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Making new kidneys – On the road from science fiction to science fact

Oded Volovelsky, MD, PhD[1,2] and **Raphael Kopan, PhD**[1]

[1]Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

[2]Division of Nephrology and Hypertension, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

Abstract

Purpose of Review—Allogenic kidney transplantation use is limited due to a shortage of kidney organ donors and the risks associated with a long-term immunosuppression. An emerging treatment prospect is autologous transplants of ex-vivo produced human kidneys. Here we will review the research advances in this area.

Recent findings—The creation of human induced pluripotent cells (iPSCs) from somatic cells and the emergence of several differentiation protocols that are able to convert iPSCs cells into self-organizing kidney organoids are two large steps towards assembling a human kidney in vitro. Several groups have successfully generated urine-producing kidney organoids upon transplantation in a mouse host. Additional advances in culturing nephron progenitors in vitro may provide another source for kidney engineering, and the emergence of genome editing technology will facilitate correction of congenital mutations.

Summary—Basic research into the development of metanephric kidneys and iPSC differentiation protocols, the therapeutic use of iPSCs, along with emergence of new technologies such as CRISPR/Cas9 genome editing have accelerated a trend that may prove transformative in the treatment of ESRD as well as congenital kidney disorders.

Keywords

kidney development; kidney organoid; progenitor; stem cell; iPSCs

Introduction

The human kidney regulates electrolyte hemostasis, hydration levels and consequently body fluid osmolality, excretes organic acids and toxins, maintains acid-base balance and excretes

Contact of the corresponding author: Oded Volovelsky, Division of Nephrology and Hypertension, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave I MLC 7022 I Cincinnati, OH 45229, Office: +1-(513)-636-8647; Fax: +1-(513)-636-7407, Oded.Volovelsky@cchmc.org.

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Conflicts of interest

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hormones that help to maintain blood pressure and regulate erythropoiesis. In order to sustain these complex processes, a unique structure (the nephron) evolved with more than 20 specialized cell types residing in a precise architecture maintained throughout life. Millions of human glomeruli filter blood; the filtrate is modified as it flows from the cortex to the medulla and back through specialized tubules, ending in the collecting ducts as urine. This complex mesodermal organ forms from week 5 to week 36 of gestation and its maintenance throughout life is critical for survival [1].

When kidney function declines, it may lead to kidney failure or end stage renal disease (ESRD). Patients suffering from ESRD have few therapeutic options. The current standard of care includes organ transplantation and dialysis-based management of comorbidities and severe complications including anemia, cardiovascular and bone disease, which are exacerbated the longer one waits until a suitable transplantable organ is identified. According to the United Network for Organ Sharing (UNOS), approximately 110,000 ESRD patients are currently waiting for a kidney donation. Each year, five percent of this population will die before receiving a transplant [2]. Transplantation restores renal function but comes at a price: most transplant recipients depend on chronic immunosuppression, placing them at increased risk of developing life threatening infections and malignancies.

Among the many reasons for ESRD are two biological facts. First, no new nephrons are generated after birth. During late gestation, en mass differentiation of all nephron-forming stem cells and coincident disappearance of the appropriate environment in which to form new nephrons terminate the nephrogenic period [3]. Second, the number of nephrons generated during human gestation varies tenfold from ~$2x10^5$ to more than ~$2x10^6$, with those at the lower end being at much greater risk for hypertension and ESRD [4–7]. Because of these realities, the kidneys lack the capacity to replace nephrons lost due to injury and are only capable of limited repair following an injury [8–10]. The presence of multipotent cells in the kidney is hotly debated, with most in agreement that each segment repairs itself from de-differentiated resident cells, with the possible exception of cells in the Bowman's capsule which may be able to regenerate podocytes [11–16]. Currently, no cells isolated from the mature human kidney possess the capacity to form a kidney-like organ. These realities suggest that increasing rates of kidney donation, improvements in building tolerance for allogeneic organs, exploration of xenograft options [17], or continuous progress in decreasing the morbidity associated with kidney disease are the only avenues that can improve outcome for ESRD patients.

One promising strategy would be creation of a new kidney from the patient's own cells, or in the case of a patient with a congenital disorder, from "repaired" patient cells or from engineered "universal donor" cells with tolerated HLA. Though still science fiction, autologous transplantation of lab-grown kidneys may become reality in the not so distant future. Among the steps on the road from fiction to practice are the Nobel-worthy discoveries of induced pluripotency/somatic cell reprogramming and CRISPR/Cas9 genome editing technologies. The availability of human iPSCs has mollified many ethical concerns regarding the use of pluripotent human cells derived from blastocysts and brought the dream of forming of new kidney anlagen ex-vivo closer to reality. Herein, we will review the

organogenesis of the kidney, and describe the latest advances in the regenerative research in this field.

The building blocks of a human kidney

Nephron formation requires the interactions of three distinct cell populations: the epithelial ureteric bud (UB), the metanephric mesenchyme (MM), and the surrounding stroma (reviewed in [18]). The MM induces UB branching, and the UB promotes MM proliferation and aggregation to form tightly associated “caps” around each UB tip (the cap mesenchyme or CM). The branched UB will form the collecting system whereas the surrounding stroma will regulate the ratio between self-renewal and differentiation within the MM as well as contribute the mesangial cells, the smooth muscle cells, the renin secreting cells, and the interstitium. Vascular components of the kidney come from both renal and extra renal origin. The molecular detail of these interactions has been extensively covered elsewhere [18–22] and will only be mentioned here in the context of iPSCs differentiation.

Importantly, the self-organizing nature of the renal anlage was noted early on: isolated MM cells undergo apoptosis, but combining them *in vitro* with the UB (or other Wnt-producing tissue like dorsal neural tube) is sufficient to induce branching morphogenesis (if UB is used) and nephrogenesis in the MM in the absence of any external supplements [23, 24]. These early observations laid the foundation for our current progress and established that once brought into proximity, MM and UB cells, through bidirectional signaling are capable of self-organizing to elaborate an organ. Thus, it appears that the first hurdle to generating *de-novo* kidney *in vitro* would be to re-create these two embryonic populations.

Creating stem cells from somatic cells

Independent of efforts to solve the impasse of ESRD, many investigators labored for decades to understand pluripotency. A major breakthrough occurred in 2006 when Dr. Yamanaka and his colleagues demonstrated that introducing four transcription factors (Oct4, Sox2, Klf4, and c-Myc, known as the “Yamanaka factors”) into mouse fibroblasts reprogrammed them into a pluripotent stem cell-like state [25]. The subsequent demonstration that human cells can also be reprogrammed to pluripotency [26–28] opened the door to the use of patient-specific human pluripotent cells in basic and translational research applications, as well as clinically. iPSCs induced by the Yamanaka factors can be generated from most somatic cell types including fibroblasts, peripheral blood and skin. Multiple investigators that independently developed protocols to coax coarse embryonic stem cells (ESC) to develop organ rudiments successfully replaced ESC with iPSCs [29–33]. However, for iPSCs to open new horizons in regenerative medicine, the original strategy using integrating retroviral or lentiviral vectors had to evolve into one that avoids the risk of insertional mutagenesis or transgene reactivation that increases the risk of malignancy [34–38]. Several methods have been developed for integration-free iPSCs generation including transfection of plasmid DNA [38], synthetic-modified mRNAs [39] or mature microRNAs [40], the use of non-integrating viral vectors (e.g. adenovirus [37] and Sendai virus [41]), or transfection of membrane-soluble recombinant reprogramming factor proteins [42]. Generation of iPSCs without the expression of established oncogenes (e.g. cMyc) is desirable, and numerous combinations of

reprogramming factors have been shown to induce pluripotency in human cells. Of note, the use of small molecules with fewer factors e.g. Oct4 alone or with Sox2 or with lineage modifiers as Sox7 (mesoderm) or Sox 1 and Sox3 (ectoderm) can induce pluripotency and reduce the risk of genetic alteration [43, 44]. Whilst the technologies used for human iPSCs generation are rapidly evolving to be more compatible with their clinical use, no single reprogramming method or donor cell type has yet been shown to consistently generate genetically stable human iPSCs. Variability in the quality and stability of human iPSCs derived from discrete cell types using multiple mechanisms [45] indicates that rigorous characterization of each iPSCs line will be necessary for clinical use of any iPSCs regardless of how it was generated.

The road from iPSCs cells to MM and UB passed through the mouse

With the advent of these technologies, the stage was set to use iPSCs technologies to generate kidney cell types. Whilst previous studies using mouse ESC to generate UB and MM provided a valuable roadmap, identifying factors directly applicable to reprogramming human iPSCs into these two cell types to create a self-organizing anlage, it has been clear that the generation of protocols for differentiation of iPSCs to renal lineages also relied on knowledge from basic studies of kidney development in model organisms (especially the mouse). For instance, the transcription factors Cited1 and Six2 were identified as important markers of undifferentiated renal progenitors [46, 47]. While the role of Cited1 is unclear, the transcription factor Six2 has a pivotal role in maintaining progenitor self-renewal: Six2-deficient kidneys lose the balance between self-renewal and differentiation, both promoted by Wnt9b-βCatnein signaling, and instead differentiate en mass into epithelia [21, 48]. The identification of Six2 and Cited1 as reliable progenitor markers, and generation of transgenic animals expressing fluorescent proteins driven by the regulatory regions of these factors, facilitated the study of renal progenitors. Understanding the processes that maintain progenitor cells in an undifferentiated state and the steps involved in secession of nephrogenesis were the next important steps.

As the number of UB branch points increase, a greater fraction of the nephron progenitors will undergo differentiation [49, 50]. Initial studies aimed at extending nephron progenitors lifespan in vitro used FGF2 (fibroblast growth factor 2) and BMP7 (bone morphogenic protein 7) as exogenous factors that can promote limited survival of renal progenitors [51]. The CM and UB express BMP7, and deletion of BMP7 leads to premature loss of the CM and reduced nephron formation [52–55]. The addition of exogenous BMP7 leads to increased proliferation rate of renal progenitor cells *in vitro* through the MAPK/JNK pathways [56]. Like Wnt, BMP7 can also promote differentiation of progenitor cells, but it does so via activation of the SMAD pathway. In contrast to BMP7, FGF2 was not expressed by the kidney anlage and FGF2-null mice undergo normal kidney development [51, 57]. As a result, investigators omitted FGF from their initial cocktails and focused on using combinations of Activin A, Wnt4, BMP and retinoic acid (RA) to promote the formation of intermediate mesoderm (IM) to allow formation of mature kidney structures from mouse ESC [58–60].

The importance of FGF ligands in maintaining the multipotency of renal progenitor cells were reestablished by demonstrating that mice lacking FGF receptors FGFR1 and FGFR2 within the CM had failed to form Six2+ progenitors [61–63]. The identity of the missing ligands was discovered by genetic analysis. Mice lacking FGF9 and FGF20, and human fetuses that lack FGF20, failed to develop kidneys. Importantly, FGF9 alone could maintain progenitor cells *in vitro,* keeping them in an undifferentiated state, for a week [64]. Building on this finding, a robust *in vitro* culture media was established soon thereafter. The main innovation was the use of SMAD inhibitors coupled with weak activation of Wnt in the presence of FGF9 [65]. This media promotes significant expansion of sorted progenitor cells in a monolayer over several passages while allowing the cells to retain their potential to differentiate into renal structures in response to high levels of Wnt [65].

In parallel with these advances, several groups developed protocols for induction of MM from ESC and iPSCs cells. Attempting to recapitulate development, the Nishinakamura group observed that the nephric duct and the mesonephros were formed from existing Osr1-expressing IM cells. By contrast, the MM emerged from Osr1-negative cells entering the embryo much later than initially thought and expressing OSR1 only after their arrival (at E8.5). These late arriving cells underwent induction *in situ* to differentiate into MM. They used these developmental insights to construct an ESC and IPSCs differentiation protocol promoting formation of a 3D kidney structure [66]. Using iPSCs cells containing an artificial bacterial chromosome (BAC) with a GFP cassette inserted into OSR1 locus to serve as a reporter monitoring the emergence of IM cells, the Osafune group used high throughput approaches and applied the principles learned from mouse ESC cells to optimize differentiation of human iPSCs cells into OSR1+ IM using a cocktail consisting of Activin A and BMP7, along with a glycogen synthase kinase-3β inhibitor CHIR99021 to achieve Wnt activation [67]. In another approach, the Laslett group has cultured human ES cells under reduced serum and lower feeder cell densities were encouraged to differentiate. Kidney cells were then sorted based on the presence of markers, including CD24, podocalyxin and GCTM2. The sorted cells expressed PAX2, LHX1, and WT1, characteristic of the embryonic kidney [68]. The Boventure group used a slight variation on these protocols to achieve renal cell differentiation from ESC or iPSCs [69], which was leveraged to create PKD models [70].

Forming kidney organoids

In addition to the encouraging results successfully creating different kidney cell types from IPSCs or ES cells, these groups succeeded in creating organ-like structures, or kidney organoids, which include connected glomerular and tubular components, by capitalizing on the self-organizing properties of the kidney anlagen [66, 70–73]. For example, the Boventure group exposed human iPSCs cells to Wnt signaling for 4 days to create primitive streak cells, followed by three days with Activin (to form posterior IM cell with high efficacy). Exposure of these IM cells to low dose of FGF9 for two days then led to the formation of Six2+ cells co-expressing other markers of renal progenitor cells. Under induction, these cells underwent development of nephron-like structures including glomeruli with podocytes connected to proximal tubules transitioning to segments resembling distal tubules. While

these organoids are too disorganized to be transplantable, they can nonetheless be used for testing the nephrotoxicity of drugs like gentamycin and cisplatin [74].

By varying the length of exposure to Wnt, and by controlling the timing of exposure to FGF9, the Little group has identified conditions supporting formation of both UB and MM which promoted the formation of self-organizing kidney-like organoids containing few hundred nephrons as well as vessels and interstitial cells [72]. Although these kidney organoids resembled a developing embryonic kidney, their size was restricted by the limited diffusion of nutrients in the absence of vascular supply. In order to overcome these obstacles, Takebe's group has independently embarked on a similar strategy to the one they used successfully to create liver organoids [29, 75]. Mixed aggregates of cells consisting of MM, UB, mesenchymal stem cells, and human umbilical cord endothelial vein cells (or iPSCs-derived endothelial cells) were grown in vitro on unique substrate matrix for a short time. The aggregates were then transplanted intracranially into immune compromised mice. Remarkably, the aggregates self-organized into urine producing, functional nephrons with glomeruli and tubular system and a robust vascular supply thanks to the inclusion of endothelial cells in the aggregate [73]. Similarly, the Nishinakamura group induced host vascularization of transplanted human iPSCs-derived kidney organoid under the kidney capsule of immunodeficient mice by including VEGF in the transplant. Ten days post transplantation, they too observed urine-producing chimeric nephrons containing human glomeruli and mouse endothelial cells [76].

Engineered autologous kidney may one day replace the current allogenic kidney transplantations. As iPSCs share the patient's genome, the autologous transplant should escape rejection (but see below). In the case of an acquired disease as diabetic nephropathy, autologous transplantation using pluripotent cells holds special promise. On the other hand, iPSCs derived from individuals with genetic forms of kidney disease also carry the disease-relevant mutations that necessitated a transplant in the first place. Therefore, these patients may not benefit from a autologous transplantation as they are almost certain to experience recurrence of the disease. One solution to circumvent this is to generate iPSCs from an unaffected matching related or non-related donor, both of which confer a risk of graft rejection. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat)/Cas9 is an emerging gene editing technology may allow correction of genetic lesions with no further changes to the genome. By introducing a double-strand break at a precise location in the genome, this technology leverages the machinery bacteria use to defend themselves against viruses to achieve precise genome editing by an RNA-guided enzyme described by Jennifer Doudna and Emmanuelle Charpentier [77]. CRISPR is dependent upon Cas9, a bacterial endonuclease capable of introducing double-stranded breaks in DNA, and a synthetic guide RNA (gRNA) matching to a desired location in the genome. The gRNA determines the position of Cas9 and thus the double-strand break by hybridization to its matching sequence [78–81]. Nearly any locus in any genome can be edited using this method. The application of CRISPR could mean that a deleterious genetic mutations or a small deletion affecting the kidney could be corrected after creation of iPSCs from patients carrying the mutation in a matter of few months. The corrected cells could then be used to build organs following the advances stated above. Importantly, a federal biosafety and ethics panel has recently approved the use of CRISPR technology in humans. The first in human study aims to

modify T cell lymphocytes to enable immunotherapy in patients with melanoma, multiple myeloma or sarcoma. If successful and risk-free, the method may be approved for broader use for patients including those with genetic forms of kidney disease.

The road ahead: remaining challenges

The development of human organoids already affords us the opportunity to study aspects of renal biology in human tissue or to complement rodent disease models. Clearly, if a disease-causing allele results in measurable effect in the organoids, it would be the system of choice to for mechanistic studies and development of therapeutic strategies. The ultimate goal however is to use iPSCs to create transplantable organs, and there are some significant obstacles yet to overcome.

First, if any pluripotent cells remain in the organoid, the risk for teratoma formation is a concern. Thus, before patients will be permitted to receive iPSC-derived organs, grafts will need to be demonstrated to be free of undifferentiated iPSCs. One strategy to achieve this is to use antibodies or small molecules [82] to selectively eliminate pluripotent stem cells from differentiating cultures. Second, there are reports that in syngeneic mice, some iPSC derived tissue may still evoke an immune response [83] raising the concern that survival of iPS derived kidneys might still require immunosuppression [84–86]. Third, good manufacturing practices (GMP) for generation, maintenance, expansion and renal differentiation of human iPSCs will have to be developed [82]. Fourth, quality assessment of the produced organoid and outcome reproducibility metrics will have to be established before such GMP produced organs can be used in the clinic. Fifth, to have a real therapeutic potential, the organ will have to develop in the recipient more than $1x10^5$ nephrons, all draining into a single collecting system. This level of organization and scale are yet to be achieved. Sixth, a functioning kidney has evolved a precise anatomical architecture including an osmotic cortico-medullary gradient which may require stromal progenitors. It is unclear if organoid-derived kidneys will be able to develop such a gradient, and thus, how efficiently will they be in urine production. Even if all that is needed is an extracellular matrix scaffold, seeding an adult decellularized organ with renal progenitor cells is unlikely to succeed since nephrogenesis proceeds at the immature periphery of a growing kidney whereas the adult ECM scaffold might be inhibitory. Alternatively, an inert synthetic scaffold may be impregnated later with interstitial cells or ECM proteins to improve kidney function [87, 88].

Conclusion

Today’s medicine is built on 20–30 year old discoveries (for example, CRISPR was discovered by Yoshizumi Ishino in 1987, [89]). Today’s discoveries – generation of pluripotent human stem cells coupled with the ability to convert these cells to kidney cell types and sophisticated gene editing – open new horizons in research, which may bring “replacement” human kidneys to the clinic in several decades.

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Key points

- Chronic shortage in transplantable kidneys motivates investigators to identify substitutes.
- Methods for growing transplantable autologous kidney will emerge from improved understanding of kidney organogenesis and from protocols directing the differentiation of iPSCs into urine-producing kidney organoids.
- Genome editing technology will allow correction of congenital mutations impacting kidney function and/or health in the iPSCs.
- Although significant advances have been achieved, many hurdles remain.