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Recapitulating kidney development: progress and challenges

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Abstract

Decades of research into the molecular and cellular regulation of kidney morphogenesis in rodent models, particularly the mouse, has provided both an atlas of the mammalian kidney and a roadmap for recreating kidney cell types with potential applications for the treatment of kidney disease. With advances in both our capacity to maintain nephron progenitors in culture, reprogram to kidney cell types and direct the differentiation of human pluripotent stem cells to kidney endpoints, renal regeneration via cellular therapy or tissue engineering may be possible. Human kidney models also have potential for disease modelling and drug screening. Such applications will rely upon the accuracy of the model at the cellular level and the capacity for stem-cell derived kidney tissue to recapitulate both normal and diseased kidney tissue. In this review, we will discuss the available cell sources, how well they model the human kidney and how far we are from application either as models or for tissue engineering.

Keywords

pluripotent stem cell; directed differentiation; kidney development; kidney organoid; transdifferentiation; human nephron progenitor

1. Introduction

Chronic kidney disease (CKD) describes the progressive deterioration of kidney function due to a variety of primary and secondary kidney diseases. The prevalence of CKD is rising worldwide and represents a significant burden of morbidity, mortality and healthcare expenditure [1–3]. In 2015, the total Medicare expenditure for all CKD and end-stage kidney disease (ESKD; requiring dialysis or transplantation) in the United States alone exceeded

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US\$98 billion [1]. For many patients CKD progresses to ESKD over years or decades, providing ample opportunity for potential therapeutic intervention. Unfortunately there exists a paucity of disease modifying treatments due to a limited understanding of disease pathobiology for most causes of CKD.

Over the last two decades there has been an interest in augmenting existing treatments for ESKD with novel regenerative medicine approaches. Indeed, a number of approaches have been proposed as options for renal replacement, including adult porcine or embryonic kidney xenotransplantation [4, 5]. Additional approaches envisaged have included bioprinting [6], renal assist devices in which primary cells were seeded on hollow fibre [7, 8], recellularization of decellularized scaffolds [9] and direct cellular therapy, including the use of mesenchymal or bone marrow derived cell types [10]. With the isolation of human embryonic stem cells in 1998 [11] came the prospect of recreating human kidney tissue from this human pluripotent stem cell type. Significant advances in cellular reprogramming, including the capacity to generate induced pluripotent stem cells (iPSC) from any adult somatic cell type [12, 13] and the capacity to enforce cellular transdifferentiation via the overexpression of key pioneer transcription factors [14–16] is beginning to provide additional approaches for the generation of cells for use in renal regeneration. In addition, continuous improvements in our understanding of kidney morphogenesis, and particularly the lineage relationships of the cells within the developing kidney, are also providing approaches for the isolation, maintenance and differentiation of progenitor cell types.

In this article, we will review the current situation with respect to potential cellular sources for renal regeneration, focussing most particularly on the directed differentiation of human pluripotent stem cells (hPSC) to kidney cell types. We will address the key questions of how accurately these recapitulate the human kidney and what challenges remain to using such cell types for disease modelling and regenerative medicine.

2. Embryological origins of the mammalian kidney

In order to recreate, maintain and expand human kidney tissue or individual kidney cell types, a detailed understanding of normal kidney development is required, including specific patterns of gene expression, key cell-cell interactions and critical signalling pathways. Renal regeneration protocols are underpinned by decades of work studying mammalian nephrogenesis predominantly in non-human models (reviewed in [17]). The permanent kidney in mammals, the metanephros, is mesodermal in origin, forming from the cells of the metanephric mesenchyme (MM) and an epithelial side branch of the nephric (Wolffian) duct referred to as the ureteric bud (UB). Both the MM and UB are derivatives of the intermediate mesoderm [18]. The metanephros is connected to the nephric duct via the ureteric bud, which branches from the nephric duct and grows towards the MM in response to the production of glial-derived neurotrophic factor (GDNF) by the MM [19]. Having reached the MM, this UB undergoes extensive rounds of dichotomous branching to form a ureteric epithelial tree. This branching occurs again in response to continued GDNF production by a restricted mesenchymal population, the cap mesenchyme. In both mouse and human, this cap mesenchyme expresses the *Six2/SIX2* transcription factor [20]. Lineage tracing studies in mouse have shown that this *Six2*-expressing cell population gives rise to all cells of the

epithelial nephrons [20]. Hence, the cap mesenchyme represents a nephron progenitor cell (NPC) population responsible for all nephron formation. In human, it has recently been confirmed that the cap mesenchyme population has a transcriptional signature similar to mouse, with the notable addition of *SIX1* expression, which is not evident in the same cell type in mouse [21]. The triggers to remain as an NPC or to commit to forming a nephron both come, at least in part, from the tips of the branching ureteric tree. More than a decade of research in mouse suggests roles for FGF9, FGF20, non-Smad BMP7 signalling and low canonical Wnt signalling as facilitating NPC maintenance [22–24] while higher levels of canonical Wnt signalling via Wnt9b and subsequently Wnt4 [25, 26], possibly coupled with the initiation of Notch signalling [27, 28], triggers a mesenchyme-to-epithelial transition (MET) initiating nephron commitment. It is this body of knowledge that has assisted in the development of methods for the isolation and culture of NPCs from mouse and human fetal tissue [29–33] as well as approaches for directing the differentiation of pluripotent stem cells to kidney [16, 34].

Following MET of induced cap mesenchyme, the morphological sequence of mammalian nephron development is well established. A pre-tubular aggregate of mesenchyme develops into a renal vesicle, an epithelial structure which becomes polarised, develops a lumen, invades the distal end of the ureteric tip forming a connecting segment and subsequently elongates away from it [35, 36]. Morphological evidence of proximal-distal nephron patterning becomes evident as it elongates it forms into a ‘comma shape’ and an ‘S-shape’ body [37]. The primitive glomerulus and distal tubule juxtapose and the tubular loop between them extends almost clonally into the medulla from an *Lgr5+* cell cluster within the medial S-shaped body [38]. Proximal-distal expression patterning has been characterised for many genes in both the pretubular aggregate [39] and the renal vesicle prior to the formation of the connecting segment [36, 40]. A decreasing Wnt signalling gradient, strongest at the ureteric tip and progressively decreasing towards the primitive glomerulus interacts with Notch signalling to pattern the nephron.[41, 42]

Around the branching UB and forming nephrons is the forming vasculature and the renal interstitium, which gives rise to mesangial and perivascular cell types [18]. While less is known about the lineage relationships and origins of these non-epithelial components of the kidney, there is strong evidence that the patterning and morphogenetic events in the kidney are influenced by these cell types. Notably, the loss of expression of the *Foxd1⁺* cortical stroma resulted in what is referred to as the ‘stromaless’ mouse in which the NPC population is greatly expanded and sustained in an undifferentiated state [43]. The recreation of human kidney tissue is also, therefore, likely to require an appropriate stromal population, some of which will also arise from the MM. Again, our understanding of the relevance of murine markers of the interstitial compartment in human kidney development is only just beginning to be investigated. In three landmark publications, the morphology, protein and gene expression of the human trimester 1 and early trimester 2 human kidney have been characterised [21, 44, 45]. What these initial studies show is that while many markers characteristic of cell type or state in developing mouse kidney are present in the developing human kidney, other genes are either not present or mark a potentially subtly different cell population [21, 44, 45]. While incomplete for all cellular components and stages of

development at this point in time, these are critical datasets upon which the renal regeneration field must rely.

2.1. Formation of the pronephros and mesonephros

As the embryological origin of the mature adult kidney, the metanephros represents the focus of renal regenerative science. However, the metanephros represents the third pair of excretory organs arising from the IM in a rostrocaudal sequence as the body plan elongates. Both the pronephros and mesonephros form epithelial tubules similar in histological structure to the nephrons that form within the metanephros. Mouse mesonephric tubules undergo patterning and segmentation similar to that seen in metanephric tubule formation [46]. While there is evidence that mesonephric tubules play a role in early filtration and solute transport [47], both the pronephric and mesonephric tubules degenerate as the nephric duct extends along the elongating body plan.

Within an embryo the distinction between mesonephric and metanephric tubules is ultimately made on anatomical grounds. Even in mouse, there are few defining features, other than the absence of a mature loop of Henle, that distinguish mesonephric from metanephric tubules. Whilst the formation of a *Six2*⁺ NPC population appears to be unique to the metanephros [20], the repetition of form between tubules arising from the pronephros mesonephros and metanephros provides some challenges for the identification of regenerated human kidney cell types *in vitro* (elaborated in Section 3.6). Nevertheless, many of the approaches used for isolating, recreating or maintaining human renal cell types draw heavily on our understanding of murine kidney development.

Cellular sources for renal regeneration—To recreate human kidney tissue or renal cell types for therapy, disease modelling or drug screening, there are three possible sources of kidney cell types; isolated human fetal progenitors, directly reprogrammed cells and human pluripotent stem cells (hPSCs) (Figure 1).

2.2. Isolation and maintenance of nephron progenitor populations

While the nephrons arise from a nephron progenitor population within the developing kidney, this population is terminally differentiated prior to birth in humans [48, 49]. Given the absence of a nephron progenitor population in the postnatal human kidney able to regenerate entire nephrons, there have been a number of attempts to isolate human nephron progenitors from human fetal kidney [50, 51]. This work began with studies into the transplantation of human fetal kidney [52]. Further studies into markers of the progenitor population within the developing human kidney revealed a capacity to selectively enrich for *SIX2*⁺ NPCs based on elevated NCAM and lack of CD133 surface expression [32]. By adopting culture in media developed for the maintenance of murine NPC *in vitro* [29, 30], our group has recently reported robust expansion of human NPC [31]. Single cell profiling of this population, using a restricted biomarker panel approach, showed evidence of mesenchymal uncommitted and committed NPCs, but also revealed that almost immediately upon *in vitro* culture the NPC fraction formed a substantively heterogeneous population of cellular states.

Having isolated nephron progenitors, the next challenge is their maintenance in culture (Figure 1). This requires not only evidence of maintained NPC marker expression but also evidence of a prolonged capacity to form nephrons when triggered to do so. Despite our extensive understanding in mouse of NPC markers/gene expression and the pathways involved in triggering nephron formation, the *in vitro* maintenance of this cellular population away from its niche within the developing organ has been a major challenge for the field. A significant breakthrough occurred with the identification of a role for FGF9 and FGF20 in nephron progenitor maintenance in vivo [22]. In addition, while canonical Wnt signalling had been identified as a critical trigger for the transformation of nephron progenitors to nephron epithelia, Karner et al [24] showed that low levels of canonical Wnt signalling were also required for nephron progenitor maintenance. Finally, following a number of studies examining the role of canonical and non-Smad mediated BMP signalling in kidney development [23], Brown et al recently developed media conditions in which they could maintain and expand nephron progenitors isolated from the nephrogenic zone of the developing mouse kidney by facilitating appropriate Wnt, FGF and BMP signalling [29]. At the same time, Tanigawa et al developed similar media that was shown to support isolated NPC [33, 53]. Finally, Li et al [30] reported another media combination able to robustly maintain NPC isolated from either mouse or human developing kidney. While each of these maintenance media contained unique components, there was substantial overlap (Table 1). These advances in protocols for the maintenance of NPC provide approaches for expanding appropriate cell sources for renal regeneration. The provision of a format or scaffold for the therapeutic use of such cells remains to be addressed.

2.3. Direct reprogramming to renal endpoints

Direct reprogramming refers to the direct conversion from one cellular identity to another as a result of the enforced expression of key pioneer transcription factors. This is a second possible approach for the generation of kidney tissue (Figure 1). While the proof of concept was demonstrated by Davis et al [54], where the overexpression of MyoD in a fibroblast was sufficient to reprogram this cell into a myoblast, the ultimate example of direct reprogramming is the generation of induced pluripotent stem cells from any adult somatic cell type via the enforced expression of 4 transcription factors (*OCT4*, *SOC2*, *KLF4*, *CMYC* [12] or *OCT4*, *LIN28*, *NANOG*, *SOX2* [55]). Only a small number of studies have attempted to directly generate kidney cell types using enforced transcription factor expression. Both of these studies have again drawn on the fundamental understanding of normal kidney development in the mouse.

In 2013, Hendry et al performed a lentiviral screen of 15 genes in an attempt to convert an adult human proximal tubule cell line (HK2) into a nephron progenitor state. Six genes were required to generate a cellular state in which there was spontaneous re-expression at the gene and protein level of other nephron progenitor markers and an enhanced capacity for the resulting cells to contribute to the nephron progenitor pool of the developing mouse kidney.

In a second approach to direct reprogramming to kidney, Kaminski et al [34] profiled developing mouse tissues seeking transcription factors with high absolute and relative expression in kidney compared to other tissues. They then selected genes with evolutionary

conserved expression in developing kidney and some previous evidence of a role in human or mouse kidney disease. Again using a pooled lentiviral approach, they identified a requirement for 4 genes, *EMX2*, *PAX8*, *HNF1B*, and *HNF4A*, to transform mouse embryonic fibroblasts to induced renal epithelial cells (iRECs). This transformation resulted in cell types able to integrate into dissociated mouse kidney *ex vivo* and showed evidence of renal epithelial gene expression and sensitivity to nephrotoxic agents, including gentamycin and cisplatin. However, the segment specification of the resulting cells suggested expression of genes regarded as marking both proximal and distal tubular segments of the nephron. This may therefore represent reprogramming to a more primitive renal epithelial progenitor state or an artificial cell state not present *in vivo*.

While these represent the only two examples in the literature to date of kidney-related direct reprogramming, as we increase our understanding of the transcriptional profile of the developing human kidney, it is likely that attempts will be made to recreate collecting duct, podocyte and other nephron-specific cell types. The application of such cells for the treatment of renal disease or the regeneration of renal tissue again presumes the identification of an appropriate format for bioengineering or advances in our capacity to deliver and functionally integrate such cells into the endogenous renal parenchyma.

2.4. Differentiation of pluripotent stem cells to kidney

The final source of cells for regenerating human kidney cell types are human pluripotent stem cells (hPSC) (Figure 1). In the ultimate illustration of transdifferentiation, the landmark studies of Takahashi et al [12] and Yu et al [55] demonstrated the capacity to induce the formation of pluripotent stem cells via the introduction of key transcription factors into human fibroblasts. The resulting iPSC displayed the accepted features of pluripotent cells, including a capacity to differentiate towards all three germ layers. While the potential to differentiate such cells to any lineage has been the great promise of the field, this has required the development of protocols specific to each target cell type. While some early studies reported the direct and uniform conversion of human pluripotent stem cells to podocyte [56] and proximal tubule [57], the majority of protocols in the kidney field have applied carefully designed multistep differentiation approaches based upon our existing understanding of kidney development in mouse from the fertilised egg onwards. Successful outcomes have been assessed based upon markers of cell identity defined primarily in mouse. There have now been many protocols described for the directed differentiation of hPSC to kidney tissue [58–68]. While some protocols were developed initially using murine pluripotent stem cells [64], many went directly to hPSC. We will focus here on the most recognised and well characterised protocols, all of which rely upon defined, stepwise differentiation and report the formation of complex multicellular tissues containing cell types and morphological features recognisable as kidney (Figure 2). Many of these protocols involve the formation of 3D structures with multiple cell types. Most describe the formation of segmenting nephrons. All draw on an understanding of embryogenesis (Figure 2). The most recent of these (2017) focusses specifically on the formation of the human ureteric epithelium (Figure 2).

As noted previously, the kidney is mesodermal in origin and hence, from an embryological perspective, kidney development commences with the formation of posterior primitive streak (PPS) which in turn gives rise to the trunk mesoderm. The mediolateral patterning of this mesoderm gives rise to paraxial, intermediate and lateral plate mesoderm, with the coincident expression in mouse of *LHX1*, *OSR1* and *PAX2* marking the IM. What is evident when comparing the protocols in Figure 2 is the congruence in signalling pathways / growth factors being applied for these early patterning events, with the activation of BMP/activin A or canonical Wnt signalling predominant. This is not surprising given prior approaches for mesodermal patterning *in vitro*, as is required for cardiomyocyte or blood differentiation [69, 70]. While the outcomes from these protocols overlap, there is substantial variation in the format of hPSC culture from monolayer culture to embryoid body. However, all protocols involve induction of canonical Wnt signalling and most use FGF9 to pattern to MM. The format used in the Freedman protocol [58] was distinctly different. After an initial expansion of pluripotent cells within Matrigel to form epiblast-like structures comprising pluripotent cells, these cultures were exposed to approximately 1.5 days of CHIR induction followed by culture without added growth factors for 11.5 days allowing for the spontaneous formation of epithelial structures across the monolayer. These were similar to what was previously reported by Takasato et al [66].

The term organoid has now been widely applied to self-organising multicellular tissues generated from hPSC [71]. Our group first shifted from the spontaneous formation of structures across a monolayer [66] to the dissociation and reaggregation of all cells in the culture to form a 3D cellular micromass that was subsequently cultured at an air-media interface [67]. This approach was an homage to the decades of developmental biology studies of the mouse kidney in which the fetal kidney was grown in such a confirmation as an *ex vivo* tissue. The fact that an embryonic tissue can continue to pattern even after enzymatic dissociation and reaggregation was shown for the embryonic mouse kidney in the 1950s [72] and has been an approach employed by ourselves [16, 73] and others [74] to examine the capacity for exogenous cell types to integrate into the developing kidney. The term kidney organoid is now more widely applied to a number of the published protocols.

2.5. Anterior versus posterior IM derivatives

Cells contributing to the trunk mesoderm do so across time. As the embryo extends, cells patterned as IM include those which are anterior, (early IM), and posterior, (late IM) [64, 75]. The most anterior IM (AIM) gives rise to both the nephric duct (Wolffian duct) and the pronephros whereas the metanephric mesenchyme forms from more posterior IM (PIM). As the collecting duct is a side branch of the nephric duct, this could either be viewed as an AIM structure or an epithelial side branch arising alongside the PIM, depending upon whether one views nephric duct extension as occurring via elongation or rearrangement of IM cells. However, lineage tracing supports the view that the UB arise from the AIM lineage [64]. Hence, there is a clear dichotomy of viewpoint between Taguchi et al [64] and Takasato et al [67] with the former suggesting it is not possible to form both the nephron and the collecting duct elements of the metanephros simultaneously while the latter has chosen an intermediate patterning to achieve both. The tubular structures arising from the protocol of Taguchi et al [64] required induction by co-culture with spinal cord and formed strongly

proximalised nephrons. Takasato et al describe the presence of glomerular (NPHS⁺WT1⁺), proximal tubule (LTL⁺ECAD⁻), distal tubule (ECAD⁺GATA3⁻) and collecting duct (GATA3⁺ECAD⁺) segments [67]. There is some contention in the field as to whether GATA3 is restricted to the collecting duct or also present in the connecting segment, as is the case for other markers in the mouse [76]. Indeed, our own single cell analysis of E17.5 mouse kidney shows a collecting duct expression pattern for a subset of the distal tubules [77]. Taguchi et al [65] have now reported a method for the targeted generation of AIM for the patterning to collecting duct, describing intervening gene expression marking Wolffian/nephric duct and ureteric bud. This protocol involves a CXCR4⁺KIT⁺ enrichment step at day 6.25 in order to enrich for UB-forming cells. The latter can be cultured in Matrigel and shows the formation of a branching epithelium with RET⁺ tips. Using a protocol developed for mouse, they combined ureteric bud cultures with nephron progenitor cultures and interstitium to recreate a 3D structure with collecting duct and nephrons. This was described as a 'higher order' kidney. Other groups have also described protocols for generating collecting duct, including Mae et al [61] and Xia et al [68]. While these vary slightly, Mae et al [61] and Taguchi et al [61] draw upon the published requirement for FGF and RA for Wolffian duct induction and GDNF for RET upregulation and branching. While the approach of Taguchi et al [65] to recombine collecting duct with nephron progenitors and stroma worked for mouse, similar results were not achieved using hPSC. However, the proposed recombination of UB with MM would provide a capacity to more accurately control the placement of each cell type in order to form a more anatomically accurate and hence potentially functionally superior organ model. In contrast, most other methods available allow for stochastic patterning to occur within culture formats that are poor models of the embryo. As a result, while there is evidence in the protocols described here for the formation of structures that mimic aspects of the developing human kidney, there is substantial inherent randomness in the morphology of what arises.

2.6. Cellular diversity and identity within kidney organoids

All protocols currently available for directing differentiation to human kidney show the presence of cellular structures reminiscent of the fetal kidney. Takasato et al [63] was also the first to report the simultaneous patterning of nephrons, collecting duct, endothelium, perivascular and renal stroma within organoids. Several other groups subsequently showed evidence for endothelial cell types [63]. While Takasato et al [61] showed some evidence of invasion of the developing renal corpuscles with endothelial progenitors, as would occur at the capillary loop stage of nephron maturation, this was not a common event. Given the large numbers of protocols published for the generation of kidney cell types from hPSC, there has been little objective characterisation of the individual cell types present within hPSC-derived kidney organoids. This has been compounded by the lack of an accurate point of reference given the paucity of data available on gene expression within the normal human kidney, both adult and fetal. Takasato et al [67] reported the global expression profiling of hPSC-derived kidney organoids in comparison to a panel of human fetal tissues, revealing the highest congruence with trimester 1 human kidney. Of note, profiling of organoids prior to nephron formation showed a closer alignment with gonad, the only other IM-derived tissue available at that time. The complexity of the kidney organoids generated using this method at the level of immunofluorescence would suggest >10 identifiable cell types. However, here as with

other protocols, individual cell types have been recognised based upon anticipated protein markers from mouse. What this global profiling does not thoroughly investigate is the presence or prevalence of ‘off-target’ populations or the reproducibility or robustness of the protocol. Organoids are 3D models of embryogenesis and hence may be viewed as ‘targeted’ rather than ‘perfect’ models of development. Single cell transcriptional profiling data from hPSC organoids is just now becoming available [77, 78] and will be critical both to better identify and validate the individual cell types present. This will allow us to not only understand what is being accurately modelled but also unintended end-points. With the recent data from Lindstrom et al characterising the structure and gene expression of normal human kidney [21, 44, 45], there is now a reference point for comparison. In addition, we [79, 80] and others [61, 65] have described a number of hPSC reporter lines which will enable the isolation and transcriptional profiling of individual cell types from within the kidney.

2.7. How well do hPSC-derived kidney models represent the human kidney?

The recent comprehensive studies of histology and gene expression during early (first and second trimester) human kidney development [21, 44, 45] has begun to provide a more secure basis upon which to evaluate and potentially improve protocols for the generation of human kidney tissue from hPSC. While these studies show strong congruence with mouse, they also note some differences between mouse and human both at the level of anatomical structure and gene expression. What is of great promise is that most of the landmarks that have been used to identify kidney cell types formed from hPSC *in vitro* are conserved. There remain many human kidney cellular components yet to be fully analysed at the transcriptional level and such studies will continue to form the foundation for our understanding of what we hope to regenerate. However, these studies did raise specific questions around the accuracy of what is present in our hPSC-derived kidney models to date.

Lindstrom et al [21, 44, 45] estimated that the transition of a renal vesicle to a capillary loop stage nephron takes in the order of 3 to 10 days. This is consistent with the time taken for a nephron to arise and segment within a kidney organoid generated using the Takasato et al [67] protocol. Here, epithelial elements are evident from around Day 12 and reach capillary loop stage from Day 18. While this timing is consistent with what is observed in the developing human kidney, the human metanephros itself does not begin to arise until week 5 of human gestation. Most protocols for directed differentiation to kidney describe the formation of PPS / IM and MM within 10 days from commencement of differentiation (Figure 2). The human pronephros is reported to arise from 3–4 weeks of gestation (22–28 days) while the mesonephros arises from day 33 (Carnegie stage 14). Hence, the pace at which nephrons are arising *in vitro* is premature for any of these three excretory structures. Should we anticipate a chronology *in vitro* reflective of human gestation? There are now protocols for the directed differentiation of hPSC to many tissue types, including those derived from ectoderm (brain, optic cup), endoderm (intestine, stomach, colon, pancreas) and mesoderm (cardiomyocyte, blood). It would be difficult to argue that any of these protocols replicate a gestational clock. There are also a growing number of protocols in which either adult somatic cells (often fibroblasts) or pluripotent stem cells are directed to adopt another lineage via the enforced expression of key pioneer transcription factors. This

clearly does not involve an accurate embryological clock. Additionally, it has yet to be established whether *in vitro* nephron structures follow a similar developmental morphological sequence as that which has been well characterised *in vivo*, that being polarising renal vesicle, comma shaped body, S shaped body, and so on. What *is* fair to say is that the pluripotent stem cell field believes the accuracy of what they have generated based on their understanding of the tissue they wish to build, even when that tissue has not yet been comprehensively analysed using human fetal material. The proof will reside in the functional utility of such cells.

As noted above, while arising in a temporal sequence across developmental time in mammals, there are no particular anatomical or histological distinctions between pro-, meso- and metanephric tubules. In our own experience, prolonged culture of hPSC-derived structures results in eventual accumulation of regions of extracellular matrix (ECM), expanded stroma and occasionally the overgrowth of off target cell populations such as cartilage. This mispatterning with prolonged *in vitro* culture is most likely due to the inappropriate format of the culture itself (that being a tissue mass >7 mm in diameter at an air-media interface without a patent vascular supply). However, are we sure we are not generating mesonephros? As described above, the murine mesonephric tubules appear to form via a similar series of histological changes as those seen in the metanephric tubules. Hence it is not apparent within an hPSC-derived tissue how to distinguish between a pro-, meso- or metanephric tubule. It has previously been proposed in mouse that mesonephric tubules lacked loops of Henle [81] and that glomerular-like structures are only visible in the cranial mesonephric tubules [82, 83], however expression of genes eventually expressed within the metanephric loop of Henle and distal tubules are expressed within mesonephric tubules [46, 84]. Initiation of the MET required to form the mesonephric tubules is also not well understood, although it is thought that a similar Wnt-mediated mechanism is likely to be involved based on the loss of such tubules in Wnt9b mutant mice. There is also evidence of a requirement for FGF8 / Fgfr1/2 signalling which may differ from metanephros [85]. The use of a canonical Wnt signalling trigger in most currently published protocols (either via addition of CHIR [63, 67] or co-culture with spinal cord [64] to initiate MET within their differentiation protocols) makes metanephric and mesonephric protocols indistinguishable.

One solution to this dilemma would be to look for the expression of unique markers. However, there is almost no information about pronephric development in the human and little information, even in mouse, regarding distinguishing markers of pro-, meso- and metanephros. Indeed, nephron patterning at the level of gene expression in mouse mesonephric tubules is very similar to that observed in metanephric nephron morphogenesis [46]. Knockout / transgenic models in mouse showing loss of some or all mesonephric tubules has been reported for many genes also required for metanephric development. For example, loss of *Wtl* and *Six1* in mouse results in the loss of the caudal mesonephric tubules as well as a loss of metanephric development while a loss of *Gata3* or *Osr1* results in a loss of all mesonephric tubules as well as defects in nephric duct extension [85]. Conversely, studies have sought markers unique to the metanephric mesenchyme. Early cDNA array profiling of mouse mesonephros versus metanephros performed to identify specific markers of metanephric mesenchyme identified *CD24a* (EST: BG076069) and *Cdh11* as restricted to the metanephric mesenchymal region. *Cdh11* expression persisted in

the stroma of the developing kidney while *Cd24a* was evident in the forming nephrons and collecting ducts in mouse [86]. This, however, is not informative for human tissue as *Cd24a* is a murine specific gene (distinct from CD24 in human tissue).

Taguchi et al [64] performed microarray and QPCR analysis of presumptive mesonephric (E9.5) and metanephric nephron progenitors (E10.5–11.5), showing common expression of *Osr1*, *Pax2* and *Six2*, despite the fact that *Six2* had previously been regarded as specific to metanephros. What they did show was increased expression of *Hoxa10*, *Hoxa11* and *Hoxd12* in metanephric progenitors. This concurs with the previous reports of a requirement for Hox11 paralogs for metanephric development [84, 87]. Studies in mouse suggest expression of *Hox11* paralogs (*Hoxa11/c11/d11*) is restricted to the caudal mesonephric and metanephric mesenchyme [84] and, more recently, lineage tracing studies in mouse suggest that this lineage boundary also represents a boundary in which caudal mesonephros / metanephric mesenchyme is *Eya1*-derived [64]. In mouse models, *Brn1* (*Pou3f3*) is proposed to maintain metanephros-specific expression [84, 88] which might assist in differentiating between meso- and metanephric outcomes *in vitro*. Lineage tracing studies of Kobayashi et al [20] in mouse suggest that the expression of *Brn1*, which is conserved in human metanephros, is unique to the MM as a result of regulation by *Hoxd11*. [87] The co-expression of these genes should therefore indicate a metanephric identity. However, this specification in gene orthologue expression to the metanephric tubule has not been established in human models.

As noted above, there has been limited transcriptional analysis of hPSC-derived kidney tissue to date. Takasato et al [67] reported the presence of HOXD11 protein within patterning mesenchyme based upon antibody staining. While expression of all *HOX11* paralogs within these organoids was very low, it increased with time as did expression of *HOXA10*. *CDH11* expression was also present throughout organoid formation while the metanephric-specific tubular genes previously described by Georgas et al [46] (*GCNT1* and *HALPNI*) increased across tubule formation within organoids. A human specific expression of *SIX1* in the nephron progenitors of human kidney is a notable difference between the mouse and human developing kidney, although human nephron progenitors also expressed *SIX2* and *CITED1*. In our kidney organoids [67], expression of all these genes is present at the day of aggregation and declines with time. Morizane et al [89] has reviewed the relative prevalence of *SIX2*⁺ cells between hPSC kidney differentiation protocols. What is still required is a systematic comparison of protocols at the single cell level. The challenge here is that greater insight into gene expression of these earlier stages of nephric development is not likely to be forthcoming due to a lack of access to such early human material. Human material acquired at spontaneous or elective termination is week 8 of gestation at the earliest, whereas the human pronephros arises at week 4 and the mesonephros at week 5 and regresses by week 8. Indeed, resolving this issue definitively will likely require *in vitro* lineage tracing within organoids, similar to have been performed in studies of human pancreatic patterning using CRISPR-Cas9 edited iPSC lines [90].

2.8. Lessons from *in vivo* organoid transplantation

A final yardstick to evaluate the tissue identity of what has been generated *in vitro* is function.

Taguchi et al [64] transplanted hPSC-derived nephrogenic mesenchyme along with spinal cord under the kidney capsule of immune deficient mouse, with the transplanted tissue forming a kidney-like tissue along with vascularized glomeruli. Sharmin et al [91], using the same differentiation protocol, developed an approach for transplanting hPSC-derived nephrons under the renal capsule along with a source of recombinant VEGFA to assist in attracting underlying endothelial sprouting. More recently, van den Berg et al [80] demonstrated that kidney organoids generated using the Takasato protocol [21, 44, 45] were producing endogenous VEGF (Figure 3). Transplantation of these structures under the renal capsule of immunocompromised mice resulted in substantial maturation of all segments of the nephrons. Wide field transmission electron microscopy revealed the formation of glomerular capillaries lined with fenestrated endothelial cells together with evidence for peritubular capillary formation (Figure 3). While prior to transplantation, renal tubules showed a pseudostratified epithelial morphology reminiscent of that observed by Lindstrom et al [44] in early human kidney, 2 weeks of *in vivo* maturation resulted in resolution of the tubular epithelium into a tight monolayer of polarised cells with ultrastructural features identifiable as characteristic of proximal tubules (mature brush border) and distal tubule / collecting duct (low cuboidal epithelium with evidence of occasional intercalated cells) (Figure 3). Of note, the same ultrastructural evidence of maturation was not achieved with extended *in vitro* culture. Imaged via a transabdominal window using intravital imaging, it was also possible to visualise murine blood (labelled with FITC-dextran injected into the tail vein) flowing through capillaries within human organoid glomeruli, the latter readily identifiable by virtue of a blue fluorescent protein CRISPR-Cas9 engineered into the MAFB gene locus of the starting iPSC cell line [80]. Using a second reporter line in which mCherry was expressed from the SOX17 locus, it was also possible to see evidence of murine blood flowing through human iPSC-derived endothelial cells, suggesting that at least a portion of the host derived vascular plexus joined with human endothelial progenitors present within the organoids. A second study has transplanted kidney organoids under the skin and also showed evidence of vascularisation from the host [92]. Together these studies would suggest that the presence of a patent vasculature improves the maturation of the hPSC-derived tissue. In summary, while the debate may continue around the specific identity of the cell types present in hPSC-derived kidney tissues, there is growing evidence that these structures do represent a model of kidney with potential utility. It is likely that with time these approaches will undergo continued tweaking and modification, both to improve the reproducibility and identity of the tissue being generated, reduce the formation of ‘off target’ cell types and modify the culture format to better facilitate applications in disease modelling, drug screening of tissue engineering.

3. Disease modelling using hPSC-derived kidney cell types

Chronic kidney disease can take years or even decades to progress to end stage renal disease, providing ample opportunity for potential therapeutic intervention. In spite of this, there

exists a paucity of disease modifying treatments. This is in large part due to a limited understanding of molecular pathobiology for most causes of CKD. To date, the use of immortalized cell lines or model animals has underpinned research into kidney disease pathobiology. Mouse models offer a mature and functional mammalian organ within a whole organism providing the opportunity to examine both the renal and non-renal phenotypes. However, as described above, considerable interspecies variation exists in development, anatomy, gene orthologue function and physiology [93, 94]. This variation impedes the translation of findings back to the treatment of human kidney disease in clinical practice. The *in vitro* study of primary patient-derived cells is thus preferable, but is limited to cell types that are readily cultured from patient biopsies, such as dermal fibroblasts or peripheral blood mononuclear cells. This cannot provide a model where cell-type specific gene expression or phenotype is required and thus the study of kidney disease necessitates a kidney model. Techniques for the isolation of human urine-derived renal epithelial cells (HURECs) have proven useful for the study of ciliopathies [95, 96], however HURECs have a limited capacity for self-renewal and represent a unicellular, two-dimensional model of a complex, multicellular organ.

Compared to these traditional animal and cell-based models of kidney disease, hPSC-derived kidney models, such as kidney organoids, present distinct advantages as a disease modelling platform. Kidney organoids derived from patient iPSC cells allow regeneration of unlimited amounts of patient-specific, multicellular, three-dimensional kidney tissue. As multicellular tissues, kidney organoids also provide the opportunity to interrogate the contribution of different cell types to disease, assisting in the targeting of novel therapies. In addition, compared to two-dimensional culture systems, three-dimensional culture systems demonstrate differences in proliferation, cell-cell and cell-junction interactions, morphology and gene expression that are likely to be highly relevant to the study of human disease [97–99]. Kidney organoids represent multicellular models of the developing organ in question. While this can presumably result in appropriate cell-cell signalling and maintenance of improved identity of individual component cell types, the utility of such structures for disease modelling needs to be evaluated.

Transcriptional profiling of kidney organoids has suggested greatest similarity to trimester 1 human fetal kidney [67]. This would infer that diseases with early- or antenatal-onset phenotypes represent better disease modelling candidates than late-onset diseases.

However, successful disease modelling of age-related diseases such as Parkinson's and Alzheimer's diseases in iPSC-derived neuronal models [100], as well as the observation of antenatal phenotypes for classically adult onset diseases such as autosomal dominant polycystic kidney disease (ADPKD) [101, 102] does suggest that modelling of late-onset diseases may be feasible. Indeed, the first use of kidney organoids for inherited renal disease modelling used iPSC in which CRISPR-Cas9 gene editing generated a homozygous *PKDI*^{-/-} gene knockout in an attempt to model ADPKD. Whilst not patient-derived, these organoids demonstrated epithelial cystic dilatation after extended culture [58] and subsequent manipulation of culture conditions was reported to alter cyst formation metrics [103]. This suggests that the use of hPSC represents a potential model for the investigation of cystogenesis in ADPKD [103] to which patient derived epithelium could be applied.

3.1. Application of hPSC-derived kidney models to genetic renal disease

Genetic renal diseases (GRD) currently represent the strongest likely utility for kidney organoid disease modelling. Whilst advances in genomic diagnostics are increasing the overall prevalence of GRD in both adult and paediatric ESKD, many GRDs are rare and thus difficult to study in a clinical context [104, 105]. With the increasing discovery of rare, novel, genomic variants of unknown significance (VUS) by next generation sequencing, kidney organoids represent a ready platform for validation of VUS as being disease-associated. We have demonstrated that the genetic ‘correction’ of a candidate VUS using CRISPR/Cas9 technology can be performed concurrently with reprogramming to generate patient iPSC and gene corrected (or ‘isogenic’) controls from one transfection event [106]. Isogenic clones are currently the gold standard control in the field and provide a comparison whereby any phenotypic variation detected is highly likely to be related to the gene correction event rather than a shift in genomic background between lines. Our own laboratory has utilised this approach to generate iPSC from a patient suffering from the heritable ciliopathic cystic kidney disease, nephronophthisis (NPHP), arising in this patient due to compound heterozygous *IFT140* variants. (Figure 4B) As well as generating patient-lines in which point mutations in the *IFT140* gene were present in both alleles, CRISPR-Cas9 gene correction was applied to one mutant allele, resulting in a gene-corrected heterozygous (*IFT140*^{+/-}) control [107]. When compared to gene corrected organoids, patient organoids demonstrated primary ciliary dysmorphology (Figure 4C) and a downregulation of gene expression involved in apico-basal polarity, cell adhesion and dynein motor assembly [107]. Whilst this genotype is not novel, the observation of previously validated cellular and transcriptional phenotypes for the condition within a patient derived organoid demonstrates the fidelity of the disease modelling platform.

3.2. Modelling glomerular diseases using hPSC-derived kidney tissue

While modelling of cystic kidney diseases and nephronophthisis bode well for the application of hPSC-derived kidney tissues for modelling heritable kidney diseases that affect tubular epithelium, an accurate model of the glomerulus is more challenging. Congenital nephrotic syndrome frequently arises as a result of mutations in podocyte-specific genes, which encode proteins regulating the podocyte cytoskeleton and the formation of foot processes and the slit diaphragm. There is growing evidence that a subset of steroid sensitive as well as steroid resistant nephrotic syndrome is the result of an as yet unidentified circulating factor. To model this, or any other immune mediated glomerulonephritis, accurately would require a patent circulatory system. Whilst iPSC derived glomeruli can be relatively easily isolated from whole organoids (Figure 4A and D), they lack a functional vascular system.

Glomerulopathies in which the underlying disease occurs as a result of mutations in the extracellular proteins of the glomerular basement membrane (GBM) will also not be feasible to model without the formation of a mature GBM. This requires the presence of the fenestrated endothelial cells of the glomerular capillaries. In many of these disease, the GBM defect arises due to the lack of an appropriate switch in collagens (Alport syndrome) or laminins (Pierson syndrome) that only occur during glomerular maturation and hence may not occur in an *in vitro* model. As discussed above, several groups have now

demonstrated that hPSC-derived kidney tissue can be transplanted *in vivo*, including under the renal capsule or subcutaneously, resulting in the ingrowth of host-derived vasculature and the formation of patent glomerular capillaries [80, 91]. A requirement for *in vivo* transplantation for disease modelling makes this a long and slow process that is not tractable to compound screening. Hence, advances are required to facilitate not only an improved glomerular capillary formation *in vitro*, but the delivery of flow. One approach to this has been the seeding of hPSC-derived podocyte-like cells and primary human glomerular endothelial cells on either side of a laminin coated membrane within a dual channel microfluidic device capable of producing strain on the membrane [108]. In this model, cyclical mechanical strain of the membrane during microfluidic flow increased collagen IV and VEGF production by cultured podocytes as well as interaction between podocyte foot processes and the endothelium [108]. Adriamycin toxicity was modelled by increased permeability of the system to albumin [108] advocating an *in vitro* platform more amenable to compound screening.

3.3. Applications for hPSC-derived kidney tissue in precision medicine.

One anticipated prospect of regenerated, patient-derived kidney tissue is that of high-content compound screening to inform personalised therapeutic decisions for patients. No such example currently exists and this is likely to be due to deficiencies in the understanding of disease pathobiology and the ensuing difficulty in designing assays that can provide a rapid and clear output. As a prelude to patient-derived high throughput compound screening, forskolin-treated 3D spheroid cultures of *PKDI*^{-/-} knockout mouse inner medullary collecting duct (IMCD3) cells utilised a series of spheroid metrics to identify compounds that reduced cyst growth [109]. Potential targets included mTOR, IGF-1 receptor and cyclin dependent kinases, all of which have been corroborated in animal models [109]. With the advent of a variety of approaches for generating nephron-like structures, or even specific kidney cell types, coupled with the increasing utility of gene editing to develop reporter-based readouts amenable to automated quantitative imaging, we may soon see high content approaches being applied to iPSC-derived kidney tissue. This may include siRNA screens to examine the effect of pathway modification on disease phenotype and drug repurposing screens to identify potential treatments. Czerniecki et al have recently published a high throughput kidney organoid model using a liquid handling robot which performed both culture and phenotyping of 384 well plates to streamline analysis of ideal culture conditions for new hPSC lines and using a CRISPR edited PKD1 knockout line identified a potentially novel treatment to attenuate cyst growth [110]. Advances in bioprinting techniques and microfluidic chips have regenerated tubular structures capable of receiving flow, examining the epithelial barrier and trans-epithelial transport [97, 111]. Beyond toxicity screening applications described above, it is yet to be seen whether integrating regenerated, patient-derived renal tubular cells into these systems will advance the maturity of *in vitro* nephrons for downstream disease modelling applications.

3.4. Limitations and challenges to modelling of renal diseases in vitro

Despite the potential application of hPSC-derived kidney tissue for modelling genetic renal disease, current differentiation protocols will remain limited in their ability to model the majority of adult onset kidney disease using patient-derived iPSC. Whilst the addition of

4. Applications of PSC-derived kidney tissues for regenerative medicine

Over the past 20 years, there have been brave pioneers in nephrology preparing the way for alternative approaches to renal replacement. These have included those advocating porcine kidney xenotransplantation [4], fetal xenotransplantation [5], the Charleston Bioengineered Kidney Project [6], recellularisation of decellularised scaffolds [9] and the renal assist device of Professor David Humes [7, 8]. Substantial challenges have arisen with these complex models, which could be attributed to problems with the cell types used as much as with the approach. The regeneration of human kidney cell types using hPSC represents a novel approach with theoretical capacity for the development of autologous or universal renal replacement therapies. Two major approaches to the use of such cells can be envisioned; i) cell therapy, and ii) tissue engineering.

4.1. Cellular therapy

A capacity to generate specific kidney cell types either via direct reprogramming of the directed differentiation of hPSC raises the prospect that delivery of such cells back into a patient may result in functional renal improvement via functional integration. Indeed, given the capacity to generate hPSC from any individual, this may represent a viable source of autologous cell types for therapeutic purposes. As such, this approach should avoid immune rejection. Only a handful of studies have investigated the utility of delivering hPSC-derived cell types into models of renal injury. The direct injection of undifferentiated iPSC into the renal artery during ischemic acute kidney injury (AKI) was reported to reduce damage resulting from oxidative stress [113]. hPSC-derived renal progenitor cells, reportedly expressing *OSR1* and *SIX2*, were shown to reconstitute 3D proximal renal tubule-like structures *in vivo* when injected under renal capsule of ischemic AKI mice [114]. It was reported that the presence of such structures resulted in the formation of renoprotective factors like Ang-1, VEGF and HGF [114]. However, neither of these studies showed evidence of engraftment of injected cells. Imberti et al reported that intravenous injection of iPSC-derived renal progenitor cells during cisplatin induced AKI robustly engrafted into damaged tubular epithelium and restored renal function and structure [115]. This is the only report to date describing the integration of introduced cells resulting functional regeneration of the injured kidney. Given the discrepancy with respect to the fate of the injected cells, differences in the injury model employed and little standardisation of characterisation of the cell types being delivered, further studies are required.

One major challenge lies in the choice of a route for cell delivery. Intravenous injection of cells in murine injury models is frequently performed via the tail vein. Injection into this site will mean the immediate delivery of the injected cells into the microvasculature of the lungs and potential removal from circulation within the spleen. As a result, it is likely that only a few injected cells will reach the kidney. Arriving via the vasculature represents a further challenge with respect to how such cells can enter the renal parenchyma. Alternatives such as direct renal parenchymal injection is suboptimal due to the risk of excessive bleeding and the long term risk of interstitial fibrosis. Transplantation under the renal capsule does not necessarily facilitate tubular integration, but may provide a better route of access than a vascular route. However, injection into the periphery of the renal cortex can result in cellular

integration. This was first shown with the injection of MSC-like cells into the parenchyma of neonatal (day 1) mice [116]. A similar approach was used in recent studies in which fetal murine nephron progenitor cells (NPCs) were injected into neonatal day 1 mouse kidney resulting in the successful engraftment into the tubular epithelium [30]. Injected cells contributed to the formation of chimeric nephrons within the developing kidney suggesting onward differentiation from the nephron progenitor state. Injection of same nephron progenitor cells under the kidney capsule of adult mice during cisplatin induced acute kidney injury (AKI), showed protection in survival and reduced the complication of AKI. Of note, there was no evidence of integration of injected nephron progenitor cells into the injured areas of the kidney [30]. This suggests that the integration of NPCs by adult kidney is completely different compared to the developing neonatal kidney. While this does show the relative utility of NPCs, what remains to be seen is whether a more mature renal cell type will be able to functionally integrate into a postnatal kidney. As protocols for directed differentiation improve or become more targeted towards generating specific cell types, it will be possible to more definitively evaluate the utility of delivering more mature versus progenitor cell types as well as investigating the most amenable temporal or developmental window for successful integration.

4.2. Bioengineering

Tissue engineering refers to the possibility of combining cells and materials to create a tissue substitute that can replace some or all aspects of the function of the original tissue. The source of cells can vary from primary cells harvested from donors to stem cell-derived cell lineages. An ability to generate kidney cell types, from progenitors to specific mature kidney cell types, should allow for the bioengineering of renal replacement tissue. A wide variety of materials have been investigated for their capacity to support cell growth either *ex vivo* or *in vivo*. This has included synthetic polymers [117], natural products such as silk [118] and protein hydrogels [119]. Biological scaffolds such as decellularised organs can also be used as the support for cell seeding. As such, material scaffolds can vary in porosity and stiffness and may both support as well as instruct the cells to maintain or adopt a particular identity. Materials have also been evaluated for their capacity to induce an immune response and/or biodegrade over time. Significant advances in bioengineering tissues have been made for tissues such as artificial skin for the treatment of burns [120], cartilage to implant in articular joint disease [121] and engineered functional heart valves to treat cardiac complications [122]. Progress is also being made on bioengineering organs using pluripotent stem cell-derived cell types cultured within artificial biomaterials [123]. However, complex organs like kidney will need very sophisticated engineering techniques to achieve a fully functional and transplantable organ. The major challenge remains the reintroduction of the epithelial and stromal elements of the organ. As the adult human kidney has in excess of 26 distinct cell types, each with a very specific anatomical location within the organ, this poses a major challenge. Either each cell type or their progenitor/s will be required.

5. Major challenges to kidney cellular therapy and bioengineering

While advances in directed differentiation or transdifferentiation will provide a source of cells to drive such developments, there remain considerable challenges. Three key issues include scale, structure and function.

Scale:

At present in our own directed differentiation protocol an organoid of 500,000 cells (5–7mm in diameter at day 25 of culture) contains 50 to 100 early nephrons (Figure 5). It is also likely to contain a proportion of off target cell populations and, as a multicellular tissue, may vary in relative cellular composition to a much larger extent than an appropriately patterned organ developing *in vivo*. A healthy adult kidney contains approximately 900,000 to 1 million nephrons [124], and an average gross volume of 134 to 146 cm³. This represents a major scale challenge. Were it even possible to recreate such a structure from a single cell type, the challenge is one of cell manufacturing and with that, quality control. The fact that the adult kidney is composed of >25 distinct cell types suggests that scale up is best done at the stage of pluripotency, with patterning and differentiation to follow. However, the utility of the cells generated for recreating a kidney will rely upon their accurate and reproducible patterning to utilisable kidney endpoints. Hence, the field will need to address how to generate these reliably, reproducibly, and at appropriate scale and cost, using suitable manufacturing processes. An alternative is to develop more controlled approaches for the uniform differentiation of specific kidney cell types. There are a number of reports of specific generation of podocytes or proximal tubular cells [56, 57], but the level of characterisation of these protocols (little or no extensive expression profiling or functional analysis) is such that it is currently insufficient to determine purity or commitment. It would be impractical to recreate an entire tissue one cell type at a time, however such approaches allow for the production of specific cell types for use in cellular therapy or for screening purposes. Any cellular production at scale will need to consider how to evaluate mutation accumulation with cell expansion [125]. This is a major topic of discussion in the iPSC field and a number of groups are now developing approaches for the engineering of suicide switches into the hPSC lines to ensure that any tissue showing oncogenic transformation can be ‘killed’ upon command [126, 127]. Even with these issues addressed, the expansion required to recreate a tissue of the scale of the human kidney may be out of reach technically and economically.

Structure:

The second major hurdle will be structure (Figure 5). Currently, the success of isolating, maintaining or generating kidney tissue *in vitro* is being measured based upon the formation of structures with markers reminiscent of early nephrons within the developing fetal kidney. What is rarely commented upon is the overall histology of such cultures. A scan of the available histological sections suggests that while there are recognisable histological features, the majority of progenitor or hPSC-derived kidney tissue contains nephrons that are poorly patterned and dysplastic [67] and parenchyma that is fibrotic [63] and sometimes contain ectopic tissue types, including cartilage [92]. While the transplantation of organoids *in vivo* shows a capacity for the nephrons present to draw in blood vessels, some of these

tissues contain largely glomeruli and even when there is evidence of a distal tubule / collecting duct-like epithelium, organoids do not necessarily have a unified nephron complement. In addition, unlike the kidney, no human kidney organoid to date has a ureteric tree feeding into a pelvis and in turn connected to a ureter.

One approach to the challenge of ‘structure’ in kidney tissue engineering has been the concept of recellularization of decellularized kidney scaffolds. Such decellularized scaffolds have been generated from a wide variety of tissues, including human lung, heart and kidney [128]. The concept here is that the tissue itself provides an accurate large-scale model of the organ of interest with the remaining ECM components providing both a supportive and an instructive environment into which a source of replacement cells can be provided. Many studies have now demonstrated the feasibility of kidney decellularization in rodent models and using human kidney tissue [9, 129, 130]. Decellularized natural scaffolds can be prepared by continuous rinsing of kidney tissue with sodium dodecyl sulphate (SDS) based solution through the renal artery until all cells are removed. This method has been successfully shown using rat [131, 132], non-human primates [133], pigs [9, 134] and cadaveric human kidneys [9, 135]. Further resin casting analysis of entire decellularized human kidney maintain a well-preserved structure and function of the vasculature, as well as growth factors that are fundamental to achieve a satisfactory recellularization [136]. It is also reported that ECM in the scaffold also instructive to the pluripotent cells to differentiate into appropriate adult cell types. Remuzzi A et al. transplanted decellularized rat kidney scaffolds *in vivo*. This resulted in no evidence of recellularization by host, but it preserved the scaffold ECM structure [137]. The major challenges lie in how to reintroduce all the required epithelial cells types into the complex narrow channels of the nephrons within the scaffolds. Indeed, to be able to redeliver renal interstitial cells is also a quandary.

Initial studies into arterial recellularization of rat kidney scaffolds with embryonic stem cells and HUVECs showed 95% retention of cells which were well distributed across the vascular network including the glomeruli [9, 131, 138]. Retrograde seeding of embryonic stem cells or neonatal rat kidney cell suspension under negative pressure, through the ureter via renal collecting duct system resulted in a complete organ recellularization, including glomeruli. Embryonic cell cytology was eventually lost over period and formed the well differentiated flattened endothelial cells, mature glomerular podocytes and tubular cells, confirming scaffold guide pluripotent cell fate [9, 131]. Further orthotopic transplantation into rat showed graft survival and also produced urine [9]. Remuzzi et al attempted to recellularize rat kidney scaffolds with mouse embryonic stem cells via renal artery, vein and ureter applying different pressure and flow systems [137]. They concluded that this resulted in very limited and inconsistent cell seeding [137], due in part to the ECM physical barriers acts as a resistant to the cell migration. Clearly there is a long way to go to recreate an organ of this scale in this way. Indeed, the gap remains between hypothesis and reality.

Function:

The final challenge lies in functional maturation. The adult kidney contains more than 20 different cell types, each with distinct functions, many of which are only achieved as a result of the appropriate arrangement of these component cells. For example, a glomerulus will not

function appropriately unless podocytes form appropriate foot processes, the endothelial cells fenestrate and, together with the podocytes, generate an appropriate glomerular basement, the capillary tuft is supported by mesangial cells and the renal corpuscle is surrounded by a Bowman's capsule with appropriately patterned parietal epithelial cells. Proximal tubules need to develop appropriate transporters and microvilli for maximum reabsorption while specialized distal tubule cells are required to create a functional macula densa. The stalks of the ureteric tree must mature to maximally reabsorb water and regulate acid/base balance whilst the medullary cells must form a water impermeable uroplakin layer. Indeed, the perivascular cells, renal fibroblasts, erythropoietin and renin producing cells are required to deliver the endocrine functions of kidney [139]. While many of these cells do arise from a small number of progenitors, much of the functional maturation of these individual cell types and nephron segments does not occur until the postnatal period. By contrast, the kidney structures being generated *in vitro* from hPSC / progenitor populations are models of the developing organ, not the postnatal structure. It is evident again from recent *in vivo* transplantation studies [80, 91, 92] that the provision of a patent blood supply facilitates substantial structural maturation. It is likely, although it is not yet proven, that this will facilitate functional maturation. The nature of what is required will depend upon the therapeutic approach being proposed. For cell therapy, integration of a progenitor cell type may be more likely than anticipating a capacity for a quiescent and highly differentiated cell to be able to home, integrate and function. Within bioengineering, the target structure does not need to exactly replicate the original tissue, as long as it achieves the desired functional outcome. That target functional outcome may also only represent a subset of the *in vivo* tissue functions and hence only require a subset of cell types.

6. Conclusions

In this review, we have provided an update on the use of human pluripotent stem cells for the generation of human kidney cell types. The progress made to date, while a major leap forward, remains preliminary in contrast to the *bona fide* organ. Indeed, the human adult kidney contains an average of 1 million nephrons, all of which are architecturally aligned to facilitate the countercurrent mechanism essential for concentration of the urinary filtrate. As yet, we have been able to create the fetal cell types and begin to perfuse such structures, but each organoid is effectively dysplastic and very immature. The key challenges include scale, structure and functional maturation, with each representing significant obstacles to renal bioengineering. However, the reproducibility with which fetal kidney cell types can be generated *in vitro* provides a capacity to re-evaluate previously proposed approaches to renal regeneration. It may be argued that the recreation of an organ as architecturally complex as the kidney *in vitro* is simply not feasible. Indeed, it is unlikely that this will be possible. However, recreation of a perfect replica of the kidney is not the only solution. At present, only 1 in 4 renal failure patients receives a transplanted organ. All other patients survive on peritoneal or haemodialysis which replaces at best 15% of normal glomerular filtration rate. The provision of a cellular replacement capable of providing better than what is currently afforded by dialysis, even if only transiently, is perhaps a more realistic target. Indeed, this was the objective of the renal assist device in which primary kidney epithelial cells from cadaveric tissues were seeded allow hollow fibres and provided in line with dialysis for the

treatment of acute kidney injury [7]. The development of that product reached clinical trial but did not proceed. Reevaluating such approaches with currently available cell sources is not without merit. And while therapeutic targets are far off, even with the imperfect kidney tissue that we can generate, we now have a window into human development and disease previously unavailable. The capacity to generate patient-derived kidney cell types for drug efficacy and toxicity screening is a major advance. It is here that stem-cell derived human kidney models are most likely to change clinical practice.

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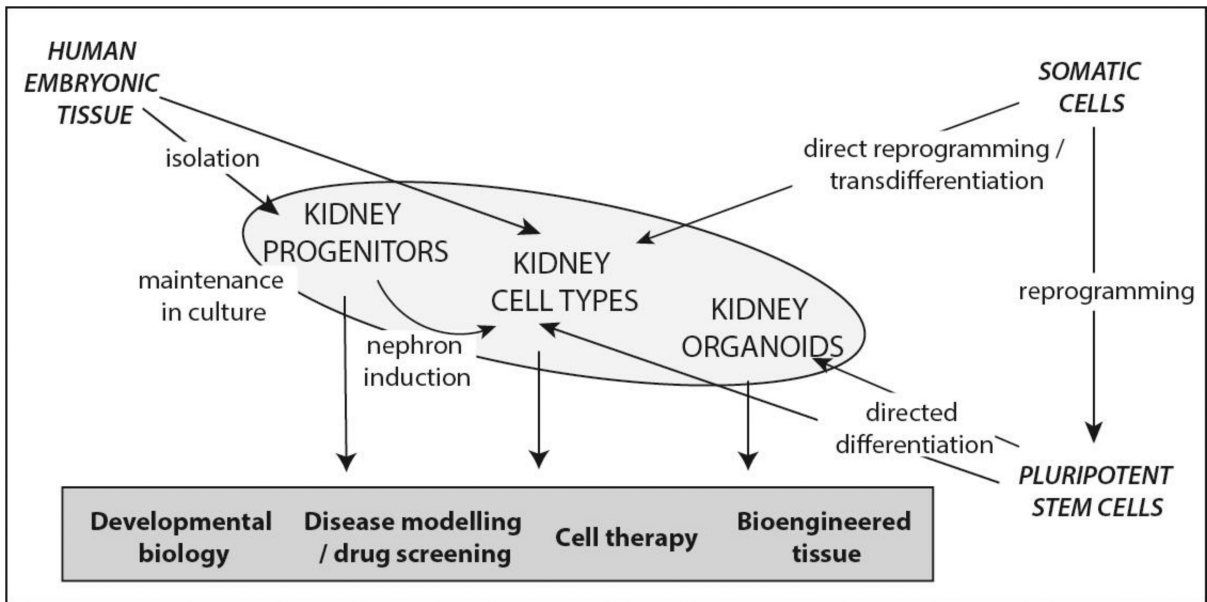


Figure 1:
 Summary of the sources of human cells and tissues available for studying kidney development, disease modelling and renal regeneration.

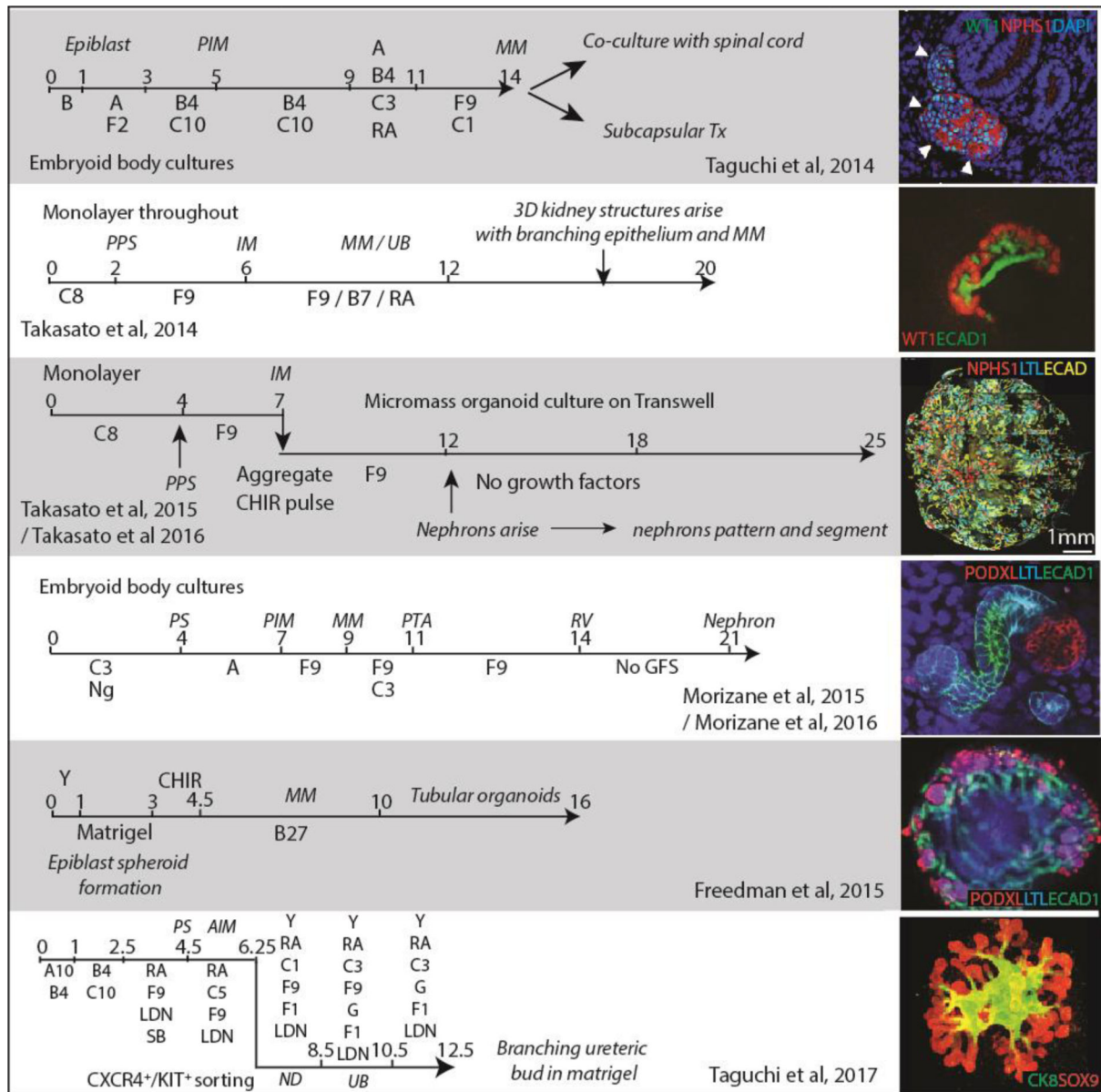


Figure 2. Published protocols for the directed differentiation of human pluripotent stem cells to kidney endpoints.

Stages of differentiation: *AIM*, anterior intermediate mesoderm; *IM*, intermediate mesoderm; *MM*, metanephric mesenchyme; *ND*, nephric duct; *PIM*, posterior intermediate mesoderm; *PS*, primitive streak; *PPS*, posterior primitive streak; *Tx*, transplantation; *UB*, ureteric bud. Compounds added: A, Activin A; A10, activating A 10uM, B4, BMP4; B7, BMP7; C or CHIR, CHIR990201 (GSK3β antagonist / canonical Wnt activator); C1, CHIR 1uM; C3, CHIR 3uM, C5, CHIR 5uM, C8, CHIR 8uM; F1, FGF1; F2, FGF2; F9, FGF9; G, GDNF; LDN, LDN193189 (BMP Type 1 receptor (ALK2/3) inhibitor); Ng, Noggin (BMP inhibitor), RA, retinoic acid; SB, SB431542 (TGFβ Type 1 receptor (ALK5) inhibitor); Y, Y27632 (Rho-kinase inhibitor).

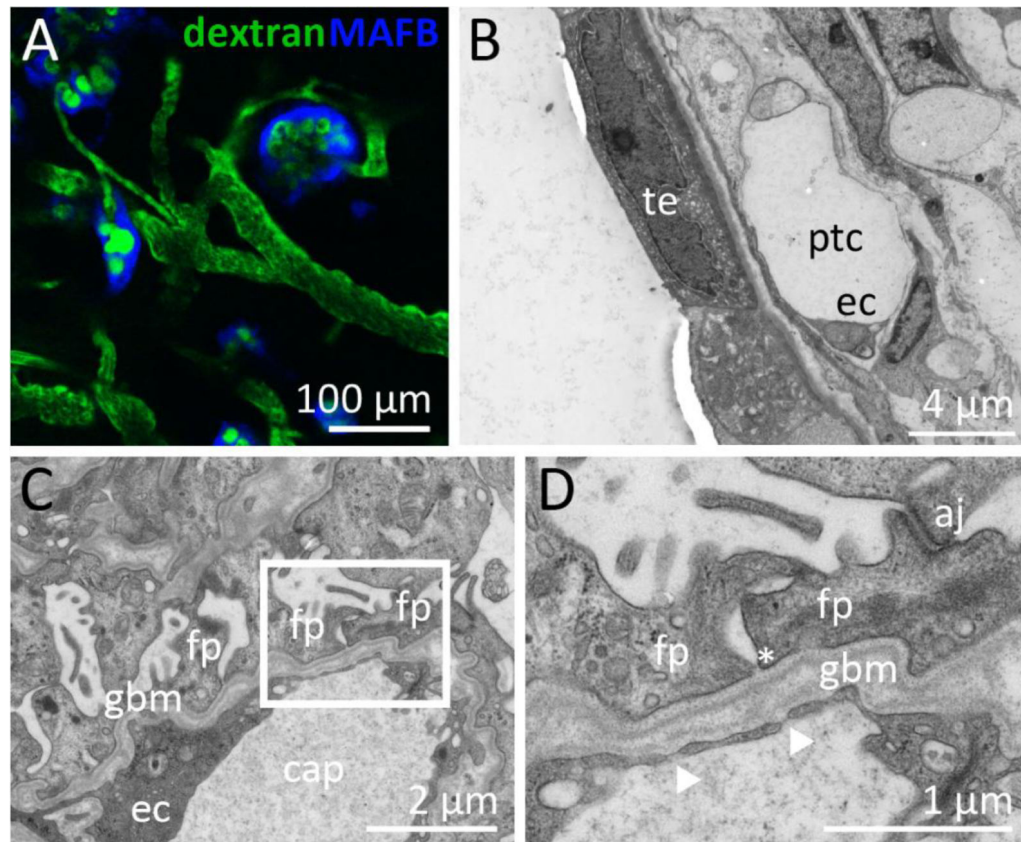


Image courtesy of C.W. van den Berg and A.J. Rabelink

Figure 3. Glomerular vascularisation and tubular maturation post *in vivo* organoid transplantation under the renal capsule.

A. Visualisation of blood flow (FITC-Dextran) through the glomerulus of an hPSC-derived human kidney organoids generated using a CRISPR-Cas9 gene edited iPSC line in which a blue fluorescent protein has been inserted into the *MAFB* gene locus in order to mark podocytes. **B.** TEM image of a proximal tubular segment within an hPSC-derived kidney organoid 2 weeks after transplantation under the renal capsule. Ec, endothelial cell; ptc, peritubular capillary; te, tubular epithelium **C,D.** Low and higher magnification TEM images of a glomerular basement membrane present between podocytes and endothelial cells of the organoid glomerulus. aj, adherens junction; cap, capillary; ec, endothelial cell; fp, foot process; gbm, glomerular basement membrane. Images courtesy of van den Berg et al [80].

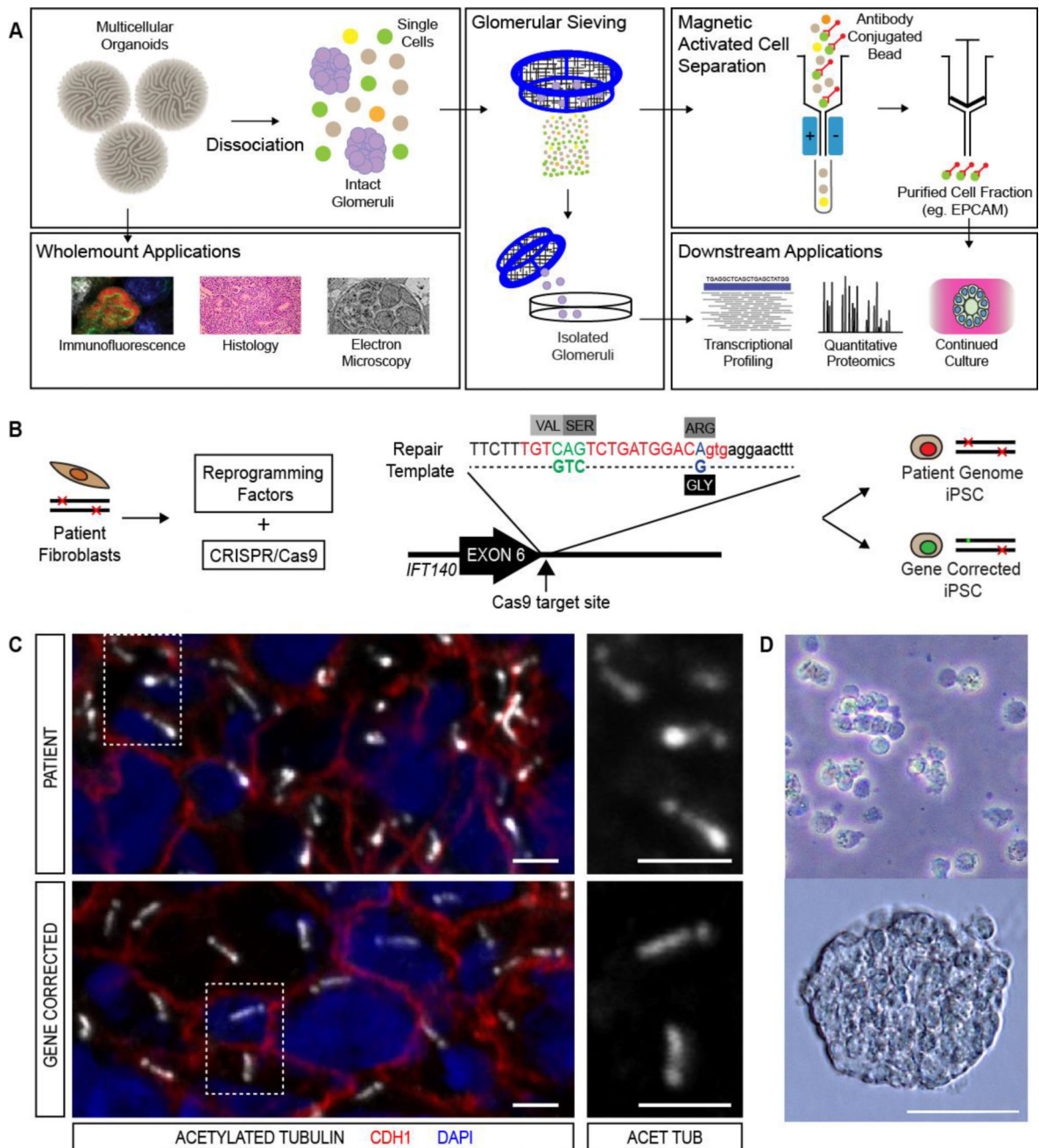


Figure 4. Application of hPSC-derived kidney tissues for disease modelling.

A. Outline for the generation of patient-derived and isogenic iPSC lines from a Maizner-Saldino syndrome patient carrying compound heterozygote mutations in the IFT140 gene [107]. The protocol for simultaneous generation of patient and gene corrected lines is described in Howden et al [79]. **B.** Approach to the characterisation of disease related changes using cells isolated from hPSC-derived kidney organoids [107]. **C.** Immunofluorescence imaging of renal tubular epithelial cells within iPSC-derived kidney organoids showing the presence of clubbed cilia in patient organoids and wildtype cilia in organoids from gene corrected lines. **D.** Bright field images of intact glomeruli isolated by

sieving dissociated hPSC-derived kidney organoids at low and high power. Figure courtesy of Dr. Lorna Hale.

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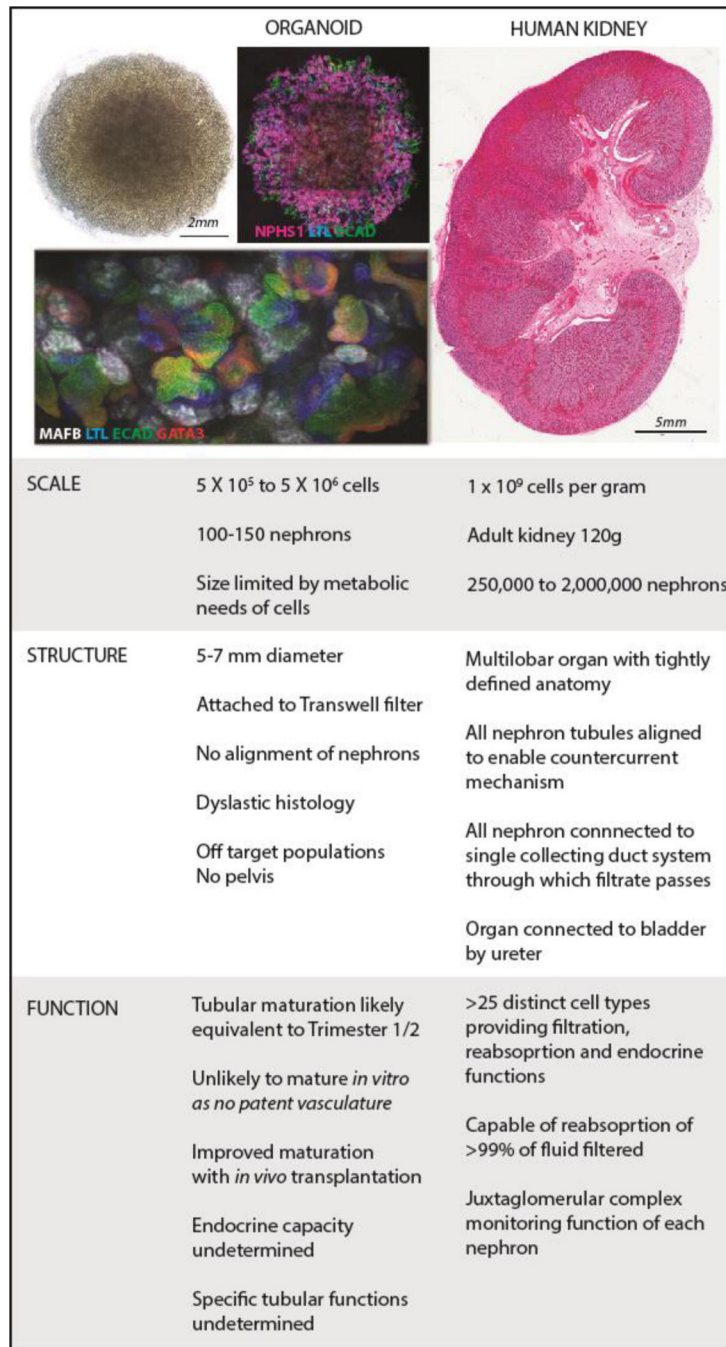


Figure 5. Scale, structure and function represent key challenges to ‘rebuilding a kidney’ using hPSC-derived models.

Images of organoids courtesy of Ms. Pei Xuan Er and Dr. Jessica Vanslambrouck, Murdoch Children’s Research Institute. Image of female human kidney (35 weeks gestation) courtesy of Prof. Mary Jane Black and Dr. Megan Sutherland, Monash University.

Table 1.

A comparison of media described for the maintenance of isolated nephron progenitors. Adapted from Li et al [30].

		NPSR		NPEM		CDBLY	
		Li et al. 2016		Brown et al. 2015		Tanigawa et al. 2016	
Culture Period		Until now, more than 17 months and more than 110 passages		Up to 10 passages were shown		Up to around 19 days	
Culture Condition	Format	3D		2D (Matrigel coating)		2D (iMatrix coating)	
	Basal Medium	DMEM/F12		APEL		DMEM/F12	
	Specific Factors	Factor	Final Concentration	Factor	Final Concentration	Factor	Final Concentration
		BMP7	50ng/ml	BMP7	30ng/ml	TGF- α	10ng/ml
		FGF2	200ng/ml	FGF9	200ng/ml	FGF2/9	50ng/ml
		Heparin	1ug/ml	Heparin	1ug/ml	LIF	5ng/ml
		Y27632	10uM	Y27632	10uM	Y27632	10uM
		CHIR99021	1uM	CHIR99021	1.25uM	CHIR99021	1uM
		LIF	10ng/ml	LDN193189	125nM	BMP7	5ng/ml
				BMP4	30ng/ml	DAPT	2.5uM
			IGF1	20ng/ml			
		IGF2	2ng/ml				