



Tissue engineering by decellularization and 3D bioprinting

Elena Garreta¹, Roger Oria², Carolina Tarantino¹, Mateu Pla-Roca³, Patricia Prado¹, Francisco Fernández-Avilés⁴, Josep Maria Campistol⁵, Josep Samitier^{6,7,8} and Nuria Montserrat^{1,8,*}

¹ Pluripotent Stem Cells and Activation of Endogenous Tissue Programs for Organ Regeneration, Institute for Bioengineering of Catalonia (IBEC), c/ Baldiri Reixac 15-21, 08028 Barcelona, Spain

² Institute for Bioengineering of Catalonia (IBEC), c/ Baldiri Reixac 15-21, 08028 Barcelona, Spain; University of Barcelona, Spain

³ Nanotechnology Platform, Institute for Bioengineering of Catalonia (IBEC), c/ Baldiri Reixac 15-21, 08028 Barcelona, Spain

⁴ Department of Cardiology, Hospital General Universitario Gregorio Marañón, Instituto de Investigación Sanitaria Gregorio Marañón, Universidad Complutense-Facultad de Medicina, c/ Dr Esquerdo 46, 28007 Madrid, Spain

⁵ Hospital Clinic, University of Barcelona, IDIBAPS, 08036 Barcelona, Spain

⁶ Nanobioengineering Group, Institute for Bioengineering of Catalonia (IBEC), c/ Baldiri Reixac 15-21, 08028 Barcelona, Spain

⁷ Department of Engineering: Electronics, University of Barcelona, 08028 Barcelona, Spain

⁸ Networking Biomedical Research Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Madrid, Spain

Discarded human donor organs have been shown to provide decellularized extracellular matrix (dECM) scaffolds suitable for organ engineering. The quest for appropriate cell sources to satisfy the need of multiple cells types in order to fully repopulate human organ-derived dECM scaffolds has opened new venues for the use of human pluripotent stem cells (hPSCs) for recellularization. In addition, three-dimensional (3D) bioprinting techniques are advancing towards the fabrication of biomimetic cell-laden biomaterial constructs. Here, we review recent progress in decellularization/recellularization and 3D bioprinting technologies, aiming to fabricate autologous tissue grafts and organs with an impact in regenerative medicine.

Introduction

Regenerative medicine holds the promise to replace or regenerate human cells, tissue or organs in order to restore or establish the normal function lost due to disease or damage [1]. By the combination of novel biomaterials with cells, one of the aims of regenerative medicine is to create autologous tissue grafts for future replacement therapies [2,3]. In the last three years, discarded human donor organs, such as kidney [4,5], lung [6], heart [7], and liver [8], have been used to obtain decellularized extracellular matrix (dECM) scaffolds, proving their potential application in tissue engineering. Despite the translational value of these advances, we are still far to generate relevant tissues for immediate clinical applications.

The use of organ-derived dECM scaffolds for bioengineering of human-scale patient-specific organs using hPSCs is envisioned as a major platform for therapeutic applications (reviewed in [9]). Interestingly, the concept of organ printing has lately taken center stage due to recent three-dimensional (3D) bioprinting advancements (reviewed in [10–13]). Current 3D bioprinting techniques have shown to simultaneously deposit combinations of different cell types encapsulated within biomimetic hydrogels via a layer-by-layer process, leading to the generation of 3D bioinspired tissue constructs (reviewed in [10,12,14,15]) [16–20]. Such approach could offer new venues when translating hPSCs-related technologies to a high-throughput 3D setup (e.g., patient induced pluripotent stem cells (iPSCs)-derived organoid screening platforms) (reviewed in [21]).

*Corresponding author: Montserrat, N. (nmontserrat@ibecbarcelona.eu)

So far, different laboratories have shown that it is possible to build up tissue- and organ-like structures either by the use of organ-derived dECM scaffolds (reviewed in [22–27]) or 3D bioprinting techniques (reviewed in [10,12,14,15]) [16–20]. However, the bioengineering of vascularized human-scale organ analogues with optimal functional activity still requires much effort from multidisciplinary research groups before this can become a reality.

Here, we review the latest advancements in the application of decellularization/recellularization technology for the generation of autologous tissue grafts taking advantage of hPSCs. We also examine how 3D bioprinting technologies may benefit from hPSCs derivatives to fabricate human organ analogues.

The advent of decellularization technology

Tissue-specific extracellular matrix (ECM) through decellularization of tissues and organs

Pioneer findings on the production of tissue-specific ECM were first reported in the 1970s and 1980s [28,29]. Despite these major steps, it was not until 10 years later that Badylak and coworkers generated intact acellular small intestinal submucosa matrices by mechanically removing all mesenteric tissues while leaving the trilaminar connective tissue layers intact [30]. Indeed, such matrices demonstrated healing capacity in a dog model for Achilles tendon repair [30]. Soon, different works on decellularization of other simple tissues such as skin [31], vascular tissue [32], heart valves [33] and bladder [34] showed promising results on the generation of biological scaffolds for biomedical applications, representing a realistic alternative to the use of synthetic biomaterial scaffolds. From that moment, further works began to apply decellularization methodologies for the fabrication of dECM slices from complex organs such as liver [35].

In 2008, the seminal work by Ott and coworkers, who successfully generated whole rat acellular hearts by means of perfusion decellularization, represented a breakthrough in the field of tissue engineering and the beginning of the era of whole organ decellularization technology [36]. Over the last years, different research groups following similar approaches have reported the possibility to derive full-scale dECM scaffolds from different organs including liver, heart, lungs, and kidneys; and multiple species including mouse, rat, pig, rhesus monkey, and human (reviewed in [22,23]) [37]. The main milestones on decellularization technology are summarized in Fig. 1.

In vivo the composition and ultrastructure of ECM is in constant remodeling by the resident cells depending on the metabolic and mechanical demands of the tissue, a concept called ‘dynamic reciprocity’ [38]. Such dynamic remodeling may be altered during injury or disease, leading to modifications in the composition and biophysical properties of the ECM, and ultimately, compromising organ function. Accordingly, it has been suggested that ‘organ specificity’, may determine why cells belonging to a specific organ exhibit an innate preference towards dECM scaffolds derived from the organ of origin [39,40]. Along this line, Nakayama and coworkers showed that human embryonic stem cells (hESCs) differentiated into renal-like cells when seeded onto rhesus monkey kidney dECM, but this was not the case when lung dECM was used instead [40]. The main hypothesis supporting such findings relies on the idea that specific ECM proteins are distinctively distributed along the different compartments of the organ as footprints from

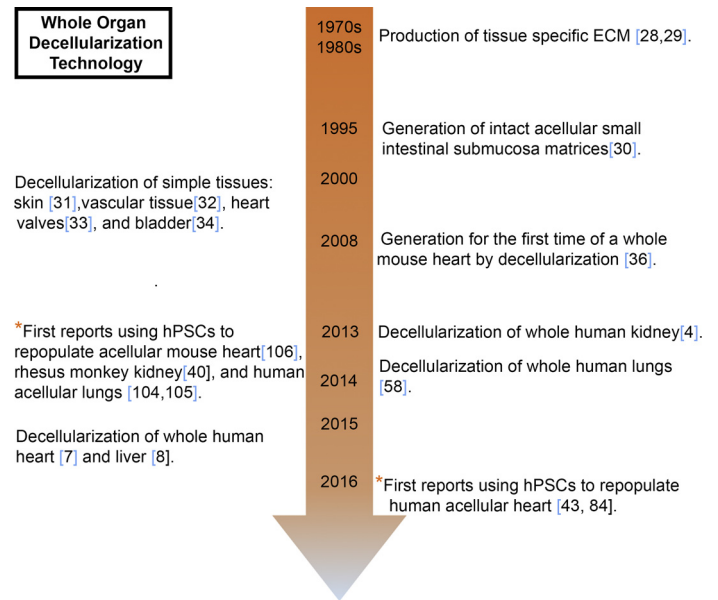


FIGURE 1

Timeline of key events leading to whole organ decellularization methodologies and major milestones using hPSC-derived cells to repopulate organ-derived dECM scaffolds (*).

resident cells. Interestingly, site-specific ECM micromechanical properties have been described to delimitate different tissue regions [41].

Overall, dECM scaffolds provide a complex site-specific combination of biochemical and mechanical cues, which have been hypothesized to guide cell adhesion, proliferation and differentiation during recellularization and further tissue formation (reviewed in [42]) [39–41,43–45]. The main advantages using dECM scaffolds for the *de novo* biofabrication of tissues and organs are summarized in Box 1.

Methods of decellularization

Decellularization techniques aim to remove all the cells from a tissue or organ while preserving the native ECM composition and architecture integrity. As such, tissue and organ decellularization, can lead to the production of 3D dECM scaffolds retaining their biological activity and mechanical properties. If effective, dECM scaffolds should not elicit immune-mediated rejection after im-

BOX 1

Potential of dECM scaffolds to recapitulate tissues and organs

- Preservation of dECM ultrastructure and composition induce favorable tissue organization and remodeling (reviewed in [23,24,36]).
- dECM can modulate cell behavior: attachment, migration and differentiation (reviewed in [23,24,36]) [39,40,43,44].
- dECM do not elicit immune-mediated rejection, since ECM components are largely and highly conserved across species [46,47].
- dECM exhibit suitable mechanical performance, similar to that of their native counterparts (reviewed in [23,24,36]) [41].
- dECM promote constructive remodeling response, as shown for a variety of tissues in both pre-clinical and clinical studies (reviewed in [42]) [45].
- The potential advantage of dECM organ specificity ensures the maintenance of selected cell functions and phenotypes (reviewed in [38]) [39].

plantation [46,47]. Extensive research has shown the mode of action and peculiarities of each kind of decellularization agents employed, as widely discussed elsewhere (reviewed in [48–50]). In general, the optimal decellularization protocol will generate a DNA-free dECM scaffold by finding the right compromise between the duration and complexity of the treatment and the conservation of the desired compositional, structural and mechanical properties of the generated dECM construct.

With respect to the techniques used to infuse the decellularization agents within all regions of the organ, perfusion through the vasculature and immersion/agitation are nowadays the most employed, though others techniques have been also described (e.g., pressure gradient [51,52], supercritical fluid [53]). Perfusion decellularization takes advantage of the innate vasculature of the organ to deliver the decellularization agents across the entire organ. Such approach is often performed in organs in which its main artery can be cannulated for perfusion with decellularization solutions under physiologic perfusion pressures. In their seminal work, Ott and coworkers developed a protocol for whole rat heart decellularization using a homemade bioreactor to perfuse 1% sodium dodecyl sulfate (SDS) through the coronary vasculature, showing that 12 hours perfusion at a physiological pressure sufficed to yield a fully decellularized rat heart [36]. The same research group subsequently applied a similar approach to decellularize whole rat lungs [54] and kidneys [55]. For lung decellularization, both the airway and vascular compartments were investigated to deliver the decellularization solutions, rendering in all cases acellular organ scaffolds with preserved ECM composition, microstructure and 3D architecture [54,56–59]. Overall, perfusion decellularization is the preferred technique to decellularize whole organs, especially in large animals or humans [4–8,58,60–63].

On another hand, in immersion-based decellularization protocols, the tissue or organ of interest is submerged into the decellularization solutions while being subjected to agitation. In this way, decellularization agents enter through the tissue by diffusion.

The duration of the protocol will then depend on the initial tissue thickness and cell density. Such approach is mainly used on tissue samples that do not have easy access to the vascular network (e.g., skeletal muscle, skin) [64–66], as well as on organ slices typically obtained from a segmental resection. Box 2 summarizes the advantages and disadvantages of the different methods of decellularization discussed in this review.

Due to the large diversity of decellularization protocols and tissue sources reported so far, there has been the necessity to establish common criteria to evaluate the effectiveness of any decellularization process. Decellularization requirements and associated methodologies are summarized in Box 3.

Although successful decellularization was achieved for many organs, still much effort should be directed on the definition of standardized decellularization protocols with the final goal to advance in the creation of biocompatible and personalized organ scaffolds for clinical applications. For that, issues including biodegradation, cytocompatibility, pathogenicity and immunogenicity should also be further studied (reviewed in [67]).

Recellularization of whole organ dECM scaffolds

One of the major issues in the field of organ bioengineering is the precise positioning of specific cell types inside their corresponding specific organ compartment. Methods of cell seeding into whole organ-derived dECM scaffolds will largely depend on the organ itself and usually will require the use of bioreactors. Complex organs including kidney, heart, lung, and liver entail the development of challenging cell seeding and culture methods to promote tissue formation and maturation. Oxygen diffusion across an engineered tissue is limited to a maximum tissue thickness of 200 micrometers (reviewed in [68]), meaning that higher tissue thicknesses must require functional vasculature to supply the cells with oxygen and nutrients as well as facilitate the removal of metabolic waste products. In this regard, bioreactor technology for whole organ engineering still needs to overcome many issues:

BOX 2

Methods of decellularization

	Perfusion	Immersion/agitation
Mode of action	• Infusion of the decellularization agents through the organ vasculature • Preferred when decellularizing large animal or human organs	• Immersion of the tissue or organ into the decellularization solutions while shaking • Used when the access to the vasculature is difficult or absent
Advantages	• Facilitates homogeneous exposure to the decellularization reagents and removal of cellular content • The possibility to apply physiological perfusion pressures would favor preservation of tissue ECM composition and architecture • Controlled perfusion conditions by the use of bioreactors enhances the robustness and efficiency of the process	• Mechanical agitation facilitates cellular content removal • Easy and fast procedure when decellularizing small animal organs or tissues, and human tissues obtained from segmental resections • Does not need overly specific bioreactor equipment
Disadvantages	• Unappropriate perfusion pressures can disrupt ECM and impact on the viscoelastic behavior of the dECM scaffold. Optimization is required for each tissue/organ • Needs cannulation of the main organ artery • This method usually needs the use of specific perfusion bioreactors	• Decellularization conditions (e.g., agitation, reagents' exposure time) have to be optimized depending on the tissue thickness. Excessive agitation can disrupt ECM • It is an unreliable method when decellularizing large animal or human whole organs • This method usually needs increasing times of exposure to the decellularization agents when compared to perfusion
References	[4–8,54,56–63]	[39,64–66]

BOX 3**Established criteria for effective decellularization**

Requirement	Methodology	Outcome for effective decellularization	References
Removal of cells and DNA content	- Decellularized tissues should accomplish (i) <50 ng of double-stranded DNA (dsDNA) per mg of dry weight dECM, as quantified by commercially available dsDNA intercalators such as PicoGreen and gel electrophoresis (ii) <200 bp DNA fragment length, analyzed by gel electrophoresis - Histological stainings (Hematoxylin and Eosin, Masson's Trichrome, Movat's Pentachrome, or Safran O) or immunohistochemistry analysis in dECM should denote the lack of visible nuclei (as stained DAPI or Hoechst). These can be used to qualitatively detect if nuclei content, cytoplasmic proteins or some extracellular components are still present after decellularization	Avoid adverse cell and host response as well as negative tissue remodeling and inflammation responses after implantation	[34] (reviewed in [48])
Quantification of residual detergents	- Quantification of remnant SDS can be determined using Stains-All reagent - Similarly residual Triton X-100 can be quantified by using derivative spectrophotometry. - Undetectable amounts of such components are desired	Avoid toxic effects from these decellularization agents, ensuring cell viability during dECM scaffold recellularization.	[67]
Preservation of ECM components	- Qualitative evaluation by immunohistochemistry analysis of the main ECM proteins including collagens, laminin, fibronectin and elastin - Quantitative colorimetric assays are employed to determine the amount of collagen, elastin and glycosaminoglycans using commercially available Sircol, Fastin and Blyscan kits, respectively - Mass spectrometry-based proteomics allows precise identification of matrix proteins and other tissue-specific proteins	Retention of the main basement membrane components and structural ECM proteins as compared to their native counterparts	[8,43,56,84]
Maintenance of 3D architecture and vascular integrity	- Micro- and nano-architecture of relevant parenchymal structures of each organ can be assessed by scanning electron microscopy (SEM) - Conservation of the hierarchical vascular bed of the organ after decellularization can be evaluated by MicroCT, dye or microbeads perfusion assays, angiography or corrosion casting	- Conservation of the main structural features specific of each organ, which will facilitate tissue organization and maintenance of specific cell phenotypes - Conservation of the hierarchical vascular bed of the organ, which is essential to further achieve an effective recellularization outcome	[4,5] (reviewed in [22,36])
Biomechanical performance	- Traditional material science and engineering techniques including uni- or bi-axial mechanical testing and atomic force microscopy (AFM) have been mainly used for mechanical testing on produced decellularized scaffolds - Lung mechanics has been assessed using pressure-volume curves and measuring force tension relationships in linear strips of decellularized lungs	Optimal dECM scaffold mechanical properties (mechanical strength and viscoelastic behavior), that following recellularization should be similar to those of their native counterparts	[4,41,56,63]

BOX 4**Bioreactor requirements for recellulization of whole organ dECM scaffolds**

- Include independent access lines for cell seeding through vascular and non-vascular routes
- Integrate pressure transducers to control flow/volume-based pressure
- Allow continuous or pulsatile perfusion through the vasculature to provide nutrients while removing metabolic waste products
- Monitoring and maintenance of physiological vascular pressure
- Monitoring and maintenance of proper gases supply
- Monitoring organ mechanics during repopulation
- Can include organ-specific biophysical stimuli (e.g. provide mechanical ventilation in lung, provide mechanical stretch and electrical stimulation in heart)
- Allow non-invasive or minimally invasive monitoring of relevant biochemical and biophysical markers indicative of organ maturation and function
- Allow automation of critical parameters
- Maintain sterility (disposable or easily sterilized components)
- Good manufacturing practices (GMP) amenable/Clinical grade bioreactors

from the definition of optimal cell seeding methodologies to the possibility to monitor biochemical and biophysical markers indicative of organ maturation and function in a non-invasive manner. Other issues are related to the application of physiological relevant stimuli that can enhance tissue formation and functionality. The principle bioreactor requirements for whole organ culture are listed in **Box 4**. Extensive discussion on recent bioreactor developments is reported elsewhere (reviewed in [69]) [70–77].

Vascular and non-vascular routes have been employed to deliver cells into whole organ-derived dECM scaffolds, mostly following dynamic seeding procedures, which consist of introducing cells at a specific concentration into the vascular perfusion line. Following this methodology, researchers have recellularized the vasculature and parenchyma of liver, heart, lung and kidney (reviewed in [22–27]). Cells introduced into the vascular flow may traverse the vascular lining through pores produced during decellularization, then reaching the organ parenchyma. In this regard, it has been proposed that the proper adjustment of flow rates may reduce shear stress on cells, while minimizing the potential damage on the dECM scaffold. Moreover, multiple inoculations of cells are preferable than a unique one with the same total cell number. Performing multiple cell infusions directly into the liver vascular circuit in a step-wise manner led to more than 85% cell engraftment [77–79], also showing a more efficient distribution of cells across all regions of the organ-derived dECM scaffold. Alternatively, direct injection of cells with a small gauge needle by performing multiple injections throughout different areas of organ parenchyma has shown less success [80]. Other non-vascular routes commonly used to reintroduce cells are the trachea in lungs [54,56–59] or the ureter in kidney [55,81–83].

So far, the large body of work regarding recellularization of whole organ dECM scaffolds has been performed in small animal models using different bioreactor settings, cell types and seeding conditions (reviewed in [22–27]). The main problems encountered during recellularization were the uncomplete re-endothelialization of the organ vasculature, and the insufficient repopulation of the organ parenchyma [55,56,60]. Notably, recently this year, Guyette and coworkers have partially repopulated whole decellularized human

hearts using a custom human heart bioreactor capable of providing coronary perfusion and left ventricle wall mechanical stimulation, showing metabolically active repopulated myocardial segments after 14 days of organ culture [84]. Also Nichols and coworkers have reported, for the first time, the development of a bioreactor system to support recellularization of whole human paediatric lung dECM scaffolds, identifying the main conditions and cell requirements necessary for bioengineering whole human lungs [85].

Moreover, many different cell types and sources have been reported for recellularization strategies of different organs (reviewed in [22–27]). Initial works made use of neonatal or fetal cells derived from the organ of interest, showing retention of their tissue-specific phenotype after seeding into the organ-derived dECM scaffold together with relevant organ-specific functionality. Overall, these works served as a proof-of-concept of the fabrication of whole organs *de novo* by decellularization/recellularization techniques. However, those findings also highlighted the necessity to find more amenable cell sources that could be easily expanded and differentiated into functional and multiple cell lineages. In this regard, hPSCs have been proposed as promising candidates due to their self-renewal capacity and the potential to give rise to any cell type in the body [86–88]. The use of hPSCs as a cell source for the development of bioengineered organs based on dECM scaffolds is further discussed in the following section.

Pluripotent stem cells: a long-standing cell source for regenerative medicine

Pluripotent stem cells

Pluripotency is defined as the ability of a single cell to divide and produce differentiated cells from the three germ layers of the embryo [86–88]. The idea to generate functional tissues and organs from pluripotent stem cells (PSCs) has been a long-standing goal in stem cell biology, representing an unprecedented opportunity to study development and even to heal degenerative diseases and aging-related disorders. In this regard, the possibility to capture and culture indefinitely hESCs from the pluripotent inner cell mass (ICM) of the blastocyst has been a major breakthrough in the area of regenerative medicine [86].

Before hESCs were first derived, seminal studies already tried to answer how shape and pattern emerge from the simple beginnings of an embryo, and even how specialized cells differentiate during embryo development becoming organized into a 3D architectural context (reviewed in [89]) [90]. Pursuing the idea to reprogram differentiated cells to an ‘embryonic’ state, Takahashi and Yamanaka in 2006 discovered that the pluripotent state found in hESCs derived from the ICM could be artificially induced in a somatic cell through the overexpression of just four transcription factors (OCT4, SOX2, cMYC, and KLF4-OSKM) [87] (reviewed in [91]). The produced cells, so called iPSCs, exhibited all the molecular and functional features of ESCs. Importantly, in the last years human iPSCs (hiPSCs) have shown to become instrumental platforms for the study of human development and disease with the identification, in some cases, of molecular and cellular mechanisms responsible for disease gestation and progression (reviewed in [92]).

Overall, one major limitation in the field of hiPSC disease modeling is the lack of a systemic context and disease-related environmental cues [e.g., disorganized ECM, insufficient biochemical signals from the niche, among others], opening new

BOX 5	
Extracellular matrix components used in hPSCs culture and differentiation	
ECM components	References
• Collagens are the most abundant ECM macromolecules found in our body. Among the 28 types of collagen that exist, collagen IV and I have been widely used in hPSCs differentiation studies demonstrating to be implicated in mesodermal differentiation. In addition, collagen's ability to self-aggregate and crosslink makes it an attractive macromolecule for biomaterial science	[97]
• Laminin is a trimeric protein found in the basement membrane, which has been largely investigated for its influence in hPSCs differentiation towards ectodermal tissues	[98]
• Fibronectin is an ECM protein highly expressed during the early stages of embryonic development, being essential for proper development of the mesoderm and the neural tube. Generally, it is widely used as cell adhesion protein due to the presence of the peptide sequence arginine-serine-aspartic acid (RGD) in its structure, which is implicated in integrin-mediated cell adhesion	[99,100]
• Matrigel is generated from the basement membrane of mouse sarcoma cells thereby containing a variety of ECM molecules and growth factors. Laminin is the major constituent although collagen IV and proteoglycans also take part of its composition. Since it is derived from basement membrane it provides a rich environment that has been largely used for the maintenance of hPSCs as well as differentiation to many lineages including cells from the three germ layers, mesoderm, endoderm and ectoderm	Reviewed in [94–96]
• Cell-deposited ECM can be extracted from different cell types by first allowing the cells to produce their own ECM and then removing them without disturbing the newly synthesized ECM structure and biochemical composition. Such cell-deposited ECM has been then used as a coating for stem cell stemness and differentiation studies	[101]

challenges when integrating biomaterials mimicking disease pathology. In this regard, it has been recently shown that the proper fine-tuning of 3D scaffolds recapitulated the abnormal contractility in hiPSC-derived cardiomyocytes from patients with long QT syndrome type 3, and not when healthy counterparts were used. Such platform also allowed for the screening of cardiotoxic compounds in different 3D settings, highlighting the potential application of these approaches for human disease modeling [93].

Differentiation strategies: from pluripotent colonies to organoids/organogenesis in a dish

One of the major areas of research in the field of hPSCs has been the development of protocols for the generation of functional cell types suitable for disease modeling and cell replacement therapies. Until recently, most protocols of differentiation relayed on the generation of single cell populations rather than complete tissues. However, in the last three years pivotal studies have demonstrated

that it is possible to generate 3D cultures of developing tissues named organoids (reviewed in [94,95]).

After the first derivation of hESCs [86], different laboratories worldwide explored hESCs capacity to undergo controlled differentiation either in monolayer, by seeding cells in the presence of different ECM protein coatings (major matrices used for the culture of hPSCs are described in Box 5) (reviewed in [94–96]) [97–101], or as spheroid-like structures named embryoid bodies (EBs) (Fig. 2). Although EBs can recapitulate several aspects of early development (reviewed in [94]), as any other methodology, EB formation still hampers the translation of this approach into a clinical setting (e.g., low reproducibility and scalability). Nevertheless, all these advances have been fundamental for the proper instruction of hPSCs to form self-organized tissue-specific organoids including the optic cup, brain, intestine, liver and kidney (reviewed in [94]).

Organoids are similar to *in vitro* derived EBs, but they can recapitulate a large number of biological processes related with spatial and temporal organization of heterogeneous tissue-specific cells within the 3D structures (Fig. 2). Even in some cases, organoids have proved to exhibit physiological functions being close to the *in vivo* setting. In this regard, kidney organoids derived from hiPSCs have been recently shown to contain multiple nephron segments surrounded by nascent blood vessels, being able to respond in front of nephrotoxic compounds [102]. Despite these findings, most hPSC-derived organoid models only represented single or partial components of a tissue, hindering the proper control of cell-cell interactions, cell-matrix interplay, and cell organization. Moreover, common hurdles in organoid technologies are being related to incomplete maturation of hPSC-derived cell types and the lack of vascularization.

Bioengineering approaches for hPSCs differentiation: dECMs as biomimetic platforms for generating tissues on-demand

Bioengineering approaches can be used to overcome major issues associated with hPSCs differentiation as maturation and functionality. In this regard, matrigel has been a fundamental matrix for organoid methodologies, including cerebral, optic cup and intestinal organoids from hPSCs (reviewed in [94,95]). However, matrigel

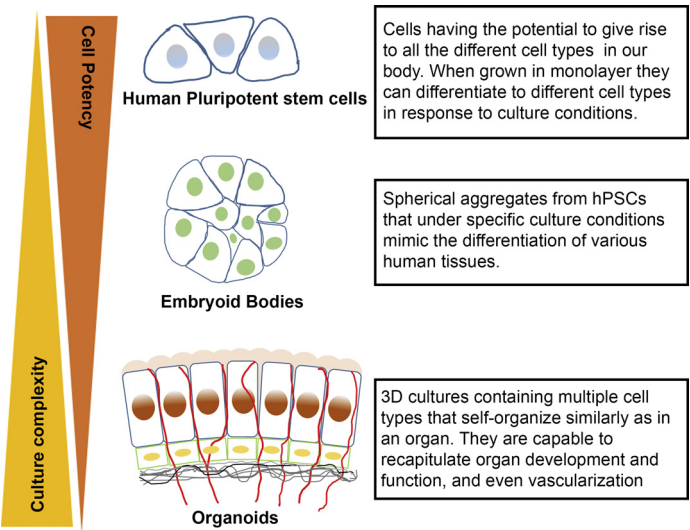


FIGURE 2 The main strategies used for hPSCs differentiation include guided differentiation in 2D monolayer cultures, the formation of embryoid bodies and the induction of 3D self-organization giving rise to organoids. The generation of organoids needs a higher cell culture complexity than the other two approaches.

BOX 6
3D bioprinting techniques

Bioprinting techniques	Additive unit	Actuation method	Cell viability	Commercial bioprinter	Bioink viscosity	Disadvantages	Advantages	References
Inkjet printing	Drop	• Piezoelectric pulse • Thermal induced pulse	>85%	Yes	Low	• Microvalves for inkjet are fragile • Requires fast material gelation/stabilization • Height of 3D constructs limited due to low bioink viscosity	• High control on the amount of material deposited (1 pL to 0.1 nL per drop)	Reviewed in [12]
Laser assisted bioprinting	Drop	• Laser induced pulse	>95%	No	Medium-high	• High cost of future commercial bioprinters • Long fabrication time • Long preparation time of material ribbons • Low diversity of bioink for ribbon preparation	• High printing resolution • Single cell deposition	Reviewed in [12]
Micro extrusion	Material strain	• Pneumatic pressure • Mechanical pressure	40–95%	Yes	Medium-high	<i>Pneumatic pressure</i> • Cells may suffer important shear stress • Medium printing accuracy • Low printing resolution <i>Mechanical pressure</i> • Cells may suffer important shear stress • Medium printing accuracy • Low printing resolution • Mechanical parts make this system more fragile • Screw based system require cleaning of mechanical parts	<i>Pneumatic pressure</i> • Abrasive materials can be used • Disposable cartridge that avoids cross-contamination • Non mechanical parts • Used by most of commercial bioprinters due to its robustness and simplicity • Allows printing cell high density <i>Mechanical pressure</i> • Abrasive materials can be used • Piston based systems uses disposable syringes • Allows printing cell high density	Reviewed in [10,12]
Stereolithography (SLA)	Cured bioink voxel	• Laser based curing • UV and visible light projection curing	>85%	Yes	Medium	• Only can be used with light crosslinkable bioinks • Multicellular structures are challenging • Printer are not specifically designed for biofabrication	• Affordable • High printing velocity	Reviewed in [10,123,127,128]

composition is not well defined and batch-to-batch differences may lead to important differences in experimental outcomes [103].

Along this line, biomaterials can be used to create stem-cell-like niches providing key elements to control the regulation of stem cell fate and function. Indeed, material properties have been often designed to mimic physiologically relevant ECM stiffness, topography, and adhesion-ligand type, density and affinity. These features, when combined with hPSCs have led to the derivation of protocols for hPSCs differentiation building personalized tissue constructs using human organ-derived dECM scaffolds [43,84,104,105], and even for partially or totally reconstructing mouse [106] and human whole organs [84,105].

Ott and coworkers recently reported for the first time the whole repopulation of decellularized human hearts with hiPSCs-derived cardiomyocytes [84]. In the same line, our group developed a rapid protocol for the generation of human heart grafts by co-culturing hPSC-derived cardiomyocytes on top of 400 micrometers-thick slices of human ventricular dECM scaffolds [43].

Importantly, rat and human lung dECM scaffolds have been also recently shown to be repopulated with endothelial and perivascular cells differentiated from hiPSCs [105]. Concerning kidney, only two works have investigated the role of rhesus monkey kidney dECM scaffolds on hPSCs renal differentiation [40,107].

Alternatively, 3D bioprinting technology have opened new venues for the bottom-up generation of tissue and organ analogues by the deposition in an additive layer-to-layer approach of differentiated hPSCs and biomaterials, specifically arranged to reproduce native 3D architectures (reviewed in [11,12,95]). Nevertheless, attempts to generate hPSCs derived bioprinted constructs are still in its infancy, with only one report on the generation of mini livers from hPSCs [16].

Tissue engineering of human organ analogues by 3D bioprinting

3D bioprinting techniques

Bioprinting techniques aim to perform simultaneous deposition of single or multiple combinations of living cells together with supportive matrices containing biochemical and biophysical cues (altogether termed as bioink). In this manner, organs or tissue analogues are constructed following a predefined architecture in 3D (reviewed in [12–14]). These methodologies are generally classified based on the technology used to generate the structures of the cell-laden material as follows:

Inkjet bioprinting, also referred as ‘drop-on-demand printers’ appeared early in 2003 [108]. Firstly developed inkjet printers modified commercially available two-dimensional (2D) ink-based printers by replacing the ink in the cartridge by a biological material, and the paper, by an electric-controlled elevator that moves on the z direction providing three-dimensionality (reviewed in [12]) [109]. Nowadays, inkjet printers make use of nozzles that generate isolated droplets of cell-laden material by means of piezoelectric [110] or thermal (reviewed in [111]) actuation systems. In this manner, by means of either acoustic waves or thermal forces, respectively, liquid drops are ejected onto a substrate. In the last years, inkjet bioprinting has allowed for the efficient introduction of gradients of cells or growth factors along the 3D constructs by the modification of drop densities and size [17,112,113]. Despite this major advances, one common drawback

in inkjet printing is the need to work with biological materials in liquid forms, which in most of the cases demand a fast polymerization procedure post-printing (e.g., by either chemical, pH or ultraviolet mechanisms, among others). All these procedures directly affect the bioprinting process, compromising the chemical and mechanical properties of ECM-derived materials. As reviewed elsewhere, other disadvantages are related to the impediment of building 3D cell-laden constructs with general tissue size, nozzle clogging when using solutions with high cell densities, and cell viability constraints (related to the use of cross-linkers) (reviewed in [12]). Nevertheless, inkjet-based bioprinters have become a massive used technology reducing costs and facilitating the use of free designs and softwares worldwide. Current research is now focused in the development of novel technologies allowing the use of multiple cell types and materials.

Microextrusion systems appeared as a modification of inkjet printers. Here the extrusion of the material takes place through micro-metric apertures (usually a needle with inner diameters that range from few to hundred micrometers). By applying a continuous force, this technique allows to print uninterrupted cylindrical lines. Commonly, these systems robotically extrude biological materials by pneumatic or mechanical dispensing systems onto a substrate. It has been reported that almost all kind of hydrogels with varying viscosities, as well as aggregates with high cell density can be printed with this approach (reviewed in [10,12]). Compared to inkjet printing, microextrusion printing enables the deposition of large amount of cells, allowing the generation of constructs with general tissue size. So far, high concentrations of hydrogels such as alginate, fibrin and Pluronic F-127, among others, have proved to be effective when producing stable 3D cell-laden structures [114–120]. Importantly, microextrusion systems have been particularly effective when printing multicellular tissue spheroids that further self-assemble into the desired 3D structure (reviewed in [15]). Although all the advantages described here, one of the major limitations of this technique is the decreased cell viability resulting from the shear stress when cells are in viscous fluids during the extrusion process [121]. Thus, one of the main challenges consists in the retention of cell viability and printing speed without decreasing pressure or reducing nozzle size. Several tissues have been fabricated using this system, including branched vascular trees, aortic valves, and *in vitro* tumor models (reviewed in [12]). Recently, Atala and coworkers have lead the first work on the fabrication of bioprinted tissue functional constructs *in vitro* and *in vivo* for mandible bone, ear-shaped cartilage and organized skeletal muscle at human-scale [18].

In *laser-assisted bioprinting (LABP)* drops of cell-laden biomaterials are generated after laser pulses. The falling bioink droplet is further collected on the substrate and crosslinked, avoiding shear stress and resulting in high cell viabilities, even when using highly viscous materials. This recent methodology relies on the use of a laser pulse that creates a high-pressure bubble on a ribbon containing the material to be printed, thereby generating a bioink droplet. LABP is nozzle-free, thus minimizing clogging-related issues. Moreover, the achieved resolution allows the delivery of single-cells on each drop. Since this system generates scaffold-free 3D cell constructs through a layer-by-layer manner, lately LABP is becoming used for the deposition of different living cells and biomaterials in a well-defined 3D structure. Besides these advantages, still possible side

effects of laser exposure to cells remain elusive. Other limitations are related to the preparation of cell-laden ribbons (specific for each cell type and hydrogel, hindering scaling-up procedures) and the deposition of metallic residues in the final bioprinted construct. Similarly, targeting and positioning cells becomes difficult because of the nature of the ribbon cell coating (reviewed in [12]). To date, few works took advantage of this system in regenerative medicine [19,122], and the high cost of LABP precludes their use for many researchers worldwide.

Stereolithography (SLA) was initially used to create cell-free scaffolds but the increasing development in the formulations of new cross-linkable materials allowed the use of SLA for 3D bioprinting (reviewed in [123]) [124]. In SLA, the laser focusing point moves on the X/Y axes along the uncured bioink, while the stage where the material is polymerized lowers allowing polymerization. Lately, *direct light projection (DLP)* has emerged as an affordable and versatile variant of SLA. In DLP, the light from a digital micro mirror device or projector is used for curing photocrosslinkable hydrogels in a layer-by-layer approach. By curing the structures plane-by-plane, DLP offers enormous advantages in front of SLA (e.g., printing time is not depending on the design complexity of each plane). DLP has been recently used for generating biocompatible

scaffolds [125–127], and more recently Wang and coworkers demonstrated that DLP enabled for the fabrication of 3D cell-laden structures with resolutions of 50 micrometers, and reaching 85% cell viability when encapsulating human fibroblasts [128]. DLP and SLA offer new alternatives for the fabrication of 3D bioconstructs with precise micro- and nano-architecture, being affordable systems in terms of costs.

A summary of the aforementioned bioprinting techniques is presented in Box 6.

3D bioprinting of cell-laden hydrogels

Since 2000, when rapid prototyping technologies were first adapted for the deposition of cell-laden hydrogel 3D structures in cell-compatible printing conditions, researchers all over the world have tried to produce biological tissue-like constructs using different cell types and hydrogel formulations. The basic 3D bioprinting equipment needs when aiming to fabricate cell-laden 3D structures are described in Box 7.

Current efforts are devoted to develop novel biomaterial formulations that can mimic the complexity of the native ECM—a concept called biomimicry—with an impact for bioprinting applications. ECM composition and rigidity have proved to dictate cell

BOX 7

3D bioprinting of cell-laden tissue constructs

3D bioprinter needs

- Allow combination of micro-extrusion, fused deposition and inkjet printing at the same time during a printing procedure
- Have at least two hydrogel-compatible printing heads, which facilitates deposition of different cell types in the same printed construct
- Possess temperature control systems for printing heads and substrate
- Allow optical monitoring of the printing process
- Allow the interchange of material cartridges during the printing procedure
- Precise control of the pressure applied during the printing process
- Allow the use of different substrates to print the desired 3D tissue construct (e.g. petri dish, multiwall plates)
- Posses an accurate calibration system for needle tip positioning
- Posses a source of light (UV, blue light) for light-crosslinking hydrogels
- Work under sterile conditions

Key material properties for 3D bioprinting

- *Printability* defines the suitability of a material for a specific printing process and largely depends on the material physicochemical properties (viscosity, shear thinning, yield stress, hydrogel crosslinking mechanism) under the conditions provided by the bioprinting instrument
- *Biocompatibility* refers to the ability to perform as a material that will support the appropriate cellular activity, including the facilitation of molecular and mechanical signaling systems, in order to optimize tissue regeneration, without eliciting any undesirable host responses
- *Biodegradability* describes the biological processes inside the body that cause a gradual breakdown of a material. Degradation kinetics should be matched to the novo tissue formation and the byproducts generated should be nontoxic
- *Biomechanical properties* of the material should provide sufficient structural integrity to the printed construct and match tissue specific biomechanic requirements
- *Biomimicry* refers to the ability of reproduce tissue-specific endogenous material compositions

3D bioprinting processing parameters

- The four main variables that need to be balanced to optimize the printing procedure and ensure the design fidelity are: the needle/tip size, the distance from the tip to the surface, the material flow rate and the linear write speed
- These variables are balanced when the leading edge of the printed bioink is continual with the needle, being possible to dispense a uniform strand
- These aforementioned variables need to be optimized for every different material used
- Changes on environmental conditions such as humidity and temperature may influence printing fidelity
- As printed structures increase in complexity, the incorporation of support structures within the fabricated pattern is necessary
- The printing time should be considered, since extended periods of time at non-optimal temperature, humidity or material biocompatibility will affect both material properties and cell viability

fate and function (reviewed in [129]). In the human body, tissue rigidity ranges from 0.2–5 kPa in soft tissues as brain, to 15,000 kPa in bone, being an important parameter to be considered when aiming to design 3D tissue and organ analogues.

Besides the need to be biocompatible and biodegradable, a biomaterial formulation for bioprinting must possess suitable physicochemical properties in order to fabricate 3D constructs with high resolution and printing fidelity – a characteristic named printability (Box 7) (reviewed in [130–132]). In addition, it must also be optimized in order to minimize stress-induced damage to the cells and biological components, which occur during the deposition process (reviewed in [130–132]). The ideal hydrogel formulation should reach a compromise between preserving cell viability and matching optimal printability.

Viscosity, shear thinning and yield stress of a defined bioink will directly affect printing fidelity during the biofabrication process. Similarly, the specific processing parameters will define the shear stress that cells will suffer during the deposition and the time required for the fabrication of a given 3D construct (e.g., tip size, flow rate, temperature; Box 7). Another important outcome is the maintenance of sufficient structural integrity by the bioprinted 3D construct, which will be mainly dependent on the gelation of the

hydrogel formulation. Natural and synthetic hydrogels applied to 3D bioprinting and their correspondent gelation mechanism are further reviewed elsewhere (reviewed in [130–133]).

Elegant works have demonstrated the feasibility of 3D bioprinting for the generation of several tissues, including bone [18], skin [19], vascular grafts [118,120,134], tracheal supports [135], heart [20,117,136] and cartilage [18,20] tissue structures, using several cell sources such as human umbilical vein endothelial cells, mesenchymal stem cells, human meniscus cells and fibroblasts (reviewed in [10,12]). However, the fabrication of human size organ analogues with complex architectures requires a more elaborate 3D bioprinting strategy, usually involving the co-deposition of other material components (such as supportive and sacrificial materials), together with cell-laden hydrogels, ensuring sufficient structural integrity to the printed construct while maintaining an internal porosity (Fig. 3). In this regard, the recent work by Atala and coworkers provides a 3D bioprinting platform for the production of tissues for human applications, paving the way for future building of solid organs [137].

Acellular organ-specific dECM hydrogels for 3D bioprinting

As decellularization protocols emerged, hydrogels made from decellularized tissues including urinary bladder [138], heart [139], liver [140], dermis [141], adipose tissue [142], bone [143], and lung [144], among others, were developed and reported to support growth and function of different cell types. However, it is not until very recently that tissue-specific dECM hydrogels have been envisioned as a new class of hydrogels for 3D bioprinting [20,145–147]. Nowadays, one of the main hurdles when using dECM hydrogels as bioinks relies on their low viscosity, which inevitably compromise shape fidelity of the bioprinted 3D construct, worsening printing resolution.

To date, only few studies have used dECM hydrogels for 3D bioprinting, applying different strategies to improve their printability [20,145–147]. Pati and coworkers were the first to successfully apply dECM hydrogels for 3D bioprinting. Their strategy consisted on the co-deposition of an open porous structure of polycaprolactone (PCL) as a supportive material, together with the cell-laden dECM hydrogel made from cartilage, heart or adipose porcine tissues [20]. Recently, the same group used skeletal muscle-derived dECM hydrogels for 3D printing of muscle constructs [147].

Following a different strategy, Skardal and coworkers elegantly developed modular hyaluronic acid and gelatin-based hydrogels supplemented with porcine liver, cardiac and skeletal muscle dECM solutions. Following a two-step crosslinking procedure, the authors achieved printable bioinks with different stiffness ranging from 100 Pa to 20 kPa, thus allowing the possibility to mimic the mechanical characteristics of different tissues in the body [145]. Other recent work by Jang and coworkers took advantage of pig heart dECM to prepare cardiac-specific hydrogels that in combination with human cardiac progenitor cells were used to fabricate 3D bioprinted cardiac constructs. The gelation of cardiac dECM hydrogels was based on thermal and chemical crosslinking using vitamin B2 via UVA activation [146].

Future outlook

A major limitation when generating artificial organs on demand stands in the development of techniques to properly reintroduce

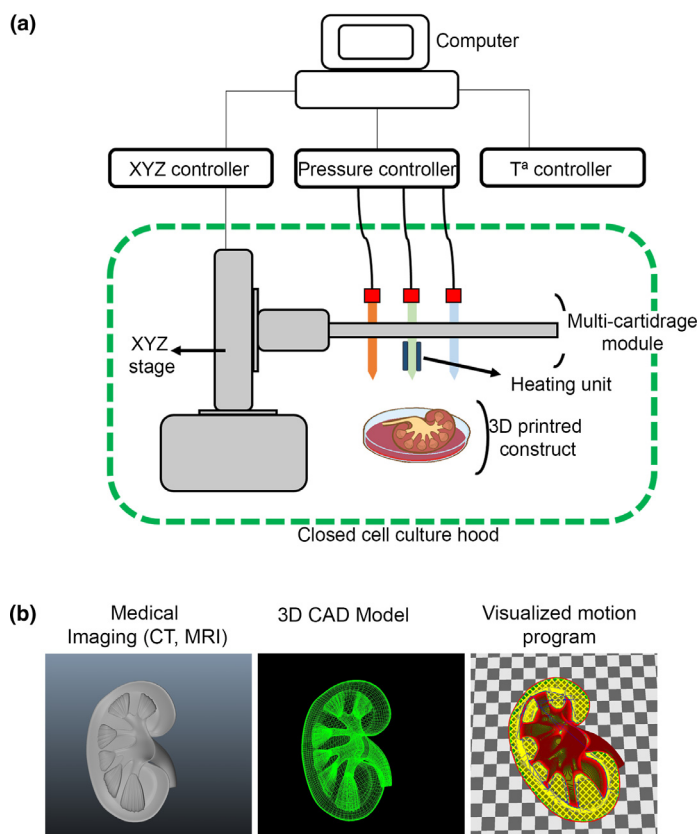


FIGURE 3

Schematic representation of a 3D bioprinting system consisting of a computer aided 3-axis stage controller and a deposition module including three different print heads connected to a pressure controller (a). Computer aided design and computer aided manufacturing (CAD/CAM) process for 3D bioprinting of a human size kidney. A 3D CAD model generated from medical imaging data (CT: computed tomography; MRI: magnetic resonance imaging) produces a visualized motion program which dictates the XYZ stage movements to generate the 3D bioprinted kidney prototype (b).

cells into the organ-specific dECM scaffolds, assessing both complete organ re-endothelialization and functional activity. Due to their intrinsic characteristics, hPSCs have been envisioned as an optimal cell source for the generation of complex tissue structures like the organ parenchyma and the vascular system, offering major advantages when compared with adult somatic or stem cells for the same purposes. Moreover, targeted genome editing, as CRISPR platform, is a powerful tool to manipulate and correct disease related genes in patient-derived hiPSCs, allowing for the generation of autologous-corrected cells suitable for disease modeling and drug screening [148]. We believe that targeted genome editing approaches combined with recent progress in the formation of patient-specific hiPSCs-derived organoids could provide an unprecedented source of organ-specific cell types suitable for cell replacement therapies. In this regard, common efforts on the definition of chemically defined conditions to culture hPSC-derived organ-specific cells has led to the examination of novel approaches guiding hPSCs maturation. Following these questions, different works have relayed on the use of human dECM scaffolds together with hPSCs in order to generate human tissue grafts

[43,84,104], and even to reconstruct whole organs [84,105], revealing the impact of organ-derived dECMs on the proper instruction of hPSCs fate and function [40,43,84].

Alternatively, 3D bioprinting represents a formidable technology for artificial organ generation. Besides the different limitations of this nascent technology (e.g., printing resolution and time, combination of different bioinks simultaneously, among others), the possibility to print human-scale tissues has been recently demonstrated [18]. In this regard, seminal studies have already proved the feasibility to print 3D tissue constructs using organ-specific dECM hydrogels as biomimetic bioinks [20,145–147], opening the door to the fabrication of novel bioink formulations matching cytocompatibility and mechanical strength requirements for 3D bioprinting.

Overall, we believe that recent advancements in the fields of hPSCs differentiation together with organ-derived dECM scaffolds or novel dECM-based hydrogels aimed for 3D bioprinting represent a step forward in the fabrication of autologous functional tissues on-demand (Fig. 4 and Box 8). To this end, multidisciplinary research in the field of engineering, biomaterials science, stem

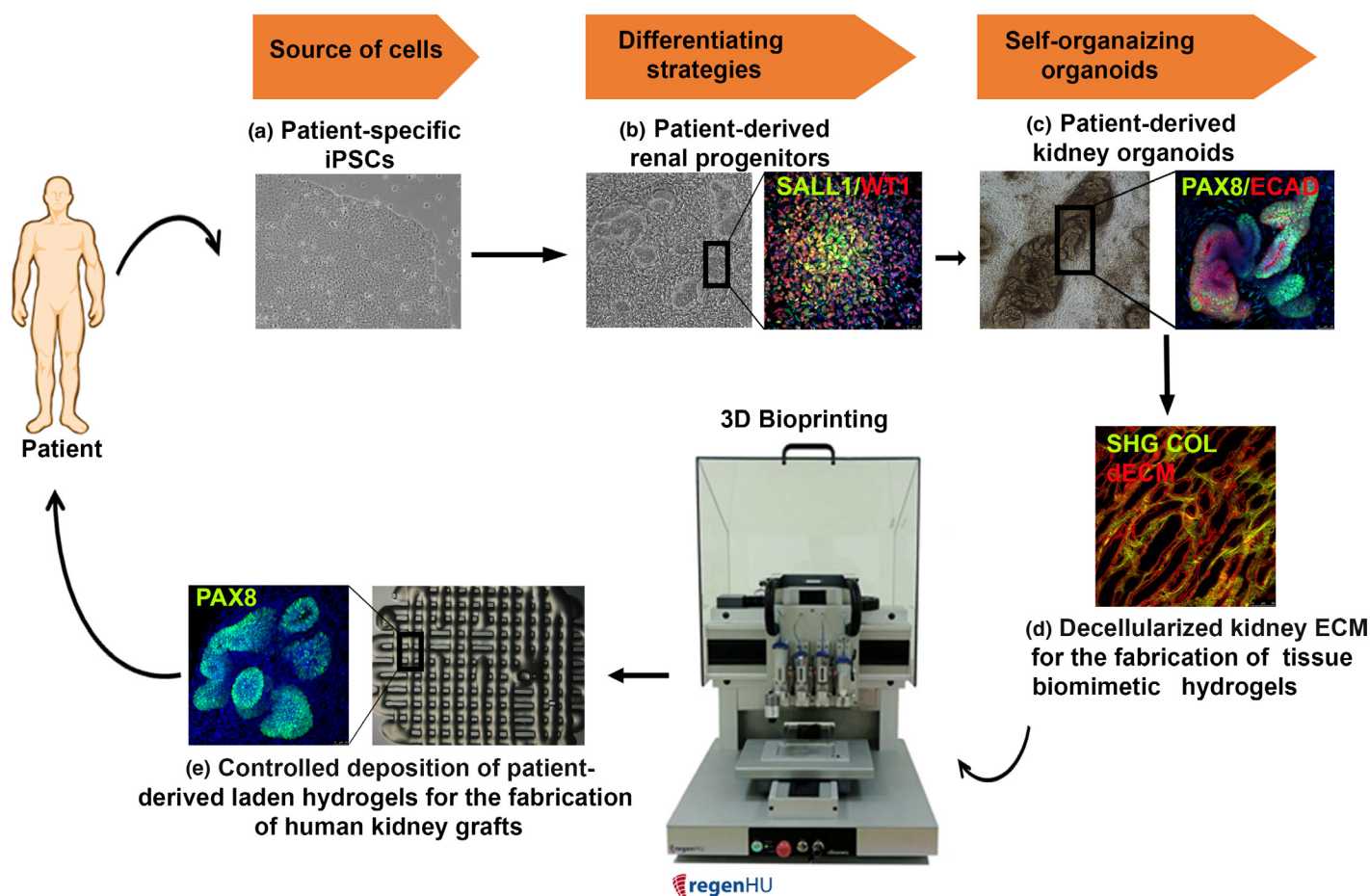


FIGURE 4

3D bioprinting approach for the development of patient-specific organ analogues such as kidney. Patient-specific iPSCs (a) are differentiated into renal progenitor cells in 2D monolayer cultures (representative image for SALL1 and WT1 double positive renal progenitors by immunofluorescence) (b) that are further induced to develop into kidney organoids (representative image for PAX8 and E-cadherin positive 3D renal-like structures by immunofluorescence) (c). The combination of kidney dECM-based hydrogels (d) with patient iPSC-derived renal progenitor cells could be used for the biofabrication of specialized 3D renal constructs (representative image for PAX8 renal structures developed into printed kidney dECM-based hydrogels) (e). This approach renders to the generation of tissue and organ analogues suitable for regenerative medicine applications (e.g., disease modeling, drug screening, cell replacement therapies, among others).

BOX 8**Challenges and future perspectives**

Research area	Specific challenges	Future outcomes
Decellularization	- Standardization of decellularization protocols for each specific organ - Systematic evaluation of immunologic response and the possibility of using dECM scaffolds from different species	- Robust production of organ-derived dECM scaffolds - Determination of the need of immunosuppressive drugs after transplantation. Increase the tissue source, overcoming donor shortage
Recellularization	- Improved seeding methodologies for complete re-endothelialization of the organ vascular bed and parenchyma recellularization - Novel bioreactor systems with optimal control over the culture conditions (oxygen delivery, biophysical stimulation, continuous monitoring of functional parameters)	- Optimal vascularization - Determination of organ biochemical/biophysical parameters during recellularization, and end-points for each organ - Definition of common guidelines for the assessment of organ mechanics Preservation of bioengineered organs before transplantation - Functional evaluation in large animal models
Human pluripotent stem cells	- Standardized protocols for expansion and differentiation to large cell numbers - Purification of hPSC-differentiated cells and identification of optimal cell culture conditions (growth factors, cytokines) for cell phenotype maintenance - Identification of hPSC-derived progenitor cell types suitable for engraftment and maturation into dECM scaffolds	- Repopulation of organ-derived dECM scaffolds with multiple hPSC-derived progenitor cell types specific for each organ compartment, and in sufficient cell number to achieve comparable native organ cell densities - Generation of personalized human bioengineered organ equivalents with optimal functional activity
3D bioprinting	- Development of novel cell-compatible hydrogels with tailored viscosity and biochemical composition - Monitoring of bioink flow rate with automatic adjustment of printing conditions - Present alignment capability on previously printed constructs - Improve bioprinting full automation - Development of bioreactors for tissue/organ growth and maturation post-processing	- Bioprinting of cell-laden structures with increased mechanical strength, bioactivity and cell viability - Increased printing fidelity - Step-wise fabrication of complex multicellular structures - Rapid production of 3D printed constructs, thereby improving cell viability - Generation of functional tissue/organs

cell biology and medicine will be essential to further succeed in the biofabrication of autologous organs for future clinical replacement strategies.

Acknowledgements

We thank SOLIDCAM STUDIO for 3D modelling, printing and rendering. E.G was supported by StG-2014-640525_REGMAMKID. R.O was founded by a FI fellowship (Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitat de Catalunya). C.T was supported by StG-2014-640525_REGMAMKID. P.P was partially supported by MINECO (SAF2014-59778). F.F.A. was supported by Instituto de Salud Carlos III-ISCII (MINECO: PI10-00141 and PI10-02038), Red de Investigación Cardiovascular (RIC) and Red TerCel from ISCII (Ministry of Economy and Competitiveness, Spain), and CAM: S2010/BMD-2420 and has been financially supported by the Commission for Universities and Research of the Department of Innovation, Universities, and Enterprise of the Generalitat de Catalunya (2014 SGR 1442) and developed in the context of ADVANCE(CAT) with the support of ACCIÓ (Catalonia Trade & Investment; Generalitat de Catalunya) and the European Community under the Catalanian ERDF operational program (European Regional Development Fund) 2014-2020. This work also was partially supported by the project MINDS (TEC2015-70104-P), awarded by the Spanish Ministry

of Economy and Competitiveness, and by CERCA Programme / Generalitat de Catalunya. N.M was supported by StG-2014-640525_REGMAMKID, MINECO, (SAF2014-59778, RYC-2014-16242), and by CERCA Programme/Generalitat de Catalunya, and CardioCel (TerCel, Instituto de Salud Carlos III) and 2014 SGR 1442.

References

- [1] C. Mason, *Regen. Med.* 3 (2008) 1–5.
- [2] P. Bajaj, et al. *Annu. Rev. Biomed. Eng.* 16 (2014) 247–276.
- [3] A.S. Mao, *Proc. Natl. Acad. Sci. U. S. A.* 112 (2015) 14452–14459.
- [4] G. Orlando, et al. *Biomaterials* 34 (2013) 5915–5925.
- [5] A. Peloso, et al. *Transplantation* (2015) 1–10.
- [6] S.E. Gilpin, et al. *J. Heart Lung Transplant.* 33 (2014) 298–308.
- [7] P.L. Sánchez, et al. *Biomaterials* 61 (2015) 279–289.
- [8] G. Mazza, et al. *Sci. Rep.* 7 (2015) 13079.
- [9] P.T. Moser, H.C. Ott, *Curr. Opin. Organ Transplant.* 19 (2014) 603–609.
- [10] D.-H.K. Christian Mandrycky, Z. Wang, K. Kim, *Biotechnol. Adv.* (2015)[Epub ahead].
- [11] D.Y.C. Cheung, B. Duan, J.T. Butcher, *Essent. 3D Biofabrication Transl.* (2015) 351–370.
- [12] S.V. Murphy, A. Atala, *Nat. Biotechnol.* 32 (2014) 773–785.
- [13] J. Groll, et al. *Biofabrication* 8 (2016) 013001.
- [14] B. Derby, *Science* (80-) 338 (2012) 921–926.
- [15] F. Marga, et al. *Biofabrication* 4 (2012) 022001.
- [16] J.K. Alan Faulkner-Jones, et al. *Biofabrication* 7 (2015) 044102.
- [17] J.A. Phillippi, et al. *Stem Cells* 26 (2008) 127–134.
- [18] H.-W. Kang, et al. *Nat. Biotechnol.* 34 (2016) 312–319.
- [19] S. Michael, et al. *PLoS ONE* 8 (2013).
- [20] F. Pati, et al. *Nat. Commun.* 5 (2014) 3935.

- [21] W. Peng, U. Derya, I.T. Ozbolat, *Trends Biotechnol.* 34 (2016) 722–732.
- [22] M.E. Scarritt, N.C. Pashos, B.A. Bunnell, *Front. Bioeng. Biotechnol.* 3 (2015) 43.
- [23] S.F. Badylak, D. Taylor, K. Uygun, *Annu. Rev. Biomed. Eng.* 13 (2011) 27–53.
- [24] J.J. Song, H.C. Ott, *Trends Mol. Med.* 17 (2011) 424–432.
- [25] A.A. Khan, et al. *J. Artif. Organs* 17 (2014) 291–300.
- [26] D. Rana, et al. *J. Tissue Eng. Regen. Med.* (2015).
- [27] N. Montserrat, E. Garreta, J.C. Izpisua Belmonte, *FEBS J.* (2016).
- [28] J.T. Hjelle, E.C. Carlson, K. Brendel, *Kidney Int.* 15 (1979) 20–32.
- [29] M. Rojkind, et al. *J. Cell Biol.* 87 (1980) 255–263.
- [30] S.F. Badylak, et al. *J. Biomed. Mater. Res.* 29 (1995) 977–985.
- [31] R.-N. Chen, et al. *Biomaterials* 25 (2004) 2679–2686.
- [32] C.E. Schmidt, J.M. Baier, *Biomaterials* 21 (2000) 2215–2231.
- [33] R.C. Elkins, et al. *Ann. Thorac. Surg.* (2001).
- [34] D. Schultheiss, et al. *J. Urol.* 173 (2005) 276–280.
- [35] T.W. Gilbert, T.L. Sellaro, S.F. Badylak, *Biomaterials* 27 (2006) 3675–3683.
- [36] H.C. Ott, et al. *Nat. Med.* 14 (2008) 213–221.
- [37] J.P. Guyette, et al. *Nat. Protoc.* 9 (2014) 1451–1468.
- [38] M.J. Bissell, J. Aggeler, *Prog. Clin. Biol. Res.* 249 (1987) 251–262.
- [39] J. Cortiella, et al. *Tissue Eng. Part A* 16 (2010) 2565–2580.
- [40] K.H. Nakayama, et al. *PLoS ONE* 8 (2013).
- [41] T. Luque, et al. *Acta Biomater.* 9 (2013) 6852–6859.
- [42] R. Londono, S.F. Badylak, *Ann. Biomed. Eng.* 43 (2015) 577–592.
- [43] E. Garreta, et al. *Biomaterials* 98 (2016) 64–78.
- [44] G. Yang, et al. *Biomaterials* 34 (2013) 9295–9306.
- [45] S.F. Badylak, et al. *Biomaterials* 103 (2016) 128–136.
- [46] M.P. Bernard, et al. *Biochemistry* 22 (1983) 5213–5223.
- [47] M.I.S. Chung, et al. *Matrix Biol.* 25 (2006) 492–504.
- [48] P.M. Crapo, T.W. Gilbert, S.F. Badylak, *Biomaterials* 32 (2011) 3233–3243.
- [49] M. He, A. Callanan, *Tissue Eng. Part B: Rev.* 19 (2013) 194–208.
- [50] T.J. Keane, I. Swinehart, S.F. Badylak, *Methods* (2015).
- [51] C.V. Montoya, P.S. McFetridge, *Tissue Eng. Part C: Methods* 15 (2009) 191–200.
- [52] L.N. Sierad, et al. *Tissue Eng. Part C: Methods* 21 (2015) 1284–1296.
- [53] K. Sawada, et al. *J. Chem. Technol. Biotechnol.* 83 (2008) 943–949.
- [54] H.C. Ott, et al. *Nat. Med.* 16 (2010) 927–933.
- [55] J.J. Song, et al. *Nat. Med.* 19 (2013) 646–651.
- [56] T.H. Petersen, et al. *Cell Transplant.* 20 (2011) 1117–1126.
- [57] A.P. Price, et al. *Tissue Eng. Part A* 16 (2010) 2581–2591.
- [58] J.E. Nichols, et al. *Tissue Eng. Part A* 19 (2013) 2045–2062.
- [59] J. Niles, et al. *Tissue Eng. Part A* 16 (2010) 2565–2580.
- [60] G. Orlando, et al. *Ann. Surg.* 256 (2012) 363–370.
- [61] R. Katari, et al. *Nephron Exp. Nephrol.* 126 (2014) 119–124.
- [62] P.M. Baptista, et al. *Methods Mol. Biol.* 1001 (2013) 289–298.
- [63] D.E. Wagner, et al. *Biomaterials* 35 (2014) 2664–2679.
- [64] A.R. Gillies, et al. *Tissue Eng. Part C: Methods* 17 (2011) 383–389.
- [65] J.E. Reing, et al. *Biomaterials* 31 (2010) 8626–8633.
- [66] B.D. Elder, D.H. Kim, K.A. Athanasiou, *Neurosurgery* 66 (2010) 722–727.
- [67] K.H. Hussein, K.M. Park, K.S. Kang, *Mater. Sci. Eng. C: Mater. Biol. Appl.* 67 (2016) 766–778.
- [68] J. Folkman, M. Hochberg, *J. Exp. Med.* 138 (1973) 745–753.
- [69] B.M. Bijonowski, W.M. Miller, J.A. Wertheim, *Curr. Opin. Chem. Eng.* 2 (2013) 32–40.
- [70] J.S. Uzarski, et al. *Tissue Eng. Part C: Methods* 21 (2015) 1032–1043.
- [71] J. Hülsmann, et al. *J. Artif. Organs* 16 (2013) 294–304.
- [72] Y. Barash, et al. *Tissue Eng. Part C: Methods* 16 (2010) 1417–1426.
- [73] J. Hülsmann, et al. *Methods Mol. Biol.* (2016) 257–284.
- [74] M.S. Raredon, et al. *Biorec. Mol. Access* 5 (2016) 72–83.
- [75] J.M. Charest, et al. *Biomaterials* 52 (2015) 79–87.
- [76] M.R. Ebrahimkhani, et al. *Adv. Drug Deliv. Rev.* 69–70 (2014) 132–157.
- [77] H. Yagi, et al. *Cell Transplant.* 22 (2013) 231–242.
- [78] A. Soto-Gutierrez, et al. *Tissue Eng. Part C: Methods* 17 (2011) 677–686.
- [79] B.E. Uygun, et al. *Nat. Med.* 16 (2010) 814–820.
- [80] N. Shirakigawa, T. Takei, H. Iijima, *J. Biosci. Bioeng.* 116 (2013) 740–745.
- [81] B. Bonandrini, et al. *Tissue Eng. Part A* 20 (2014) 1486–1498.
- [82] M. Caralt, et al. *Am. J. Transplant.* (2014) 64–75.
- [83] E.A. Ross, et al. *Organogenesis* 8 (2012) 49–55.
- [84] J.P. Guyette, et al. *Circ. Res.* (2015).
- [85] J.E. Nichols, et al. *J. Tissue Eng. Regen. Med.* (2016).
- [86] J.A. Thomson, et al. *Science* 282 (1998) 1145–1147.
- [87] K. Takahashi, et al. *Cell* 131 (2007) 861–872.
- [88] J. Yu, et al. *Science* 318 (2007) 1917–1920.
- [89] P.B. Armstrong, *Crit. Rev. Biochem. Mol. Biol.* 24 (1989) 119–149.
- [90] P. Weiss, A.C. Taylor, *Proc. Natl. Acad. Sci. U. S. A.* 46 (1960) 1177–1185.
- [91] S. Yamanaka, *Philos. Trans. R. Soc. Lond. B: Biol. Sci.* 363 (2008) 2079–2087.
- [92] G. Tiscornia, E.L. Vivas, J.C. Izpisua Belmonte, *Nat. Med.* 17 (2011) 1570–1576.
- [93] Z. Ma, et al. *Biomaterials* 35 (2014) 1367–1377.
- [94] M.A. Lancaster, J.A. Knoblich, *Science* 345 (2014) 1247125.
- [95] X. Yin, et al. *Cell Stem Cell* 18 (2016) 25–38.
- [96] L.G. Villa-Diaz, et al. *Stem Cells* 31 (2013) 1–7.
- [97] Y. Liu, et al. *PLoS ONE* 7 (2012).
- [98] J.S. Meyer, et al. *Stem Cells* 29 (2011) 1206–1218.
- [99] E.L. George, et al. *Development* 119 (1993) 1079–1091.
- [100] R.A. Oldershaw, et al. *Nat. Biotechnol.* 28 (2010) 1187–1194.
- [101] Y. Yan, et al. *Biomaterials* 73 (2015) 231–242.
- [102] M. Takasato, et al. *Nature* 526 (2015) 564–568.
- [103] H.K. Kleinman, et al. *Biochemistry* 21 (1982) 6188–6193.
- [104] S.E. Gilpin, et al. *Ann. Thorac. Surg.* 98 (2014) 1721–1729.
- [105] X. Ren, et al. *Nat. Biotechnol.* 33 (2015) 1097–1102.
- [106] T.-Y. Lu, et al. *Nat. Commun.* 4 (2013) 2307.
- [107] C.A. Batchelder, M.L. Martinez, A.F. Tarantal, *PLOS ONE* 10 (2015).
- [108] R.S. Tuan, G. Boland, R. Tuli, *Arthritis Res. Ther.* 5 (2003) 32–45.
- [109] J.P. Mattimore, et al. *Conf. Proc. IEEE SOUTHEASTCON*, 2010, 510–513.
- [110] Y. Fang, et al. *Tissue Eng. Part C: Methods* 18 (2012) 647–657.
- [111] X. Cui, et al. *Recent Pat. Drug Deliv. Formul.* 6 (2012) 149–155.
- [112] E.D.F. Ker, et al. *Biomaterials* 32 (2011) 8097–8107.
- [113] P.G. Campbell, et al. *Biomaterials* 26 (2005) 6762–6770.
- [114] N.E. Fedorovich, et al. *Tissue Eng. Part A* 14 (2008) 127–133.
- [115] K. Jakab, et al. *Proc. Natl. Acad. Sci.* 101 (2004) 2864–2869.
- [116] R. Landers, et al. *Biomaterials* 23 (2002) 4437–4447.
- [117] Y.S. Zhang, et al. *Biomaterials* 110 (2016) 45–59.
- [118] W. Jia, et al. *Biomaterials* 106 (2016) 58–68.
- [119] K.A. Homan, et al. *Sci. Rep.* 6 (2016) 34845.
- [120] D.B. Kolesky, et al. *Proc. Natl. Acad. Sci. U. S. A.* 113 (2016) 3179–3184.
- [121] R. Chang, J. Nam, W. Sun, *Tissue Eng. Part A* 14 (2008) 41–48.
- [122] V. Keriquel, et al. *Biofabrication* 2 (2010) 014101.
- [123] K.C. Hribar, et al. *Lab Chip* 14 (2014) 268–275.
- [124] V. Chan, et al. *Lab Chip* 10 (2010) 2062–2070.
- [125] G. Mapili, et al. *J. Biomed. Mater. Res. Part B: Appl. Biomater.* 75 (2005) 414–424.
- [126] A.P. Zhang, et al. *Adv. Mater.* 24 (2012) 4266–4270.
- [127] S.P. Grogan, et al. *Acta Biomater.* 9 (2013) 7218–7226.
- [128] Z. Wang, et al. *Biofabrication* 7 (2015) 1–29.
- [129] W.L. Murphy, T.C. McDevitt, A.J. Engler, *Nat. Mater.* 13 (2014) 547–557.
- [130] J. Malda, et al. *Adv. Mater.* 25 (2013) 5011–5028.
- [131] D.L.K. Rod, et al. *ACS Biomater. Sci. Eng.* (2016).
- [132] A. Skardal, A. Atala, *Ann. Biomed. Eng.* 43 (2015) 730–746.
- [133] T.L. Panwar, *Molecules* 21 (2016).
- [134] D.B. Kolesky, et al. *Adv. Mater.* 26 (2014) 3124–3130.
- [135] D.A. Zopf, et al. *N. Engl. J. Med.* 368 (2013) 2043–2045.
- [136] R. Gaetani, et al. *Biomaterials* 33 (2012) 1782–1790.
- [137] H.W. Kang, et al. *Nat. Biotechnol.* 34 (2016) 312–319.
- [138] A.L. Brown, et al. *Macromol. Biosci.* 6 (2006) 648–657.
- [139] R.M. Wang, K.L. Christman, *Adv. Drug Deliv. Rev.* 96 (2016) 77–82.
- [140] A. Skardal, et al. *Biomaterials* 33 (2012) 4565–4575.
- [141] M.T. Wolf, et al. *Biomaterials* 33 (2012) 7028–7038.
- [142] D.A. Young, et al. *Acta Biomater.* 7 (2011) 1040–1049.
- [143] M.J. Sawkins, et al. *Acta Biomater.* 9 (2013) 7865–7873.
- [144] R.A. Pouliot, et al. *J. Biomed. Mater. Res. Part A* (2016).
- [145] A. Skardal, et al. *Acta Biomater.* 25 (2015) 24–34.
- [146] J. Jang, et al. *Acta Biomater.* 33 (2016) 88–95.
- [147] Y.J. Choi, et al. *Adv. Healthc. Mater.* (2016).
- [148] F. Gonzalez, *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* (2016).