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STATE-OF-THE-ART REVIEW

Regenerative strategies for kidney engineering

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The kidney is the most important organ for water homeostasis and waste excretion. It performs several important physiological functions for homeostasis: it filters the metabolic waste out of circulation, regulates body fluid balances, and acts as an immune regulator and modulator of cardiovascular physiology. The development of *in vitro* renal disease models with pluripotent stem cells (both human embryonic stem cells and induced pluripotent stem cells) and the generation of robust protocols for *in vitro* derivation of renal-specific-like cells from patient induced pluripotent stem cells have just emerged. Here we review major findings in the field of kidney regeneration with a major focus on the development of stepwise protocols for kidney cell production from human pluripotent stem cells and the latest advances in kidney bioengineering (i.e. decellularized kidney scaffolds and bioprinting). The possibility of generating renal-like three-dimensional structures to be recellularized with renal-derived induced pluripotent stem cells may offer new avenues to develop functional kidney grafts on-demand.

Introduction

Kidney-related diseases constitute one of the major health challenges in modern society. Chronic kidney disease (CKD) is a leading cause of mortality and morbidity in western countries that is estimated to affect 11% of the adult population. It can progress toward end-stage renal disease (ESRD), which is incurable, often requiring dialysis or preferably renal transplantation. Of note, dialysis treatments alone account for 2% of national healthcare budgets, and this parameter is set to double in the next few years. If this trend were to continue, national governments would need to spend between 3% and 5% of their annual healthcare budgets on renal replacement therapies without taking into account its wider costs in terms of additional medical expenses.

Currently in the USA there are 122 403 people waiting for lifesaving organ transplants; of these, 101 189 await kidney transplants [1,2]. The median wait time for an individual's first kidney transplant is 3.6 years and can vary depending on different parameters such as health status, compatibility and organ availability [2]. Of note, in 2014, 17 105 kidney transplants took

Abbreviations

BMP4, bone morphogenetic protein 4; CKD, chronic kidney disease; CRISPR, clustered regularly interspaced short palindromic repeat; ECM, extracellular matrix; ESC, embryonic stem cell; ESRD, end-stage renal disease; FGF, fibroblast growth factor; GDNF, glial-cell-derived neurotrophic factor; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; hPSC, human pluripotent stem cell; IM, intermediate mesoderm; iPSC, induced pluripotent stem cell; MM, metanephric mesenchyme; NPC, nephron progenitor stem cells; OSR-1, Odd-skipped related 1; PSC, pluripotent stem cell; UB, ureteric bud.

place in the USA. Of these, 11 570 came from deceased donors and 5535 came from living donors [1,2]. The US Department of Health and Human Services Organ Procurement and Transplantation Network estimates that over 3000 new patients are added to the kidney waiting list each month and that in 2014 4270 patients died whilst waiting for a kidney transplant. Thus, there is a serious need for the development of new therapeutic strategies that could overcome these issues.

Recent progress in stem cell based methodologies and tissue engineering approaches have led to the definition of novel regenerative medicine strategies for the treatment of kidney disease. First, the feasibility to emulate important kidney developmental cues in the culture dish has shown that under certain conditions human pluripotent stem cells (hPSCs) can be differentiated *in vitro* towards the main renal cell types compromised during kidney disease (e.g. podocytes and tubular cells, among others) [3,4]. Second, emerging technologies including decellularization/recellularization and bioprinting technologies have revolutionized the field of tissue engineering, opening the possibility of creating complex 3D organs such as kidneys (reviewed in [5,6]).

Here we review the current advancements in regenerative medicine strategies to restore kidney function with a main focus on (a) the directed differentiation of hPSCs towards renal-like cells amenable to being used for kidney engineering and (b) the use of decellularization/recellularization and bioprinting technologies. All these methodologies are envisioned as promising tissue engineering strategies for kidney bioengineering and here we discuss the major challenges for the future translation of these promising technologies into the clinics (Fig. 1).

Stem cell therapies for kidney regeneration: challenges and current status

Kidney-related diseases constitute a major health issue worldwide [7–9]. The incidence and poor prognosis of the kidney and its associated diseases reveal the urgent need not only for development of therapeutic approaches but also for expanding our understanding of kidney development and repair potential, especially nephrons' generation, differentiation and self-renewal capacity. Interestingly, the adult mammalian kidney has the capacity, upon injury, to repair certain cell types, including tubular and epithelial cell compartments, but not glomerular structures [10–12]. This seems to take place by proliferation of resident cells, in the absence of progenitor populations or newly formed multilineage structures, a process termed 'cellular regeneration' [13]. So far, cellular regeneration has been traditionally evaluated by lineage tracing pinpointing the cellular origin of these proliferating cells [12–14], whereas progenitor cell populations can be identified by the ability of the putative candidate cells

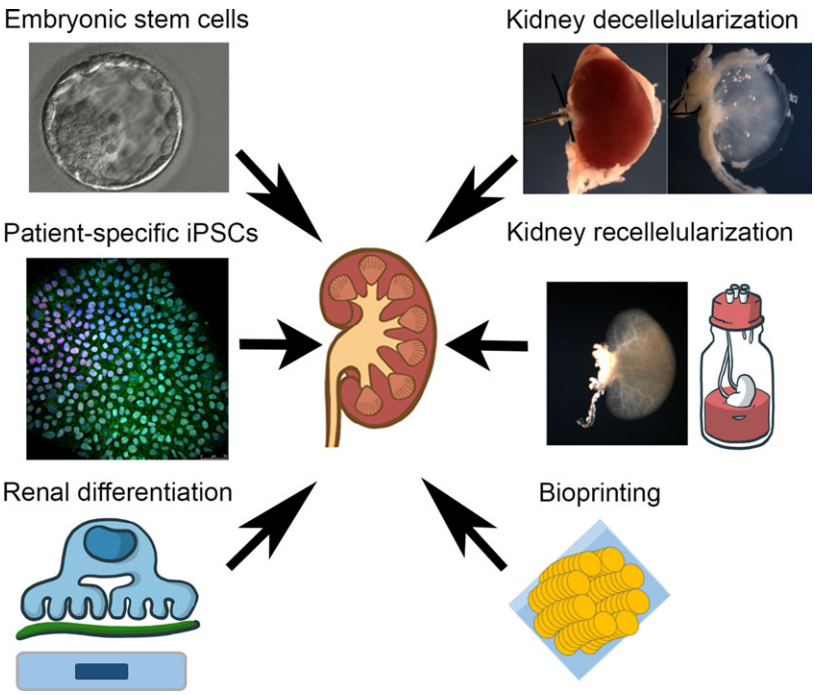

Fig. 1. The use of PSCs (both embryonic stem cells and induced pluripotent stem cells) opens the door for the identification of the proper conditions sustaining adult kidney cells for cell therapy. Similarly, kidney bioengineering aims to use kidney scaffolds as biological matrices for further recellularization, and lately bioprinting has emerged as a major strategy to build organ-like structures in three dimensions.

to differentiate into multiple lineages [15]. Contrary to other organisms, such as zebrafish and insects, in which the formation of new nephrons elicits regeneration of lost nephrons, in mammals the formation of new nephrons is restricted to embryogenesis and completed by the time of birth [16].

Pluripotent stem cells (PSCs): a potential source in regenerative medicine

Early in 1998 it was demonstrated that pluripotent stem cells (PSCs) could be derived from the inner cell mass of the embryonic blastocyst, resulting in embryonic stem cells (ESCs) [17]. Human ESCs (hESCs) represented then a new source for the study of tissue development and disease modeling *in vitro*. From that initial moment, intense research has been devoted to the definition of robust protocols for hESC differentiation aiming to generate functional cells able to restore the loss of function arising during the development of degenerative diseases. Nevertheless, the molecular mechanisms involved in lineage restriction of hESCs to adopt specialized cell types are still under investigation. Importantly, the most critical impediments to the use of hESCs in the clinics are the need for human embryos, which raises ethical concerns, and tissue rejection after transplantation.

There are other methods to generate PSCs (hPSCs) from somatic donor cells; these involve cell reprogramming by various methods, including somatic cell nuclear transfer into an enucleated egg [18], fusion with a PSC [19], exposure to small chemical compounds [20] and transduction of reprogramming factors [21]. In the cases of somatic cell nuclear transfer and cell fusion mediated reprogramming, somatic cell nuclei are exposed to totipotent or pluripotent environments, respectively [22]. On the other hand, the transduction of the reprogramming factors POU domain class 5 transcription factor 1 (POU5F1, also known as OCT3/4), SRY (sex determining region Y) box 2 (SOX2), Krüppel-like factor 4 (KLF4) and myelocytomatosis oncogene (c-MYC) (together referred to as OSKM, the Yamanaka factors) results in the generation of autocompatible induced pluripotent stem cells (iPSCs), which share self-renewal and differentiation potential capacities when compared to hESCs. Most of the initial studies on patient-iPSC generation have relied on the use of integrative systems overexpressing c-MYC and KLF4, two well-known oncogenes [23,24]. To overcome these issues, different laboratories, including ours, searched for different cell types that could be reprogrammed in the absence of the two oncogenic-related factors, taking advantage of the intrinsic properties from the donor cells of choice (e.g. cells expressing relatively high levels of any Yamanaka factor could be reprogrammed in the absence of that particular gene, such as neural stem cells in the absence of SOX2). In this regard, iPSCs were efficiently generated in the absence of KLF4 and/or cMYC from keratinocytes [24], cord blood stem cells [25] and even neural stem cells [26]. Although these discoveries revealed the possibility of reducing the number of factors for iPSC generation, they were still relying on the use of integrative systems leading to undesired effects related to random transgene insertion (e.g. risk of transgene induction after differentiation, incomplete reprogramming, among others). Very rapidly, different laboratories worldwide developed novel methodologies in order to generate transgene-free iPSCs [27–32], also incorporated important changes in culture systems (e.g. matrices/media) in order to produce feeder-free human iPSCs (hiPSCs) [33–35], and identified soluble compounds replacing Yamanaka factors for iPSC generation [20]. Together with these important challenges in the field of iPSC derivation, intense research has been published on patient-specific iPSC generation and subsequent differentiation into relevant tissues compromised during disease progression, providing a unique scenario for disease modeling [36–41] and even to screen patient-specific drugs [42,43].

In the same manner, one area that has drawn great interest is correction of genetic based diseases in patient-specific hiPSCs with a prospect of precise medicine. PSCs are amenable sources for genome editing because they can undergo extensive tissue culture manipulations (e.g. drug selection and clonal expansion) while retaining their pluripotency signature and genome stability [44]. Different research groups have demonstrated the generation and correction of patient-specific hiPSCs [38,39,45–49]. These studies have demonstrated functional correction of the disease-associated phenotype upon differentiation of patient-specific hiPSCs, and even opened the door for the screening of compounds reverting disease phenotype [49,50], an approach that seems poised to become the first application of the potential of hiPSCs in regenerative medicine and human health [51].

Generation of kidney cells from PSCS: PSC based methodologies model renal development and disease

Kidney is composed of thousands of nephrons that arise during development through reciprocal inductive interactions between two different cell types: the

metanephric mesenchyme (MM) and the ureteric bud (UB) derived cells [16]. The MM differentiates into the different epithelial cell types within the kidney (from the glomerulus through the connecting segment), and the UB generates the calyceal system. In the last few decades many reports have defined culture conditions for the isolation and *in vitro* expansion of these cell populations with limited success (reviewed in [13,14,52]). An alternative methodology for the generation of unlimited quantities of kidney-related cell types is the differentiation of PSCs. In the last few years independent research groups, including us, have described for the first time the possibility of generating different kidney populations from PSCs. In this regard, Song and colleagues were the first to report on the derivation of podocyte progenitors from hiPSCs that were shown to be functional and efficiently integrated in mouse metanephric tissues. However, the question of which embryonic progenitor cells during development are equivalent to Song's iPSC-immature podocytes remains to be answered [3].

Later in 2013 Mae and colleagues differentiated, for the first time, monolayers of hESCs towards intermediate mesoderm (IM), the embryonic tissue that gives rise to both MM and UB [53]. For this purpose the authors generated different reporter hiPSC lines in which GFP was targeted into Odd-skipped related 1 (OSR-1, a gene transiently expressed in the IM during embryogenesis). Under specific cell culture conditions IM-differentiated iPSCs showed signatures of other kidney mature cell types and limited tubular structures. Interestingly, the same authors pursued the definition of a chemically defined medium for the derivation of IM-differentiated iPSCs and recently described the identification of two retinoid-like molecules inducing IM derivation from iPSCs. In their particular setting, the authors demonstrated that the resulting IM-differentiated iPSCs exhibit the capability to differentiate into multiple kidney cell types, also developing 3D renal-tubule-like structures in *ex vivo* organ culture settings [54].

Along the same line, our group reported for the first time the possibility of generating UB progenitors from IM cells from hESCs and from hiPSCs derived from patients affected by polycystic kidney disease. This was accomplished with a two-step protocol inducing first mesodermal specification by bone morphogenetic protein 4 (BMP4) and fibroblast growth factor 2 (FGF2), and second IM and renal-like lineages by the exposure of differentiated cells to retinoic acid, activin A and BMP2. After 4 days UB progenitors expressed HOXB7, RET and GFRA1, rather than markers from MM. Moreover, when UB-like-hiPSC-derived cells were co-cultured with dissociated E11-5 mouse metanephric cells, the generated cells only integrated into cytokeratin 8 positive (+) UB-like structures, suggesting the induction of UB lineage-committed IM cells *ex vivo* [55].

On the other hand, the Little group have developed protocols for the generation of well-characterized kidney progenitors from both hESCs and hiPSCs into IM through the posterior primitive streak, from which IM is derived during development. The authors made use of a reporter hESC line for monitoring the expression of MIXL1 (a gene transiently expressed in the primitive streak during embryogenesis) by knock-in GFP into MIXL1 locus. The Little group generated two different protocols, one for the simultaneous generation of MM and UB derivatives and the other for UB cells alone from PSCs. Noticeably, this work made use of re-aggregation assays to define the differentiation potential of the renal-like generated cells, showing that the re-aggregated differentiated PSCs alone could organize and generate nephron-like structures [56].

More recently Lam and colleagues reported a rapid and efficient differentiation protocol for the production of PAX2+LHX1+ IM cells from hPSCs that spontaneously form tubular-like structures. Interestingly, Lam showed that hPSC-derived PAX2+LHX1+ IM cells efficiently integrated into mouse metanephric cultures and differentiated into multipotent nephron progenitor stem cells (NPCs) of the cap mesenchyme expressing SIX2, SALL1 and WT1 markers [57]. In the same manner, Taguchi and colleagues have also shown that it is possible to derive NPCs that possess the developmental potential to reconstitute 3D nephron-like structures containing both glomerulus-like and renal-tubule-like structures *in vitro* upon co-culture with embryonic spinal cords from both mouse ESCs and hiPSCs [58]. In this particular work Taguchi and colleagues made use of *in vivo* lineage mouse models and examined different stages of NPC development in OSR-1-GFP reporter mice, and found that the OSR-1-GFP-Integrinα^+PDGFRα^- population exhibits the highest potential to give rise to *in vitro* forming nephrons. In this manner the authors redefined the spatial and temporal location of metanephric nephron progenitors *in vivo*, demonstrating that IM contains at least two metanephric progenitor subpopulations, anterior and posterior domains, and that posteriorly located IM gives rise to MM lineages [58]. Interestingly this last work outlined when nascent mesoderm commits to IM and how it becomes specified to MM and UB lineages [58].

Interestingly, Imberti and colleagues derived renal progenitor cells from hiPSCs following a two-step

protocol by first exposing hiPSCs to retinoic acid, RhoA and PI3K inhibitors and activin A, inducing IM generation. Next IM-committed hiPSCs were treated with FGF2, BMP7 and glial-cell-derived neurotrophic factor (GDNF) for 13 additional days in order to generate MM-derived hiPSCs. Although other authors had already demonstrated the possibility of generating MM-derived hPSCs, in this work Imberti and colleagues demonstrated for the first time that hiPSC-derived renal progenitors robustly engrafted into damaged tubuli restoring renal function [59].

Recently Morizane and colleagues have shown the possibility of developing NPCs from hPSCs. The work of Morizane elegantly shows that it is possible to recapitulate MM kidney *in vitro* and generate SIX2+-SALL1+WT1+PAX2+ NPCs with 90% efficiency after 9 days. More importantly, the authors proved that NPCs form kidney organoids in both 2D and 3D settings after 21–28 days, containing epithelial nephron-like structures expressing markers of podocytes (PODXL, NPHS1), proximal tubules (CDH2, LTL), loops of Henle (CDH1, UMOD) and distal tubules (CDH1, BRN1) mimicking nephron development. Importantly, the organoid culture system was used to study the mechanisms of proximal and/or distal tubular toxicity by exposing kidney organoids to chemical agents routinely used in animal models *in vivo* [60]. Along this same line, Freedman and colleagues have shown that kidney stem cells derived from hPSC-derived spheroids can be easily derived into kidney organoids mirroring tissue-specific epithelial physiology. In particular the authors were able to knock out podocalyxin, PKD1 (polycystin-1) or PKD2 (polycystin-2) genes by clustered regularly interspaced short palindromic repeat (CRISPR)/CAS9 RNA-guided nucleases CRISPR/Cas9, proving for the first time the feasibility of 3D renal-derived structures to model human kidney disease [61]. Very recently Takasato and colleagues [62] has gone one step further in the generation of kidney organoids containing individual nephrons that further segmented into distal and proximal tubules, early loops of Henle and glomeruli containing podocytes elaborating foot processes and undergoing vascularization. Like Freedman *et al*. [61], Takasato and colleagues also showed that proximal tubules accumulated dextran cargo and differentially apoptose in response to cisplatin [62].

Although all these reports showed the possibility of generating different kidney populations from hPSCs, they still differ with regard to the reagents used, concentrations and timing (Table 1). Importantly, the different findings on PSC differentiation towards renal lineages revealed that we are still far from identifying marker genes or surface proteins defining the different IM populations identified in mice [58].

Genome editing technologies

Recently, different gene editing platforms to increase homologous recombination efficiency, namely DNA nucleases (zinc finger nucleases, TAL effector nucleases and meganucleases), together with CRISPR/Cas9 have emerged as potential tools for gene editing in patient hiPSCs [44]. Overall, DNA nucleases and CRISPR/Cas9 technologies allow for different genome manipulations in a site-specific manner, such as gene activation/inactivation, sequence deletion, element replacement and chromosomal rearrangement [44,63]. In the context of kidney disease modeling CRISPR/Cas9 technology has been used in 3D renal-derived structures from hiPSCs in order to knock out PKD1 (polycystin-1) and PKD2 (polycystin-2) genes, leading to the possibility of modeling polycystic kidney disease in a 3D setting resembling the kidney epithelial compartment [61].

Besides the tremendous impacts of genome editing platforms on human disease modeling, other aspects recently explored include the use of such technologies for the generation of PSC reporter cell lines [64–67]. Recently the Little group has developed a new protocol for the generation of self-assembling kidney organoids from hPSCs equivalent to the human fetal kidney during the first trimester of gestation [62]. These results reveal on one hand that we are still far from developing mature kidney cells with therapeutic potential, suggesting that new strategies should be taken into account when developing mature kidney cells for clinical purposes (e.g. use of conditioned culture medium from kidney embryonic cells, use of re-aggregation co-culture systems with human fetal cells, among others). On the other hand, the mRNA expression profile in Little *et al*.'s work arises from different cell types self-assembled within the kidney 3D structures, hampering the analysis of the molecular pathways sustaining renal differentiation from the different individual cell types comprising kidney organoids. In this regard, genome editing technologies, in particular the CRISPR/Cas system, may help to generate human reporter PSC lines for the dissection of the molecular cues driving renal differentiation and thus properly to evaluate the matureness of the generated cells compared with their *in vivo* counterparts from developing embryos.

So far, kidney PSC reporter cell lines have been generated by means of bacterial artificial chromosome

Table 1. Recent literature on renal differentiation from human pluripotent stem cells. This table provides an overview of several recent publications in which human pluripotent stem cells were differentiated towards renal cell lineages. Details of the growth factors and cytokines used during differentiation are listed.

Authors and year	Pluripotent stem cells of origin	Renal differentiation protocol		
Song *et al.* (2012) [3]	Human iPSCs (mesangial cells)	Embryoid body formation (3 days): 10 ng·mL^{-1} activin A, 15 ng·mL^{-1} BMP7, 0.1 μM retinoic acid	Glomerular podocytes (adherent culture, 8 days): 10 ng·mL^{-1} activin A, 15 ng·mL^{-1} BMP7, 0.1 μM retinoic acid	
Narayanan *et al.* (2013) [4]	Human ESCs	Proximal tubular cells (20 days): 10 ng·mL^{-1} BMP2, 2.5 ng·mL^{-1} BMP7, 10 ng·mL^{-1} activin A, 0.1 μmol·mL^{-1} retinoic acid		
Mae *et al.* (2013) [53]	Human ESCs (H9, khES1 and khES3), human iPSCs (201B6, 201B7, 253G1 and 253G4)	Mesendoderm (2 days): 100 ng·mL^{-1} activin A, 3 μM CHIR99021, 10 μM Y27632	Early intermediate mesoderm (8 days): 100 ng·mL^{-1} BMP7, 3 μM CHIR99021	Intermediate mesoderm (7 days): 10 ng·mL^{-1} TGFβ1
Araoka *et al.* (2014) [54]	Human ESCs, human iPSCs (dermal fibroblasts)	Early intermediate mesoderm (2 days): 3 μM CHIR99021, 1 μM 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-	Intermediate mesoderm (3 days): 1 μM TTNBP	

Table 1. (Continued).

Authors and year	Pluripotent stem cells of origin	Renal differentiation protocol				
		naphthalenyl)-1-propenyl] benzoic acid (TTNPB)				
Xia *et al.* (2013) [55]	Human iPSCs (dermal fibroblasts)	Mesoderm progenitors (2 days): 30 ng·mL^{-1} BMP4, 50 ng·mL^{-1} FGF2, 17.5 μg·mL^{-1} insulin	Intermediate mesoderm ureteric bud progenitor-like cells (2 days): 1 μM retinoic acid, 10 ng·mL^{-1} activin A, 100 ng·mL^{-1} BMP2			
Takasato *et al.* (2014) [56]	Human ESCs	Posterior primitive streak: (a) 2 days, 30 ng·mL^{-1} BMP4, 10 ng·mL^{-1} activin A; (b) 2 days,8 μM CHIR99021	Intermediate mesoderm: (a) 4 days, 200 ng·mL^{-1} FGF9, 1 μg·mL^{-1} heparin; (b) 10 days, 200 ng·mL^{-1} FGF9, 1 μg·mL^{-1} heparin	Metanephric mesenchyme and ureteric bud progenitors: (a) 11 days, 200 ng·mL^{-1} FGF9, 50 ng·mL^{-1} BMP7, 0.1 μM retinoic acid, 1 μg·mL^{-1} heparin; (b) 6 days, no factors		
Lam *et al.* (2013) [57]	Human ESCs, human iPSCs (dermal fibroblasts)	Mesendoderm (2 days): 5 μM CHIR99021	Intermediate mesoderm (4 days): 100 ng·mL^{-1} FGF2, 1 μM retinoic acid	Cap mesenchyme (3 days): 100 ng·mL^{-1} FGF9, 10 ng·mL^{-1} activin A		
Taguchi *et al.* (2014) [58]	Mouse and human iPSCs	EB formation (1 day): 0.5 ng·mL^{-1}	Epiblast (2 days): 1 ng·mL^{-1} activin A,	Posterior nascent mesoderm (6 days):	Posterior intermediate mesoderm (2 days):	Metanephric mesenchyme (3 days): 5 ng·mL^{-1}

Table 1. (Continued).

Authors and year	Pluripotent stem cells of origin	Renal differentiation protocol					
		BMP4, 10 μM Y27632	20 ng·mL^{-1} FGF2	1 ng·mL^{-1} BMP4, 10 μM CHIR99021	10 ng·mL^{-1} activin A, 3 ng·mL^{-1} BMP4, 3 μM CHIR99021, 0.1 μM retinoic acid, 10 μM Y27632	FGF9, 1 μM CHIR99021, 10 μM Y27632	
Imberti *et al.* (2015) [59]	Human iPSCs (SC101A-1)	Intermediate mesoderm (6 days): 0.1 μM retinoic acid, 1 μM CCG1423, 5 μM LY294002; 10 ng·mL^{-1} activin A added for 2 days starting at day 2	Metanephric mesenchyma (13 days): 50 ng·mL^{-1} BMP7, 10 ng·mL^{-1} FGF2, 15 ng·mL^{-1} GDNF				
Morizane *et al.* (2015) [60]	H9 human ESCs, human iPSCs (dermal fibroblasts)	Late primitive streak (4 days): 8 μM CHIR99021 for ESCs; 10 μM CHIR99021 and 5 ng·mL^{-1} noggin for iPSCs	Posterior intermediate mesoderm (3 days): 10 ng·mL^{-1} activin A	Metanephric mesenchyme (2 days): 10 ng·mL^{-1} FGF9	Pretubular aggregate (2 days): 3 μM CHIR99021, 10 ng·mL^{-1} FGF9	Renal vesicle (3 days): 10 ng·mL^{-1} FGF9	Self-organizing nephron (7–14 days): FGF9 retrieved
Freedman *et al.* (2015) [61]	Human ESCs (H9/WA09), human iPSCs (foreskin fibroblasts and dermal fibroblasts)	Sandwiched spheroid colonies (3 days): 10 μM Y27632, matrigel	Tubular organoid differentiation containing proximal tubules, endothelial cells and podocytes (13 days): 12 μM CHIR99021 for 36 h, then retrieved; media replacement				

Table 1. (Continued).

Authors and year	Pluripotent stem cells of origin	Renal differentiation protocol			
					every 3 days thereafter
Takasato *et al.* (2015) [62]	Human iPSCs (CRL1502, clone C32)	Intermediate mesoderm (7 days) (anterior and/or posterior, depending on CHIR exposure time): 8 μM CHIR99021 (2–5 days), then 200 ng·mL^{-1} FGF9 and 1 μg·mL^{-1} heparin (2–5 days)			Kidney organoid containing nephrons associated with a collecting duct network surrounded by renal interstitium and endothelial cells (11–18 days): 5 μM CHIR99021 for 1 h, then 200 ng·mL^{-1} FGF9 and 1 μg·mL^{-1} heparin (5 days), then no cytokines (6–13 days)

based vectors [53] and lentivirus [56]. These seminal studies have revealed the suitability of reporter cell lines for the identification and amplification of the renal cell types of interest (i.e. IM cells and primitive streak cells) during the onset of differentiation. Importantly, the Osafune group has gone one step further in the use of the OSR-1-GFP reporter cell line and identified two retinoic acid receptor agonists, namely AM580 and TTNPB, that together with CHIR99021 induced the differentiation of IM cells by activating the expression of BMP4 [54]. Interestingly the new differentiation method using small compounds provided a less expensive and more consistent method of generating IM cells than the growth factor method described previously by the same group [53]. Since the identification and amplification of tissue-specific renal stem/progenitor cells with nephrogenic potential is a critical step in developing cell based therapies for renal disease, the use of kidney reporter PSCs may result in the definition of cell culture conditions to be applied in 3D renal-derived structures from hPSCs.

Novel strategies for kidney bioengineering

The field of tissue engineering applies the principles of biology and engineering to the development of functional substitutes for damaged tissue [68]. For decades, researchers have been struggling to develop a variety of techniques and methodologies to fabricate bioinspired scaffolds using natural or synthetic biomaterials [69,70]. A consensus in the field is that to be successfully used for tissue engineering such scaffolds have to fulfill several key characteristics in order to encourage the correct tissue formation and function: (a) be biocompatible; (b) possess the appropriate mechanical strength; (c) show bioactivity in order to modulate the 3D cellular microenvironment; and (d) enable diffusion of nutrients and clearance of metabolic waste. Significant achievements have occurred in the field including the successful engineering of tissue grafts such as skin [71], cartilage [72,73], bone [74], bladder [75], blood vessels [76] and trachea [77]. However, researchers are nowadays facing new challenges related to the bioengineering of more complex 3D organs like lung, liver, heart and kidney.

Complex organs require an intact vascular network that can be further reconnected to the circulatory system upon transplantation into the recipient in order to ensure adequate nutrient and oxygen supply to the whole organ. Moreover, such organs usually possess highly organized organ-specific multicellular structures responsible for performing essential functions. The

Table 2. Recent literature on kidney decellularization/recellularization strategies. This table provides an overview of several recent publications in which whole kidneys were decellularized and recellularized *in vitro* using various cell types. Details of the species from which the kidney is decellularized, main decellularization conditions used, cell types seeded, method of seeding, method of kidney scaffold culturing and main results are provided.

			Recellularization				
Authors and year	Species	Decellularization	Seeded cells	Seeding method	Scaffold culture method	Implantation	Main outcome
Ross *et al.* (2009) [79], (2012) [99]	Sprague-Dawley rat	Gravity-based perfusion (at 100 mmHg) through renal artery and ureter of (a) 3% Triton X-100, (b) DNase, (c) 3% Triton X-100, (d) 4% sodium dodecyl sulphate (SDS)	Murine ESCs	Manual injection through either the artery or ureter	Automated perfusion system designed to maintain 120/80 mmHg	No	Cells injected into artery were initially trapped in glomeruli but migrated into vasculature during extended culture. Cell injection through ureter led to uneven cell distribution
Song *et al.* (2013) [80]	Sprague-Dawley rat	Renal artery perfusion at constant pressure of 40 mmHg with 1% SDS 12 h, 1% Triton X-100 30 min	Rat neonatal kidney cells and human umbilical vein endothelial cells (HUVECs)	Seeding of HUVECs at 1 $mL{\cdot}min^{-1}$ arterial flow followed by overnight static incubation. Rat neonatal kidney cells seeded through the ureter while a negative pressure gradient was applied to the bioreactor chamber	Arterial perfusion culture at 1.5 $mL{\cdot}min^{-1}$ in a bioreactor	Orthotopic implantation into rat	Partial restoration of renal filtration and electrolyte reabsorption. Recellularization of 70% of glomeruli. No thrombi or bleeding when implanted
Bonandrini *et al.* (2014) [82]	Sprague-Dawley rat	Renal artery perfusion at 0.4 $mL{\cdot}min^{-1}$ at pressure of 62−107 mmHg	R1 murine ESCs	Dynamic seeding through renal artery at 0.2 $mL{\cdot}min^{-1}$	Bioreactor perfusion culture	No	OCT4 expression decreased while NCAM, Tie-2 and CD31 increased

Table 2. (Continued).

Authors and year	Species	Decellularization	Recellularization			Implantation	Main outcome
			Seeded cells	Seeding method	Scaffold culture method		
		with 1% SDS, 17 h					
Sullivan *et al.* (2012) [86]	Yorkshire pig	Renal artery perfusion with 0.5% SDS 36 h and DNase overnight	Primary renal cortical human cells	5 mm × 7 mm biopsies of cortex were seeded statically with cells	Static culture for 3–4 days	No	Cell proliferation. Better performance of SDS compared to Triton X-100
Nakayama *et al.* (2010) [89], (2013) [95]	Rhesus monkey	Kidney slices: 1% SDS 7–10 days	WA09 human ESCs	Kidney slices of 8 mm diameter were statically seeded with cells	Static culture for 8 days	No	Increased expression of kidney tubule gene markers
O'Neill *et al.* (2013) [96]	Yorkshire pig	0.02% trypsin 2 h, 3% Tween 2 h, 4% sodium deoxycholate 2 h	Murine kidney stem cells and mouse mesenchymal stem cells	Cells were seeded on slices of decellularized scaffolds, ECM-derived hydrogels and solubilized ECM	Static culture up to 7 days	No	Papillary ECM inhibited proliferation and increased the metabolic activity of renal stem cells
Caralt *et al.* (2014) [83]	Sprague-Dawley rat	Three methods: (a) 1% Triton X-100; (b) 1% Triton X-100 and 0.1% SDS; (c) 0.05% EDTA with 0.02% trypsin and 1% Triton X-100	Human iPSC-derived endothelial cells and immortalized human renal cortical tubular epithelial cells (RCTEs)	Manually injected or infused via bioreactor antegrade perfusion into renal artery at 25 $mL{\cdot}min^{-1}$ for 15 min	Static culture for 36 h and bioreactor perfusion culture at 4 $mL{\cdot}min^{-1}$ up to 7 days	No	Endothelial cells found in the vasculature, and RCTEs formed tubular structures. Oxygen access was limited in some areas
Peloso *et al.* (2015) [84]	Lewis rat	Aortic anterograde pulsatile perfusion with Triton X-100 1% at 70 $mL{\cdot}h^{-1}$ (1 h 25 min), then PBS at 50 $mL{\cdot}h^{-1}$ (1 h)	Human pancreatic carcinoma cells (MIA PaCa-2)	Manually injected through renal arterial vascular network	The organ was perfused in a bioreactor with specific medium for 24 h with flow rate of 1 $mL{\cdot}min^{-1}$	Orthotopic implantation into rat	Homogeneous cell distribution in the renal matrix and non-toxicity status of the scaffold. Absence of the vascular endothelial layer

Table 2. (Continued).

Authors and year	Species	Decellularization	Recellularization			Implantation	Main outcome
			Seeded cells	Seeding method	Scaffold culture method		
		followed by SDS 1% at 70 mL·h^{-1} (1 h 25 min)					after transplantation allows direct contact between the blood and the ECM, which results in intense activation of the coagulation cascade and clot formation
Guan *et al.* (2015) [85]	Wistar rat	Renal artery perfusion at 2 mL·min^{-1} with 0.01 M PBS for 15 min, 0.5% SDS for 4 h, and then PBS for 24 h to remove SDS	Mouse ESCs	Manually seeded through the renal artery and ureter	Cells were allowed to attach overnight, after which perfusion culture resumed at 1 mL·min^{-1}	Orthotopic implantation into rat	Accelerating whole kidney scaffold cell repopulation using controlled perfusion conditions. ESCs attached to ECM and proliferated. Vascular tree completely obstructed by thrombi
Guan *et al.* (2015) [88]	Ordinary pig	Renal artery perfusion: distilled water at 10 mL·min^{-1} for 2 h, 1% SDS at 10 mL·min^{-1} was for 28 h and 1% Triton X-100 for 2 h,	Mouse ESCs	Multiple puncture seeding using a 28G injector	Cells were allowed to attach for 4 h, then the kidney scaffold was connected to a bioreactor system and perfused at	No	Decellularization protocol used significantly decreased DNA content while maintaining the basic structural characteristics

Table 2. (Continued).

Authors and year	Species	Decellularization	Recellularization			Implantation	Main outcome
			Seeded cells	Seeding method	Scaffold culture method		
		final rinse with PBS at 10 mL·min^{-1} for 4 days			2 mL·min^{-1} for 7 days		and retaining cytokines
Orlando *et al.* (2013) [90]	Discarded human kidneys	Distilled water at 12 mL·min^{-1} for 12 h, 0.5% SDS at 12 mL·min^{-1} for 48 h in both the renal artery and ureter, final rinse with PBS for 5 days at 6 mL·min^{-1}	–	–	–	No	Optimized decellularization by perfusion through the artery but also through the ureter retrograde into the collecting system. Angiogenic capability of the ECM scaffold was demonstrated
Peloso *et al.* (2015) [91]	Discarded human kidneys	PBS at the rate of 12 mL·min^{-1} for 12 h, 0.5% SDS at 12 mL·min^{-1} for 48 h in both the renal artery and ureter, final rinse with DNase for 6 h at 6 mL·h^{-1} and then with PBS at 6 mL·h^{-1} for 5 days	–	–	–	No	Morphology and dimensions of the acellular glomerulus and its vascular network were relatively well preserved after decellularization and are comparable to their cellular counterparts. Vasculature retained its innate resilience. Cytokines remained within

Table 2. (Continued).

Authors and year	Species	Decellularization	Recellularization			Implantation	Main outcome
			Seeded cells	Seeding method	Scaffold culture method		
							the matrix post decellularization at significant concentrations
Abolbashari *et al.* (2016) [98]	Yorkshire pig	Renal artery perfusion with 0.5% SDS 36 h, and DNase overnight	Primary porcine renal cells	Multiple cell injections into the cortical region of renal scaffolds. Optimized cell injection method capable of repopulating 50% of upper renal pole	Bioreactor perfusion at 10 $mL{\cdot}min^{-1}$ of flow rate directly into the kidney via the renal artery	No	Cellular organization into appropriate renal tubular structures without vascular entrapment of the seeded cells. Level of hydrolase activity was comparable to that of normal kidneys. Erythropoietin production by repopulated renal cells continuously increased with time in bioreactor culture, indicating a clinically relevant functional output

kidney represents one of the most complex organs in terms of development, spatial organization and lineage specification. A human kidney comprises over 30 different cell types and thousands of intricately patterned and functionally compartmentalized epithelial structures named nephrons, which are the functional units of the kidney. Each nephron is differentiated into different regions, mainly the Bowman's capsule that encloses the glomerulus, and the renal tubule, each having different anatomical features and physiological roles [16]. Such organ complexity cannot be reproduced by traditional scaffold based tissue engineering methodologies.

Recently, the derivation of organ-specific extracellular matrix (ECM) scaffolds by decellularization/recellularization and the ability to precisely position cells and materials by 3D bioprinting technologies have been envisioned as two of the most promising organ bioengineering approaches.

Fabrication of organ scaffolds by decellularization/recellularization technology

Decellularization of whole organs has opened new perspectives on tissue engineering [78] and several laboratories have reported on the generation of renal scaffolds from rodent [79–85], pig [86–88], rhesus monkey [89] and human kidneys [5,90,91] (Table 2). A variety of decellularization protocols have been described (and reviewed elsewhere [92–94]) which generally involve the antegrade perfusion of detergents, enzymes or other cell-lysing solutions through the organ vasculature to remove the cellular components while preserving the 3D architecture and biochemical composition of the native ECM.

After decellularization, kidney scaffolds have been shown to preserve the glomerular and tubular architecture and the vascular network. Interestingly, some studies have revealed an essential role of the renal ECM in modulating cell behavior. Nakayama and colleagues showed that hESCs seeded onto decellularized rhesus monkey kidney ECM formed tubules and expressed a battery of renal-related markers, and that those findings were not observed when lung ECMs were used, indicating a certain degree of specific renal ECM function [95]. Indeed, in a recent publication by O'Neill and colleagues [96], papillae-derived renal stem cells were found to be differentially regulated when cultured in contact with the different regions of the kidney ECM, suggesting a regional-specific effect of the kidney ECM on stem cell behavior. Moreover, in a recent study by Yu and coworkers [97], rat acellular ECM kidney scaffolds were grafted in partially nephrectomized rat kidneys showing some regrowth of the excised area after 4 weeks of transplantation. Interestingly, microscopic analysis showed the migration of nestin-positive cells from the injured kidney into the grafted decellularized scaffold, indicating that intact kidney acellular scaffolds retained bioactive cues. However, no significant renal functional recovery was reported. Overall, these works support the idea that acellular kidney ECM retains renal-specific biochemical and biophysical cues that are able to modulate cell proliferation and differentiation. Although the underlying mechanisms are yet to be understood, these findings suggest that organ ECM scaffolds derived by decellularization would be an ideal scaffolding system to provide cells with the appropriate organ-specific microenvironment for the production of functioning kidneys.

Despite these encouraging data, the number of studies that have shown successful transplantation of recellularized whole kidneys has been limited [80,83–85,87]. One of the main hurdles is still the need to efficiently re-endothelialize the vascular network of the engineered kidney before implantation in order to avoid extensive thrombosis when being reconnected to the recipient vasculature. Orlando *et al.* [87] implanted decellularized porcine renal scaffolds into recipient pigs, showing sustained blood pressure during intraoperative monitoring; however, after an extended time period of 2 weeks, the authors reported extensive thrombosis, pointing out the fact that endothelialization of the scaffold vasculature is a mandatory requirement to ensure successful transplantation over prolonged time periods. Also Peloso *et al.* [84] observed the same problem of thrombus formation upon implantation of decellularized kidney scaffolds in a rat model. Remarkably, in a study led by Ott [80], whole rat acellular kidneys were obtained and recellularized with rat neonatal kidney cells and human umbilical vein endothelial cells, and further implanted orthotopically leading to some restoration of renal filtration and reabsorption. In this relevant proof-of-concept work, no evidence of bleeding or thrombus formation was found during the implantation process, highlighting the importance of re-endothelialization of the acellular scaffold vasculature. All this work proves that orthotopic transplantation of the renal scaffolds is nowadays technically feasible, and that proper recellularization of such scaffolding is needed to further achieve effective transplantation and organ function.

The optimal source of cells to repopulate acellular kidney scaffolds is another unsolved question. Current work has tried to recellularize kidney scaffolds with different cell sources including neonatal kidney cells

[80], human umbilical vein endothelial cells [80], mouse ESC [82,85], hiPSC-derived endothelial cells [83], immortalized human renal cortical tubular epithelial cells [83], human pancreatic carcinoma cell line [84] and porcine primary renal cells [98]. With the recent advancements in iPSC and gene editing technologies as well as the latest progress in defining protocols for renal differentiation of PSCs into relevant kidney cell types, it appears that renal cells derived from PSCs would be an ideal cell source for whole organ engineering (as discussed in the section Stem cell therapies for kidney regeneration above). It is believed that PSC-derived progenitor renal cells rather than more mature renal cell phenotypes would be more appropriate to produce a bioengineered kidney. Nevertheless, much work still needs to be done to precisely characterize and validate those PSC-derived cell populations in terms of cell identity, differentiation and final maturation in order to enable the proper repopulation of the different kidney compartments as well as to perform the desired renal physiological functions.

Current work typically uses renal artery [79–82,99] or ureter [79,80] as a route of cell delivery into the kidney scaffold. However, the choice of the route for cell seeding needs to be further studied given that different biochemical and biophysical cues from different ECM compartments could affect cell proliferation, differentiation and further maturation, as suggested already by some investigations [95,96]. Currently, homemade bioreactors are used to deliver cells into the kidney scaffold as well as to ensure uniform delivery of nutrients and oxygen to the recellularized kidney scaffolds. Song *et al.* [80] designed a bioreactor that allowed perfusion through both the renal artery and the ureter including a port for the withdrawal of air to generate a negative pressure environment and produce a transrenal pressure gradient which improved cell seeding through the ureter. However, a major problem is still the uneven cell distribution normally observed after cell seeding, with cells failing to reach the glomeruli in some cases and generally the inefficacy to reach cell densities comparable with the ones found in the native kidney. Therefore, further optimization of bioreactor systems will be essential in order to achieve complete recellularization and organ maturation prior to implantation [100].

Overall, the possibility of combining acellular kidney ECM scaffolds together with hiPSC-derived renal cells represents an unprecedented technology platform for kidney engineering [101]. Already a few papers have shown the possibility of translating the decellularization technology to human kidneys [5,90,91] although none of them studied their recellularization. Future directions will need to define the optimal recellularization conditions (cell density, cell seeding route and seeding methodology) in order to improve cell density, distribution and retention into the different compartments of the kidney acellular scaffold. Moreover, the scaling up of the technology to recellularize human-sized kidney scaffolds will require improved cell differentiation protocols, efficient cell delivery strategies and innovative organ bioreactor systems with physiologically relevant kidney culture conditions (volume, pressure, flow rate and mechanical stimuli) in order to produce functioning human kidneys [100,102].

3D bioprinting

3D bioprinting applies recent developments of additive manufacturing technologies (also known as 3D printing) to accurately deposit living cells or cell aggregates together with hydrogel based supporting biomaterials (altogether termed bioink) into precise geometries to build organ-like or tissue structures in three dimensions [103,104]. In recent years, novel advancements have been made in the development of different bioprinting techniques for the generation of 3D live tissue-like structures (reviewed in [6]) including those based on cellular inkjet printing, extrusion, soft lithography and laser-induced forward transfer. All of them consist of a computer-aided device that translates in three dimensions complex geometries aimed to mimic specific tissue biological features or even whole organs.

Despite the great potential of this technology in the field of tissue engineering, one of the major hurdles that first needs to be solved is the lack of adequate biomaterials to support successful bioprinting of living structures. Much research is being done in this regard in order to develop new classes of bioinks, as extensively reviewed elsewhere [105–107]. Interestingly, in recent work by Pati *et al.* [108] bioinks from decellularized adipose, cartilage and heart tissues were prepared and used for printing cell-laden constructs with controlled porosity. The authors demonstrated the possibility of generating tissue analogues *in vitro* with either adipogenic or chondrogenic potential when human adipose derived stem cells and human inferior turbinate tissue derived mesenchymal stromal cells were printed using adipose tissue derived and cartilage tissue derived ECM bioinks respectively. In the context of kidney regeneration, the successful generation of functional kidney scaffolds by bioprinting would be limited by the possibility of mimicking kidney ECM in terms of composition and spatial organization as well as of depositing different renal cell populations in an organized manner. In this regard, acellular kidney ECM-derived bioink would be an

attractive option since it may provide kidney-specific instructional cues to the printed cells.

Although 3D bioprinting technology is still in its infancy, it has already shown its potential in organ regeneration. The group of Atala has been able to design a printable bladder using modified inkjet bioprinting technology [109], showing that it is possible to print different cell types in an organized 3D structure. This seminal work opened new avenues of organ 3D printing as a whole. Other approaches such as the use of tissue spheroids as building blocks have demonstrated great success in fabricating microtissues such as vascular networks [110,111] that can be further assembled into organized macrotissues.

Although much work still needs to be done before a complete fully functional kidney could be printed, a preliminary step would be the creation of small scale organoids or functional units such as the nephron that could be useful for a number of applications such as drug screening and disease modeling. Already some recent work has been exploring the use of hPSCs for bioprinting [112,113] in order to study the possible effects of the bioprinting process parameters on cell viability and differentiation potential.

In the context of whole organ engineering, several challenges need to be taken into account for the implementation of bioprinting techniques [103,104]: (a) the development of novel bioinks (i.e. tissue-specific bioinks); (b) the improvement of bioprinting manufacturing devices with enough spatial resolution to recapitulate the hierarchical structure of complex organs and mimic organ cell densities; (c) the need for vascularization; (d) the development of adapted perfusion bioreactors suitable for organ printing and further organ maturation.

Conclusions

The past 3 years have been exciting for the field of kidney tissue engineering. Recent work indicates the feasibility not only to generate kidney cells from patient-specific iPSCs but to understand the developmental cues guiding their *in vitro* production. The variation in the different findings indicates that the 3D kidney generated structures when combining renal-differentiated cells from hPSCs have different degrees of functional potential. Thus, it has already been suggested that there is a need to develop further *in vivo* assays proving the functionality of the generated iPSC patient-derived renal cells, such as their transplantation into a nephrectomy based kidney injury mouse model. Also and in the quest to understand which molecular components are defining renal identity during human development, the generation of reporter PSCs for renal markers is essential for a comprehensive analysis of kidney development, leading to the derivation of novel protocols for the formation of mature and functional renal cells.

Other issues related to kidney engineering rely on the development of innovative approaches that can mimic such organ complexity. Recently, two different strategies have been attracting much attention among researchers: whole organ engineering by decellularization/recellularization and bioprinting of living organ-like structures, as they could become a novel source for organ-on-demand supply.

In conclusion, the continuous advances in the field of regenerative medicine would directly benefit the generation of novel strategies for kidney bioengineering: from the manufacturing of biomimetic scaffolds and 3D living constructs to the production of iPSC patient-derived renal cells in the clinical setting.

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Author contributions

N. M. performed experiments, analyzed data, contributed reagents or other essential material and wrote the paper; E.G. performed experiments, analyzed data and wrote the paper; J.I.B. contributed reagents or other essential materials and wrote the paper.

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