nephron progenitors for up to 7 months, and demonstrated their ability to differentiate into glomeruli and renal tubules. They also reported the expansion of hiPSC-derived nephron progenitors, but this process appeared less robust than that for human embryo-derived progenitors. Improved protocols, as well as the creation of frozen stocks of hiPSC-derived nephron progenitors, are required to accelerate research into kidney regeneration.

Generation of ureteric buds and higher-order kidney structures

Although the above observations represent marked progresses in research, most kidney organoids have been generated via nephron progenitors and thus lack the tissues derived from the ureteric buds. They therefore contain glomeruli and renal tubules, but no collecting ducts. As an exception to this, Takasato *et al.* [6] developed a single protocol that could simultaneously induce nephron progenitors and ureteric bud-like cells. The organoids contained numerous glomeruli and renal tubules connected to epithelia expressing some of the ureteric-bud markers. The cells surrounding these

structures expressed some stromal progenitor markers, while endothelial cells were also detected in the organoids, suggesting that the complex kidney structure might be generated *in vitro* by a relatively simple protocol. However, the induced ureteric bud-like cells did not branch, and their ability to maintain and differentiate nephron progenitors remained elusive.

Bioengineering represents one possible approach for generating the complex branching structures of the kidney. For example, Homan *et al.* [17] seeded immortalized or primary human proximal renal tubular cells into a convoluted scaffold generated by a 3D printer. Various types of hiPSC-derived cell sources may be useful for not only generating whole kidneys, but also for improving renal dialysis. In contrast, we recently proposed an alternative method. Based on our revised model accounting for the distinct origins of the nephron progenitors and ureteric buds, as described above, we developed a new induction protocol for ureteric buds using both mESCs and hiPSCs [18]. This protocol differed markedly from that for nephron progenitors, which was consistent with our model *in vivo*. We then combined the differentially induced ureteric buds and nephron progenitors from mESCs, along with stromal progenitors derived from mouse embryos, and generated mouse kidney organoids with higher-order kidney structure, including glomeruli and renal tubules located at the tips of extensively branching ureteric buds (collecting ducts). A combination of differentially induced precursors thus represents a useful strategy for creating a genuine kidney structure (Fig. 3). However, stromal progenitors were also essential for this strategy, and the generation of similar kidney organoids in humans will require the induction of functional stromal progenitors from hiPSCs. The Takasato protocol [6] showed simultaneous induction of stroma-like cells with other lineages. The Morizane protocol [7] showed proliferation of stromal cells in organoids treated with IL-1β [19]. The similarities and differences of these stromal cells in the organoids and those *in vivo* need to be examined. At the same time, better stromal progenitors need to be induced that can faithfully mimic development *in vivo*.

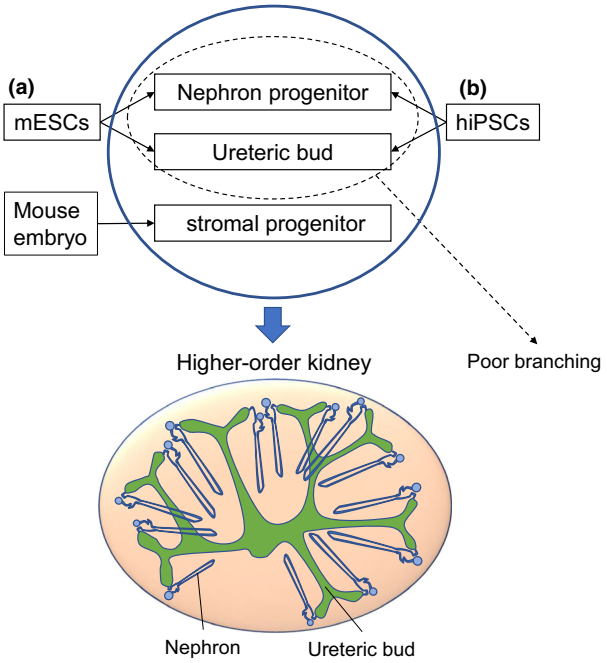

Figure 3 Strategies for generating kidney organoids with higher-order structure. (a) Combination of nephron progenitors and ureteric buds induced differentially from mESCs, with stromal progenitors isolated from mouse embryonic kidneys, generates higher-order kidney structure *in vitro*. (b) hiPSC-derived kidney organoids without stromal progenitors show poor branching of the ureteric bud. Stromal progenitors that support extensive ureteric bud branching need to be induced from hiPSCs.

Vascularization of kidney organoids

Even if human kidney organoids with higher-order structure are generated, several hurdles remain to be overcome before applying these organoids to transplantation therapy. One problem involves the insufficient formation of a vascular network in the organoids. Because glomeruli in the kidney filter the blood to generate urine, an adequate vasculature is an essential component. Although some vascular endothelial cells exist in the kidney organoids [6], most glomeruli within the organoids remain avascular *in vitro*. However, we previously reported that hiPSC-derived glomeruli were efficiently vascularized when the nephron progenitor-type organoids (excluding the ureteric buds) were transplanted under the kidney capsules of immunodeficient mice [20]. The reason for this apparent discrepancy between the *in vitro* and *in vivo* situations remains unknown, but may be related to the mechanical flow that exists in the host vasculature. Blood flow is known to play an important role in adequate organ vascularization. For example, Serluca *et al.* [21] arrested the heartbeat in zebrafish using an inhibitor of myofibrillar ATPase and showed that vascular integration into glomeruli was impaired. A similar mechanism may operate in glomerular vascularization in mammals. Interestingly, most endothelial cells in the glomeruli in the transplantation experiments were derived from the host animals rather than from the transplanted iPSCs [20,22]. In contrast, another group detected some integration of

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hiPSC-derived endothelial cells upon transplantation [23]. Further studies are needed to determine the relative contributions of the donor- and host-derived vasculatures to the transplanted kidney organoids. Takebe *et al.* [24] reported the formation of hiPSC-derived vascularized liver organoids by transplanting liver organ buds, which were combined with human umbilical cord endothelial vein cells (HUVECs) and mesenchymal stem cells, into immunodeficient mice. They also performed similar experiments using mouse kidney, by combining dissociated mouse embryonic kidney with HUVECs and mesenchymal stem cells *in vitro*. Vascularized glomeruli were observed upon transplantation, though the ureteric buds in the kidney organoids were distributed in a disconnected fashion. More efficient methods are therefore needed to integrate hiPSC-derived renal endothelial cells into the glomeruli of the kidney organoids.

Furthermore, the vasculature formed following transplantation [20] was much smaller than the renal arteries *in vivo*, which carry 20–25% of the cardiac output. It is therefore necessary to generate larger arteries both inside and outside of the kidney organoids, to allow the required substantial blood flow. However, little is currently known about the mechanisms of renal artery development, and further work is needed to elucidate this process and to recapitulate the complex architecture of the renal vasculature.

Generation of a single urinary exit tract

One of the important problems associated with artificial kidney transplantation involves the formation of a urinary exit tract. None of the currently available kidney organoids have a single ureter that elongates outside the organoids. Once blood flow from the host is supplied to the kidney organoids, the glomeruli start to produce urine, but in the absence of a ureter, the urine would not flow and would eventually cause hydronephrosis. Artificial kidney thus requires a ureter with a one-way exit, allowing the urine to flow without reflux or obstruction (Fig. 4). The ureter should subsequently be connected to the host's ureter or urinary bladder. However, ureters in the artificial kidney may be too small to be sutured to the host tissues, and technical improvements are required to resolve these issues. Nevertheless, Yokote *et al.* [25] reported a draining system using the host urinary bladder. They transplanted rat embryonic metanephros together with the cloaca, as the precursor tissue of the urinary bladder, to the para-aortic region of the host rats. Four weeks after transplantation, they removed one kidney from the host and connected the cut end of the host ureter to the newly formed bladder of the graft. The newly formed bladder served as a urine reservoir before connection to the host, allowing the graft to grow without hydronephrosis. Induction of the urinary bladder, in addition to the kidney and ureter, is needed to apply this system to kidney organoids. However, a completely different protocol will be required for the bladder, because it is derived from endoderm and formed in a different developmental path from the mesoderm-derived kidney.

Optimal transplantation sites for artificial kidneys

Conventional clinical transplantation involves placing the donor kidney in the host's iliac fossa. However, it is difficult to generate an adult-size mature kidney fully *in vitro*, and it may thus be necessary to graft the immature artificial kidney and wait for it to mature after transplantation. Therefore, it is necessary to consider the most appropriate transplantation sites for artificial kidneys. Given that blood filtration is one of the main functions of the kidney, the transplant site should be rich in blood flow, and should also be close to the host's urinary exit tract. Although no reported studies have directly compared different transplantation sites for iPSC-derived kidney organoids, some have compared sites using rat embryonic kidneys. Rogers *et al.* [26] reported that transplantation into the omentum resulted in increased weight and inulin clearance of the grafts. Marshall *et al.* [27] transplanted the grafts into retroperitoneal fat tissues adjacent to the renal vessels, circumflex iliac vessels, and aorta/vena cava, and showed that the para-aorta/vena cava area was better than the omentum, based on the glomerular filtration rates of the graft kidneys.

It is also necessary to optimize the developmental stages of the artificial kidneys used for transplantation. Dekel *et al.* [28] transplanted human and pig embryonic kidneys into immunodeficient mice and reported that kidneys at early developmental stages (7–8 weeks of gestation in humans) differentiated efficiently into renal tissues, while kidneys at later stages (10–14 weeks of gestation in humans) underwent necrosis. Kim *et al.* [29] transplanted cells isolated from rat embryonic kidneys into immunodeficient mice and reported that cells at earlier stages formed kidney structures and showed higher expression of kidney-specific genes compared with kidney cells at later stages. These results suggest that there is an optimal time window during which embryonic kidneys can regenerate kidney tissues upon transplantation.

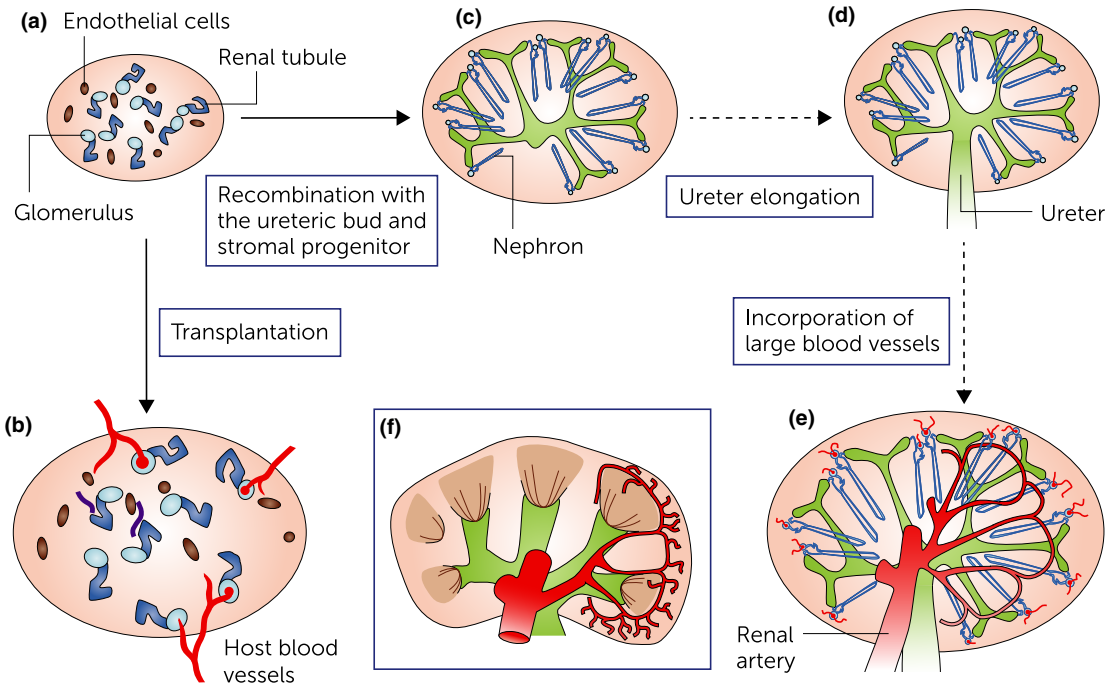

Figure 4 Vascularization of hiPSC-derived kidney organoids. (a) Most glomeruli in the nephron progenitor-derived kidney organoids remain avascular *in vitro*. (b) Vasculature invades into the glomeruli upon transplantation of the nephron progenitors. However, the overall vasculature remained primitive. (c) Combination of nephron progenitors with the ureteric bud and stromal progenitors generates higher-order kidney structure. However, the ureter remains short. (d) Ureter elongation is required to secure a urinary outlet. (e) Large vasculature should be integrated into the kidney organoids. (f) The adult kidney as the ultimate goal for artificial kidneys.

We should also assess the maturation status of the kidney organoids following successful transplantation. In addition to the size and histological structures of the organoids, it is also necessary to establish markers (for gene and protein expression) to determine the developmental stages of the organoids. The maturation processes from embryonic to adult kidneys are currently poorly known. However, developments in single-cell RNA sequencing technology will make it possible to determine the gene expression profiles of every kidney lineage at various developmental stages in humans. It will then be possible to compare gene expression profiles between the organoids (both *in vitro* and *in vivo*) and human embryonic kidneys, and assess the maturity of every cell lineage in the organoids.

In addition, quality control of human ESC/iPSC lines is essential for the development of transplantable artificial kidneys as a new source for kidney therapies. Merkle *et al.* [30] identified mutations in five of the 140 independent hESC lines, including in the tumor suppressor p53. The incidence of p53 mutations increased with passage number in standard culture, indicating a selective advantage of these mutations. Contamination of undifferentiated ESC/iPSCs in the artificial kidneys may also cause teratoma formation, and safety issues should thus be taken into consideration in future regenerative medicine procedures.

Reconstructing the kidney in animals

Some groups are currently generating human organs in large animals for use in transplantation. As a pilot study, Yamanaka *et al.* injected mouse nephron progenitors into the nephrogenic zone of host embryonic rats and eliminated host-derived nephron progenitors by drug-induced depletion. The donor mouse-derived nephron progenitors then differentiated into nephrons [31], while the other tissues derived from the host rat ureteric bud, stromal progenitors, and endothelial cells, remained. The next step will be to use hiPSC-derived nephron progenitors. Yamaguchi *et al.* [32] injected undifferentiated mESCs or iPSCs into pancreas-deficient rat blastocysts and generated mouse-derived pancreases in rats. When the generated pancreas was dissociated and transplanted into other diabetic mice, blood glucose levels were normalized for over 370 days. These strategies may be applicable to the generation of human kidneys in large animals, such as pigs. However, at least three issues must be considered. First, any residual pig cells will cause hyperacute rejection, and all the nephron

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progenitors, ureteric buds, stromal cells, and endothelial cells must thus be replaced with human cells. Second, although rat/mouse chimeras have been reported [31,32], it is currently unknown whether the generation of chimeric animals or organs between humans and pigs is achievable. Wu *et al.* [33] injected hiPSCs into pig blastocysts and implanted them into a pig uterus, and observed integration of human cells into the pig embryos, though the contribution was not marked. Third, it is not possible to control the chimeric percentages of the resultant animals, especially in the case of blastocyst complementation. Human cells may distribute not only to the kidney, but also to other organs, including the brain and germ cells. This could raise ethical issues that will require careful discussion.

Other attempts have been made to generate genetically engineered pigs that possess organs transplantable to humans. Owing to the rapid progress of CRISPR/Cas9-mediated genome-editing technology, it may be possible to eliminate the histocompatibility complexes responsible for rejection upon xenotransplantation. Längin *et al.* [34] recently succeeded in generating pig hearts lacking galactose-α1,3-galactose epitopes and expressing human CD46 and thrombomodulin by genetic engineering technology, and these pig hearts survived for up to 945 days after transplantation into baboons. Another problem of xenotransplantation involves possible infection of humans by porcine endogenous retrovirus; however Niu *et al.* [35] successfully applied CRISPR/Cas9 technology to inactivate this virus in a primary porcine cell line, and generated pigs from the cell line by somatic nuclear transfer. These technologies will contribute to realize safe xenotransplantation in the future.

Conclusion

Recent advances in the generation of iPSC-derived kidney organoids have led to great expectations in the medical field. However, many hurdles still need to be overcome. The combination of many techniques, including iPSCs, kidney organoids, genome editing, and large-animal manipulation, will accelerate scientific advances toward the generation of transplantable artificial kidneys in the future.

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Conflicts of interest

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