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Review

Construction of 3D in vitro models by bioprinting human pluripotent stem cells: Challenges and opportunities

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HIGHLIGHTS

- 3D bioprinting allows controlled deposition of cells in space at micrometric scale.
- ESCs and iPSCs can be used to generate 3D bioprinted models of several human organs.
- We discuss challenges and opportunities of bioprinting human brain cells.

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ABSTRACT

Three-dimensional (3D) printing of biological material, or 3D bioprinting, is a rapidly expanding field with interesting applications in tissue engineering and regenerative medicine. Bioprinters use cells and biocompatible materials as an ink (bioink) to build 3D structures representative of organs and tissues, in a controlled manner and with micrometric resolution. Human embryonic (hESCs) and induced (hiPSCs) pluripotent stem cells are ideally able to provide all cell types found in the human body. A limited, but growing, number of recent reports suggest that cells derived by differentiation of hESCs and hiPSCs can be used as building blocks in bioprinted human 3D models, reproducing the cellular variety and cytoarchitecture of real tissues. In this review we will illustrate these examples, which include hepatic, cardiac, vascular, corneal and cartilage tissues, and discuss challenges and opportunities of bioprinting more demanding cell types, such as neurons, obtained from human pluripotent stem cells.

1. Introduction

1.1. 3D bioprinting

The term 'biofabrication' refers to 'a process that results in a defined product with biological function' (Groll et al., 2016). This general definition encompasses the technologies that use cells as building blocks and materials as cement, to generate three-dimensional (3D) constructs. In this way, biofabrication provides the possibility to combine cells in a controlled way, to build structures that closely mimic natural tissues. 3D bioprinting, which is included among the methods of biofabrication, allows the assembly of artificial tissues by printing a suspension of cells or cell aggregates in a natural or synthetic extracellular matrix. Some methods use an ink, made of a combination of biocompatible non-living materials and living material (bioink). The bioink is deposited in 3D space using computer-numerically-controlled (CNC) machines to reproduce tissue architecture at the micrometric scale.

Three main techniques based on the deposition of a cellularized bioink have been developed so far: inkjet, laser-assisted and extrusion-based (Hölzl et al., 2016). Inkjet bioprinting, one of the first approaches to produce 3D biological constructs by printing through a nozzle (Nishiyama et al., 2009), uses inkjet cartridges and piezoelectric actuators, which exploit electricity for precise movement control, or thermal microheaters, based on temperature, to eject micrometric droplets of bioink (Fig. 1A). Inkjet bioprinters are simple to use but the choice of the bioink is limited by its rheological properties, namely density, viscosity and surface tension. Only bioinks with low viscosity can be used to avoid clogging issues. This strongly limits the possibility to tune cell density and matrices formulation, making inkjet bioprinting unsuitable for most applications. This limitation is circumvented in deposition approaches based on a laser, which represent nozzle-free bioprinting methods. Laser-assisted bioprinting (LaBP) (Koch et al., 2013) uses a planar donor slide loaded with the bioink and a laser-pen (Fig. 1B). The donor slide (mounted upside-down) is coated with a thin

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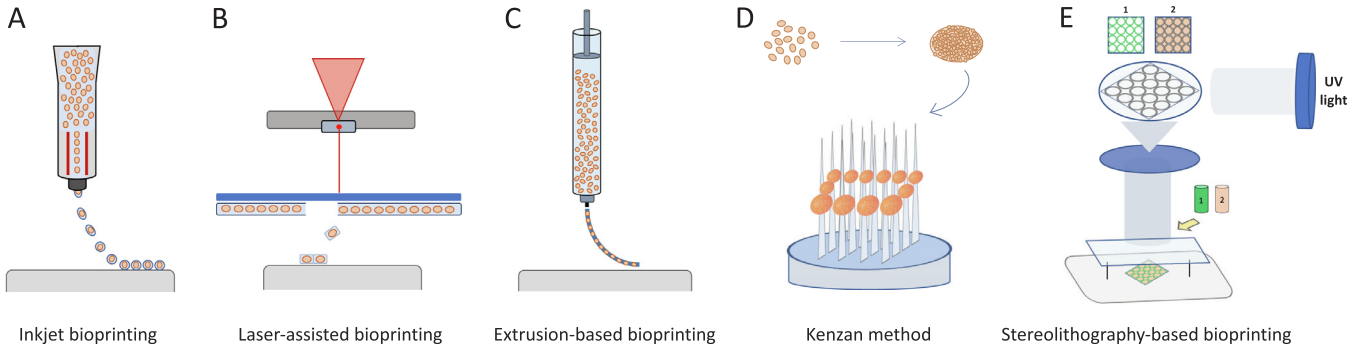

Fig. 1. 3D bioprinting strategies. The figure is a schematic representation of the methods used so far for bioprinting undifferentiated hPSCs or differentiated cells derived from hPSCs. (A) Inkjet bioprinting. (B) Laser-assisted bioprinting. (C) Extrusion-based bioprinting. (D) Kenzan method. (E) Stereolithography-based bioprinting. See text for detailed description of each method.

laser absorbing layer and a thicker layer containing cells and biomaterial (bioink) to be printed. A vapor bubble is formed upon laser focusing on the absorbing layer, triggering the downwards expulsion of micrometric droplets from the substrate. Bioink droplets are thus deposited on an underlying collector substrate layer-by-layer, allowing the formation of arbitrary patterns and stratified 3D structures. High resolution and freedom in the bioink formulation are major points of strength of LaBP, while time-consuming slide preparation, high costs and low accessibility to the general user are regarded as important limitations. Extrusion bioprinting overcomes some of the limitations of the aforementioned deposition techniques and has become the most popular bioprinting approach (Costantini et al., 2018). These bioprinters extrude the bioink out of nozzles using pneumatic or piston-driven actuators, depositing cellularized filaments with micrometric resolution (Fig. 1C). Microfiber deposition is particularly suitable to mimic tissues with elongated architectures or cylindrical symmetry, like blood vessels, hepatic lobules, muscle fibers or neuronal fascicles. Different techniques have been developed for inducing the transition of the bioink from sol (non-gelled precursor, with liquid behavior) to gel (solid-like behavior) during extrusion bioprinting. One possibility is represented by direct printing into a coagulation bath, containing a crosslinking solution that triggers bioink gelation. For instance, bioinks containing the polysaccharide alginate, derived from the cell walls of brown algae, rapidly gel by ionic crosslinking upon extrusion in a solution of calcium chloride (Andersen et al., 2015). A recent evolution of extrusion-based bioprinting consists in the implementation of the extruding nozzle with microfluidic devices. In co-axial wet-spinning 3D bioprinting, the bioink and the coagulation solution are extruded simultaneously. As a result, sol-gel transition occurs at the tip of the printing head, dispensing the hydrogel with rapid and precise control on its spatial positioning (Colosi et al., 2017; Costantini et al., 2018).

Other biofabrication techniques allow controlled generation of cellularized 3D constructs without relying on layer-by-layer deposition of biomaterials. The Kenzan method is a scaffold-free bioassembling procedure based on the initial formation of cell aggregates or spheroids in conventional cultures. During this process, cells spontaneously produce extracellular matrix. Preformed spheroids, in the absence of any bioink, are then laced by a robotic platform into arrayed steel microneedles (Fig. 1D), named kenzan ("sword mountain") from the device used in the Japanese art of flower arrangement ikebana. The kenzan provide temporary support for further cultivation, allowing fusion of spheroids into larger cellular aggregates (Moldovan et al., 2017a). Stereolithography-based bioprinting systems use light to induce polymerization of light-sensitive materials. This is achieved by exploiting the reactivity of photoinitiators, which trigger the formation of polymer chains from monomeric materials upon ultraviolet (UV) or visible light exposure (Mondschein et al., 2017) (Fig. 1E). This method avoids negative effects of nozzle-based bioprinting in terms of shear pressure, velocity and high resolution. However, exposure to ultraviolet and near-ultraviolet light may potentially be cytotoxic for the cells, inducing DNA damages, and the choice of photocrosslinkable biomaterials is limited.

A number of possible biomedical applications have been proposed for 3D bioprinting, ranging from regenerative medicine to disease modeling. The ability to produce a three-dimensional biological construct that mimics a tissue is clearly of interest for repairing, or regenerating, the same tissue in a patient. Moreover, an artificial tissue made of cells carrying pathogenic mutations might be used to study disease onset and progression and for pharmacokinetic studies in vitro (Guillemot et al., 2010). For both basic biology studies and biomedical applications of 3D bioprinting, two crucial requirements must be met: a) cells inside the printed construct must be representative of the cell types in the real tissue; b) the cytoarchitecture of the artificial tissue must recapitulate at best the physiological organization. Comparative overview of the literature shows that common bioprinting technologies differ in terms of throughput, cell viability, resolution, speed and costs, among other features (Knowlton et al., 2015; Moroni et al., 2018). It should be noted that there is always a trade between resolution, compatibility with cell deposition, cell viability as well as mechanical stability and no one of the existing 3D bioprinting methods is able to provide all advantages (Ionov, 2018). This is a recent technology and many gaps must be filled before the above-mentioned requirements can be fulfilled. An important limitation is the quality of the "building blocks", i.e. the cells. Some specialized cell types can be obtained directly from an individual if they reside in an accessible tissue, such as blood or skin. Other kinds of cells can be derived from adult stem or progenitor cells, such as mesenchymal stem cells or satellite (muscle stem) cells, which have a limited differentiation potential. However, biofabrication methods would convey their maximum potential only if a cell source is found that: a) can provide all cells of interest, including non-accessible cells such as neurons or cardiomyocytes; b) can be obtained in scalable amounts; c) can be derived directly from a patient, thus carrying the same genetic background.

In this review we will discuss the possibility of producing in vitro constructs by 3D bioprinting, using human pluripotent stem cells as biological material. We will present relevant examples in recent literature, where different bioprinting methods have been used to generate a range of 3D constructs, with the aim of reproducing human tissues.

1.2. Human pluripotent stem cells: from conventional 2D to novel 3D cultures

Human Pluripotent Stem Cells (hPSCs) include Embryonic Stem Cells (hESCs) and induced Pluripotent Stem Cells (hiPSCs). These cells fulfill all the above-mentioned requirements for the "building block": they can be virtually converted into any cell type (pluripotency) and are endowed with unlimited self-renewal capacity (stemness). Induction of

pluripotency in somatic cells by defined factors has been first described in mouse (Takahashi and Yamanaka, 2006) and then in human (Takahashi et al., 2007; Yu et al., 2007). While hESCs can only be derived by destruction of a blastocyst stage embryo, these seminal studies provided the possibility of generating hiPSCs from adult cells (e.g. skin or blood) by a "reprogramming" process. Moreover, hiPSCs with pathogenic mutations can be obtained from patients and the genetic defect can be introduced (or corrected) by genome editing (Kiskinis et al., 2014). The possibility to obtain hiPSCs carrying pathogenic mutations and their plurilineage differentiation potential make this cell system a powerful tool for advancing our knowledge on the fundamental mechanisms underlying human tissue homeostasis and disease, for drug screening and for regenerative medicine. A great drive in the generation of hiPSCs representative of human diseases has marked the past decade. As biomedical applications require hiPSC differentiation into disease-relevant cell types, development of proper differentiation methods is mandatory to fully exploit their potential. However, generating the full variety of sub-types of cells that make human organs and tissues remains a challenge.

Moving from conventional 2D cultures to 3D is regarded as a promise of significant improvement towards the production of more physiological in vitro models of human development and disease. The need for more realistic models is particularly relevant for neurological diseases (Centeno et al., 2018). Inter-species differences and human-specific features of brain development are not always recapitulated in animal models. Moreover, access to human brain tissue is limited and neurons cannot be easily cultured and expanded in vitro. Despite hPSCs provide the opportunity of generating human neurons in vitro, conventional 2D cultures of hPSC-derived neurons have several limitations: they do not mimic the complex brain tissue organization, cell-cell interactions are limited to side-by-side contacts, dynamics of nutrients and oxygen diffusion and waste removal are lacking. Notably, in the few cases in which 2D and 3D hiPSC-based models have been compared, 3D models have better recapitulated Alzheimer's disease phenotypes (Zhang et al., 2014; Lee et al., 2016). An improvement over conventional 2D models was also demonstrated for neurodevelopmental disorders. For instance, hiPSC-derived 3D brain organoids have been successfully used to model microcephaly (Lancaster et al., 2013), Miller-Dieker Syndrome (Iefremova et al., 2017), Lissencephaly (Bershteyn et al., 2017), Timothy syndrome (Birey et al., 2017) and to study the effects of Zika virus infection on human neural development (Qian et al., 2016; Dang et al., 2016; Cugola et al., 2016; Garcez et al., 2016). Human brain organoids are built by self-organization of differentiating hiPSCs along the neural lineage. Both the variety of the cell types and the main organization of the brain are recapitulated, to some extent, in a self-assembled brain organoid (Kelava and Lancaster, 2016). In addition to the self-assembled whole-brain organoids described by Lancaster et al. (Lancaster et al., 2013), other brain organoid models, based on the use of patterning cues to direct the formation of specific brain regions, have been established (recently reviewed in Arlotta and Paşca, 2019).

At present, unfortunately, reproducibility is regarded as a major issue in the field. Lab-to-lab (and sometimes individual-to-individual) variation in methodologies, cell lines, manipulation and plastics material, have been all recognized as significant sources of variability (Jabaudon and Lancaster, 2018). Moreover, the process of self-assembly often produces significant variability in quality and brain regions of different batches of organoids (Lancaster and Knoblich, 2014). Such "batch syndrome" (Kelava and Lancaster, 2016) can be partially addressed by guiding the cell self-organization with fiber microfilaments, used as floating scaffolds to produce microfilament-engineered cerebral organoids (enCORs) (Lancaster et al., 2017). More recently, the Arlotta laboratory has shown that moving from self-patterned organoids (Quadrato et al., 2017) to models involving the use of exogenous factors to influence patterning (Velasco et al., 2019) greatly improved reproducibility by reducing variability issues in terms of cell type composition.

1.3. Challenges in 3D bioprinting human PSCs

Bioprinting would represent a possible alternative to guide the assembling process of a 3D model, as it would allow combining cells and matrices into a single construct with a defined pattern at the micrometric scale. Application of 3D bioprinting to hPSCs is a field at its infancy. Only recently, advancements in biofabrication techniques have opened the possibility to apply these methodologies to hPSCs. Despite the enormous potential of hiPSCs for disease modeling and regenerative medicine, and despite the boost that biofabrication methods might provide to the cell system, only a handful of recent reports describe bioprinted human 3D constructs based on hPSCs. This gap is mainly due to some peculiar characteristics of hPSCs, which make bioprinting challenging for this kind of cells. First, hPSCs show poor survival in culture as single cells, and single cell dissociation is a necessary step in most bioprinting procedures. Second, hPSCs are highly responsive to environmental cues, due to their intrinsic nature of embryonic-like cells able to respond to developmental signals. Third, hPSCs tend to form clusters, or colonies, due to their epithelial character. This propensity must be taken into consideration when nozzle-based bioprinting methods are used.

Recent reports showed that undifferentiated hPSCs can be bioprinted using extrusion (Faulkner-Jones et al., 2015; Reid et al., 2016; Gu et al., 2017; Nguyen et al., 2017) and laser (Koch et al., 2018). Following the printing procedure, maintenance of pluripotency -i.e. the ability to differentiate into ideally any cell type- is a crucial point that must be accurately evaluated. In some cases, directed differentiation of hPSCs within the printed construct was demonstrated, namely along cartilage (Nguyen et al., 2017), cardiac (Koch et al., 2018) and neural (Gu et al., 2017) lineages. These procedures, in which hPSCs are first printed as an undifferentiated population and then induced to differentiate either to multiple lineages or into a specific cell type of interest, are defined here as "post-printing differentiation" (Fig. 2A). The reversal approach, consisting of printing lineage-committed cells, previously obtained by hPSCs differentiation, is defined here as "pre-printing differentiation" (Fig. 2B). Examples of this latter approach include hepatocytes (Faulkner-Jones et al., 2015; Ma et al., 2016; Yu et al., 2019), cardiomyocytes (Ong et al., 2017a; Ong et al., 2017b; Yu et al., 2019), endothelial and smooth muscle cells (to obtain vascular tissue; Moldovan et al., 2017b) and limbal epithelial stem cell (to obtain corneal tissue; Sorkio et al., 2018), derived from hPSCs by conventional differentiation and then used as biological material for bioprinting.

In the following sections we will describe relevant examples of both post-printing differentiation and pre-printing differentiation in recent literature (summarized in Table 1), highlighting main advantages and caveats.

1.4. Bioprinting undifferentiated hPSCs and post-printing differentiation

To the best of our knowledge, the first report of hPSCs used in biofabrication is the work by Faulkner-Jones et al. (2013). These authors used a valve-based cell printer to produce hESCs spheroid aggregates. Starting from dissociated hESCs in the conventional maintenance medium, without any additional matrix component, the valve-based printing process generated printed droplets deposited onto a plate, which was then flipped for subsequent culture of the cells inside hanging droplets. This procedure promoted cell-cell adhesion and formation of an individual aggregate within each single droplet. This resulted in spheroids of uniform size. Cell viability was maintained during and after the printing process. It is unclear if hESCs maintained pluripotency after printing, as this was only assessed by immunostaining analysis of the pluripotency marker OCT4 and no functional test was reported. An important feature of this technique is provided by the dual nozzle setup, allowing to create gradients of cells and other bioinks to

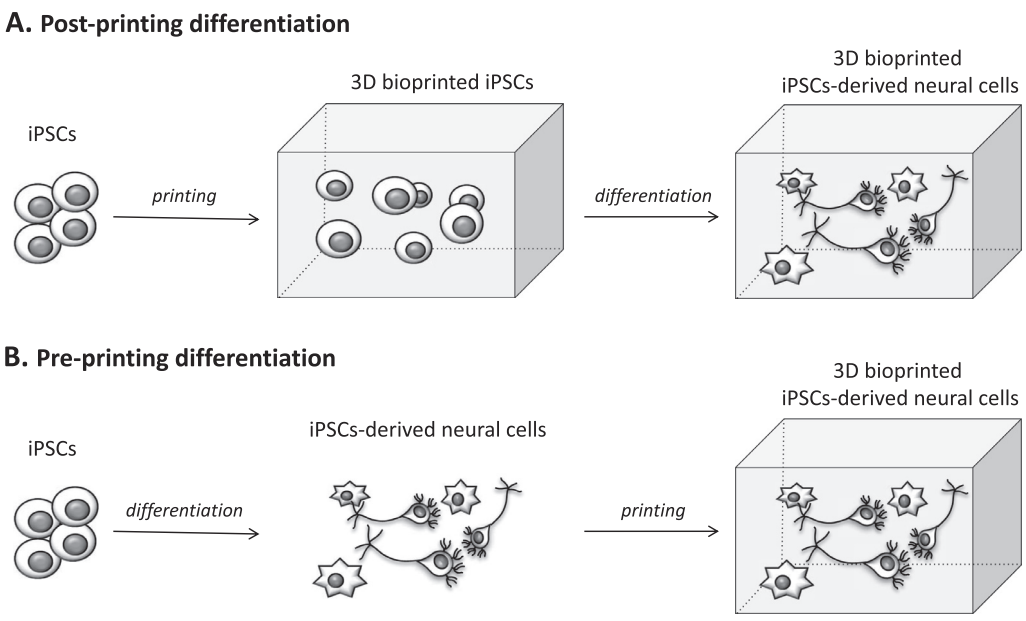

Fig. 2. 3D bioprinting and differentiation of hPSCs. Schematic representation of post-printing differentiation and pre-printing differentiation strategies. As an example, neural tissue derived from human iPSCs is represented. (A) In post-printing differentiation, hiPSCs are first printed to generate a 3D construct containing undifferentiated pluripotent cells. Subsequently, hiPSCs are induced to differentiate within the construct to obtain neural cells (e.g. neurons and astrocytes). In this case there is no control on the relative position and number of the cells in the final neural model. (B) In pre-printing differentiation, hiPSCs are first induced to differentiate to a neural lineage with conventional culture methods. Then hiPSC-derived neural cells are bioprinted to generate the final neural model. In this case, the cytoarchitecture of the neural model can be controlled.

generate cellular aggregates with variable size. This would allow testing a number of different aggregate sizes in a faster and more standardized way than that achievable by manual pipetting. Generation of embryoid bodies, or spheroids, is often used as a preliminary step in differentiation of hESCs and hiPSCs towards diverse lineages. Homogenous aggregate size and density of cells within individual spheroids in the culture are important parameters to ensure reproducibility and efficiency during subsequent differentiation. The increased speed of the technique would also be beneficial in order to reduce cell stress upon dissociation. An improved procedure, based on the same valve-based approach and without supporting biomaterial, was then used by the same group with both hESCs and hiPSCs (Faulkner-Jones et al., 2015). In this work, the authors assessed the effect of different nozzle lengths on post-printed viability. Both increased pressure and nozzle length significantly affected cellular viability, probably due to higher shear forces applied to the cells during printing, confirming the fragile nature of hPSCs. Marker analysis, but no functional assay, was used to confirm maintenance of pluripotency. Reid et al. (2016) used an extrusion system that was optimized in order to have minimal shear and pressure related effects on cells. In this case, pluripotency of hiPSCs was tested post-printing by a functional assay, consisting of formation of embryoid bodies (EBs) in serum-containing medium and subsequent analysis of endoderm, mesoderm and ectoderm markers (Reid et al., 2016).

Ability of bioprinted human hiPSCs to differentiate into multiple lineages was also functionally assessed by Gu et al. (2017), who performed extrusion-based printing of cells within a polysaccharide-based bioink made of alginate, carboxymethyl-chitosan and agarose. Cross-linking was performed by immersing scaffolds in calcium chloride after printing, producing a stable and porous construct in which cells could proliferate. hiPSCs were then differentiated in situ to self-assembling 3D EBs that expressed markers of the three germ layers, thus demonstrating maintenance of pluripotency after printing. In the same work, hiPSCs within the bioprinted construct were induced to differentiate along the neural lineage, generating neurons and glial cells (see below).

A possible application of hiPSCs printing and subsequent differentiation is the generation of a 3D construct mimicking a human tissue for transplantation. In this case, the composition of the bioink is important not only to ensure long-term hiPSCs viability and maintenance of 3D structure, but also to form a proper physiological mimetic environment upon differentiation. As an example, two alternative bioinks have been tested for post-printing differentiation of cartilage tissue from hiPSCs: nanofibrillated cellulose (NFC) with alginate (NFC/A) or

Table 1
Human 3D tissues obtained from pluripotent stem cells by post-printing differentiation and pre-printing differentiation.

Lineage	Cell source	Bioprinting method	References
Post-printing differentiation			
Plurilineage	hiPSCs	Extrusion-based	Reid et al. (2016)
Plurilineage	hiPSCs	Extrusion-based	Gu et al. (2017)
Neural	hiPSCs	Extrusion-based	Gu et al. (2017)
Cartilage	hiPSCs; iChons	Extrusion-based	Nguyen et al. (2017)
Plurilineage	hiPSCs	Laser-assisted	Koch et al. (2018)
Cardiac	hiPSCs	Laser-assisted	Koch et al. (2018)
Pre-printing differentiation			
Liver	hESCs; hiPSCs	Extrusion-based	Faulkner-Jones et al. (2015)
Liver	hiPSCs; hASCs; HUVECs	DLP	Ma et al. (2016)
Liver	hiPSCs	DLP	Yu et al. (2019)
Cardiac	hiPSCs	DLP	Yu et al. (2019)
Cardiac	hiPSCs; HUVECs; HCFs	Kenzan method	Ong et al. (2017a,b)
Vascular	hiPSCs	Kenzan method	Moldovan et al. (2017b)
Cornea	hESCs, hASCs	Laser-assisted	Sorkio et al. (2018)

Plurilineage indicates that the capacity to differentiate into cells of the three germ-layers (ectoderm, endoderm, mesoderm) was tested, after bioprinting, by a functional assay. hiPSCs: human induced Pluripotent Stem Cells; hESCs: human Embryonic Stem Cells; iChons: mitotically inactivated human chondrocytes; hASCs: adipose-derived stem cells; HUVECs: human umbilical vein endothelial cell line; HCFs: human adult ventricular cardiac fibroblasts. DLP: digital light processing.

hyaluronic acid (NFC/HA) (Nguyen et al., 2017). Specifically, proteoglycan and collagen in the cartilage would be simulated by NFC and alginate, respectively. For effective induction of chondrogenic differentiation, hiPSCs and mitotically inactivated chondrocytes (iChons) were printed together in NFC/A bioink (proven to be the best bioink in terms of cell viability) and crosslinked. Printed constructs were maintained 7 days in pluripotency medium and then differentiated, giving rise to hyaline-like cartilaginous tissue after four weeks (Nguyen et al., 2017). These works suggest that bioinks containing alginate (subsequently crosslinked by immersion in calcium chloride) in combination with either carboxymethyl-chitosan and agarose (Gu et al., 2017) or NFC (Nguyen et al., 2017) are both suitable for extrusion-based bioprinting of undifferentiated hiPSCs. It is currently unknown, however, if hiPSCs printed in NFC/A retain plurilineage differentiation.

The other method used so far for undifferentiated hiPSCs is laser-assisted bioprinting. A recent paper suggests that hiPSCs are quite amenable to laser printing itself, but very sensitive to the applied biomaterials (Koch et al., 2018). Extensive test of biomaterials indicated that a bioink composed of 85% E8 (a commercial pluripotency medium) and 15% hyaluronic acid (which allows adjusting the bioink viscosity), used in combination with Matrigel as substrate coating, make the best conditions for hiPSCs survival and proliferation after LaBP. Moreover, Matrigel resulted the best gel substrate for retain the specific printed pattern over time. Matrigel is a widely used basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma and containing extracellular matrix (ECM) proteins (including laminin, collagen IV, heparin sulfate proteoglycans, entactin/nidogen) and growth factors. Its rather undefined composition represents a possible source of variability and development of more standardized synthetic matrices would be desirable to overcome this major limitation. Maintenance of pluripotency was tested by spontaneous multilineage differentiation, resulting in no difference between printed and non-printed cells (in the same bioink). Moreover, directed differentiation showed that laser-printed hiPSCs retain the ability to form beating cardiomyocytes (Koch et al., 2018).

1.5. Bioprinting differentiated cells derived from hPSCs (pre-printing differentiation)

Several lineages of potential interest for biomedical applications have been so far derived from human PSCs and then bioprinted into 3D constructs. They include liver, cartilage, cornea, cardiac and vascular tissues (Table 1).

The first attempt to produce PSC-derived bioprinted models of human liver was reported by Faulkner-Jones et al. (2015), who used the above-mentioned valve-based dual nozzle setup. hESCs and hiPSCs were induced to differentiate into hepatocyte-like cells (HLCs) with a multi-step protocol, which included an endoderm priming phase followed by hepatic differentiation and maturation. Differentiating cells were dissociated during the hepatic differentiation step of the protocol and printed. After printing, differentiation was allowed to proceed through the maturation stage before marker analysis. All hepatic markers were expressed in printed cells at a comparable level with unprinted control, suggesting that the differentiation process and cell lineage were not altered by the procedure. In the same work, the authors tested the hypothesis that a 3D environment could promote maturation of hPSC-derived hepatocytes in vitro. They used a hydrogel containing an alginate solution to print multilayered circular structures. In this approach, the bioink made of hepatocyte-like cells suspended in the alginate solution was dispensed by one of the nozzles, while the other dispensed the crosslinker solution. Despite the resulting tube-like constructs maintained their structure over time, significant decrease in viability was reported immediately after printing and for the first 24 h. Moreover, compared to conventional 2D cultures, a delay in albumin secretion, used as a readout of hepatic differentiation and maturation, was observed during subsequent time points.

A more complex model of human liver was described by Ma et al. (2016), who used a digital light processing (DLP) 3D printing method based on photopolymerization. Notably, in addition to hiPSC-derived hepatic progenitors (HPCs), this liver model included umbilical vein endothelial cells (HUVECs) and human adipose tissue derived stem cells (hASCs), representing supporting nonparenchymal cells from endothelial and mesenchymal origin. These cells were resuspended in two different bioinks, specifically: gelatin methacrylate (GelMA), with stiffness similar to healthy liver tissues, for hiPSC-derived hepatic progenitors, and a mix of Glycidal methacrylate-hyaluronic acid (GMHA)/GelMA, known to support vascularization, for the supporting cells. In DLP, UV light triggers photopolymerization of the hydrogel solutions. The application of two digital masks (complementary hexagonal patterns) in a sequential manner produced a 3D model, in which HPCs and supporting cells organization resembled an array of liver lobules with a hexagonal architecture and physiological dimensions. The designed pattern was generally maintained, but over time HPCs spontaneously reorganized in aggregates. Liver marker expression analysis suggested increased maturation of HPCs in the construct. This observation was also supported by functional analyses using anabolic (albumin secretion) and catabolic (urea production) readouts (Ma et al., 2016). Further implementation of this technique could rely on the development of improved photocrosslinkable bioinks. To this regard, the same group recently reported the use of a novel bioink formulation for DLP-based 3D bioprinting of hiPSC-derived hepatocytes (Yu et al., 2019). In this case, photocrosslinkable GelMA was mixed with a solution of decellularized ECM (dECM) from porcine liver, as a first step to develop a more physiological bioink reproducing the complex biochemical composition of the liver. Such favorable environment is supposed to provide “natural” cues to improve cell phenotype, viability, function and maturation in the bioprinted construct. dECM-based photocrosslinkable bioinks can also be used for DLP bioprinting of other hiPSC-derived cell types of interest. For instance, dECM obtained from porcine heart left ventricle was used for cardiomyocytes, which were printed in parallel lines to favor formation of striated myocardial tissue (Yu et al., 2019). Tuning the light exposure time during printing could be used to modulate the mechanical properties of heart and liver dECM bioink formulations, producing environments that recapitulate the peculiar stiffness of normal liver and developing heart. This feature could promote hiPSC-derived hepatocytes and cardiomyocytes differentiation and maturation (Mueller and Sandrin, 2010; Young and Engler, 2011; Yu et al., 2019). More in general, since the stiffness of the biomaterial can affect stem cell differentiation potential (Engler et al., 2006), the possibility to tune matrix elasticity could be of interest for modulating subsequent differentiation of hPSC-derived cells within bioprinted constructs.

The Kenzan method, which does not require bioink, has also been recently used to generate 3D constructs from hiPSCs. One example is represented by hiPSC-derived vascular-like structures (Moldovan et al., 2017b). Endothelial progenitors (endothelial colony forming cells, ECFC) and smooth muscle forming cells (SMFC), both derived from hiPSCs, were first assembled into vascular cell spheroids, which were then implanted in stainless steel micro-needles. This assembling promoted spheroids fusion in a vascular tissue-like structure, in which cell translocation and further matrix secretion was observed. The same biofabrication method was used to generate cardiac tissue (Ong et al., 2017a,b). In this protocol, spheroids were made of hiPSC-derived cardiomyocytes mixed with HUVEC endothelial cells and a human cardiac fibroblast cell line. The bioprinter was set up in order to select only spheroids of appropriate size before assembling them in the needle array. The result, upon subsequent removal from the needle array, was a beating cardiac patch. Notably, 3D bioprinted cardiac patches engrafted and showed signs of vascularization upon implantation onto rat hearts (Ong et al., 2017b).

Human corneal tissues were generated by laser-assisted bioprinting using limbal epithelial stem cells (LESCs), derived by differentiation of

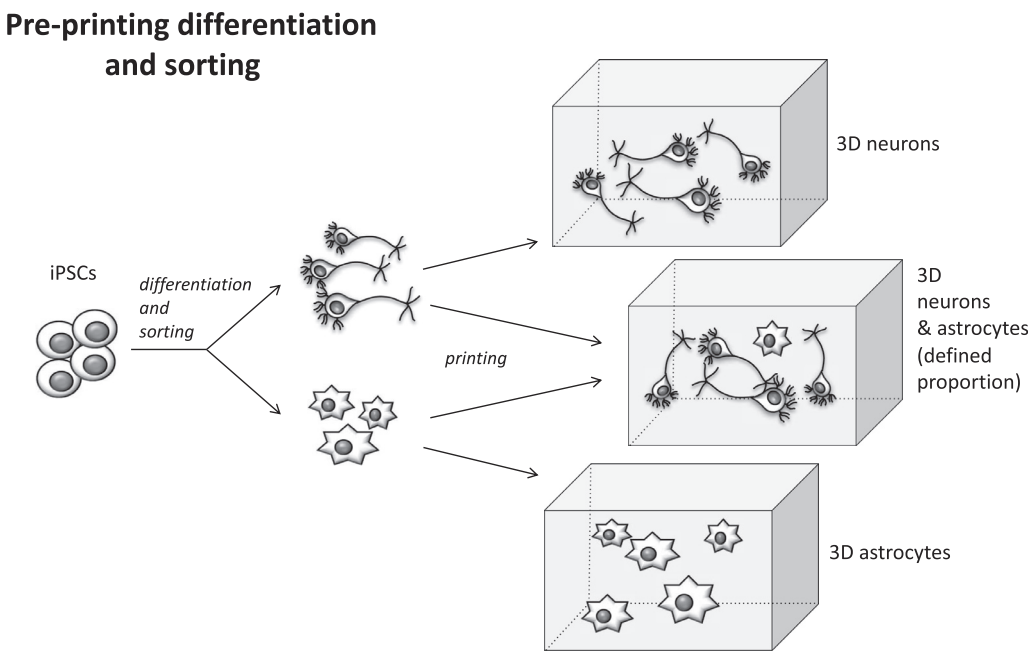

Fig. 3. Pre-printing differentiation and sorting. Schematic representation illustrating the possibility to control the cell composition of the 3D construct by differentiation of hiPSCs, followed by sorting of individual cell populations and bioprinting. Cytoarchitecture and relative proportions of each cell type can be controlled. This approach also allows to include other cell lineages, e.g. microglia, in the neural construct.

hESCs, and hASCs, which are able to differentiate to corneal keratocytes reproducing corneal stroma (Sorkio et al., 2018). In this work, hESC-derived LESCs in a laminin-containing bioink were printed alone, forming a stratified corneal epithelium, or in multiple layers onto hASCs stromal structures, mimicking the cytoarchitecture of the uppermost part of the native human cornea.

1.6. Construction of 3D brain models by bioprinting stem cells

In the last years, successful generation of hiPSC-derived self-assembled brain organoids has shown the advantages of 3D neural cultures (Lancaster et al, 2013, 2017; Quadrato et al, 2017; Renner et al, 2017). The 3D environment might potentially favor the formation of synaptic connectivity and circuits, needed for brain function and dysregulated in brain pathologies, such as neuropsychiatric disorders. As discussed above, self-organization of these structures represents the major cause of variability between different batches of neural organoids (Lancaster and Knoblich, 2014). 3D bioprinting is emerging as a possible alternative strategy to generate neural constructs in a more reproducible and controlled way. However, due to the complexity of the nervous system microenvironment, 3D bioprinting of neural tissues remains a challenging task. In particular, development and optimization of proper matrices for 3D neural cultures represents a limiting critical point (Frampton et al, 2011; Lam et al, 2015). So far, to the best of our knowledge, only two works have reported 3D bioprinted constructs generated from neural cells of human origin (Gu et al., 2016, 2017).

In the first paper (Gu et al., 2016), Gu and colleagues generated a human 3D neural mini-tissue construct (nMTC) by a microextrusion printing technology. Human neural progenitors (the commercial ReNcell CX immortalized cell line) were encapsulated in a defined and well-characterized bioink made of alginate, carboxymethyl-chitosan and agarose, which was subsequently crosslinked with calcium chloride. The nMTCs showed a homogeneous cell distribution and consistent cell viability, promoting cell survival and proliferation. Following the printing process, in situ differentiation was evaluated by analyzing several neural identity markers. Interestingly, a marked increase of some of them (i.e. the neuronal protein beta-tubulin III, TUJ1; the Glial Fibrillary Acidic Protein, GFAP; the synaptic vesicles protein synaptophysin, SYP) was reported in 3D constructs compared to conventional 2D cultures. Finally, spontaneous and bicuculline-induced calcium analysis demonstrated functional maturation of in situ differentiated neural progenitors.

The same group later reported a successful attempt to print hiPSCs by using the aforementioned bioink and extrusion-based approach (Gu et al., 2017). Printed cells were differentiated along the neural lineage in situ. Marker analysis suggested that, compared with conventional 2D cultures, neural maturation was enhanced in hiPSC-derived 3D bioprinted neural constructs. Functional tests were performed 40 days after printing by evaluating bicuculline-induced calcium flux and cell migration. Direct comparison of bicuculline-induced activity between printed and unprinted cells was not reported, leaving unsolved the question of whether the 3D construct improves also functional properties in addition to markers expression. Moreover, as suggested by gene expression analysis, in situ post-printing differentiation generated a very heterogenous population.

Collectively, these works demonstrate that human neural progenitors can be bioprinted and suggest that bioink formulation is a crucial point to promote cell survival and spatial distribution inside printed constructs.

1.7. Future perspectives

Bioprinting allows combining multiple cell types in a defined 3D architecture, providing the possibility of recapitulating tissue composition and cytoarchitecture by overcoming lack of reproducible cellular structure, which is one of the major limitations in the emerging brain organoid field. This represents an essential requirement for disease modeling and also for the development of new cell-based therapies.

In conventional hPSC differentiation, mixed populations containing different lineages are usually obtained. In each experiment, the fraction of each cell type in the mixed population could be greatly variable, hampering disease modeling, drug screening and regenerative medicine applications. For this reason, strategies for the purification of desired cell types during hPSC differentiation have been set up. These include fluorescence, or magnetic, sorting and differential adhesion (Amos et al., 2012). Notably, purification would not be possible in post-printing differentiation, leaving little control on the composition of the construct. Moreover, during post-printing differentiation, hiPSCs proliferate and migrate, resulting in the partial or total loss of the original printed pattern (Koch et al., 2018). Pre-printing differentiation holds several advantages, including: better control on the cells that will be

included in the construct, possibility to combine different cell types with precise stoichiometry, determining the relative position of each cell type within the construct (Fig. 3).

We can foresee increasing interest in producing 3D constructs by pre-printing differentiation of hPSCs. At present, both bioink-based and scaffold-free approaches have been used. It is likely that no individual method would be universally suitable for the wide variety of cell types and tissues that can be derived from hPSCs. Similarly, for the methods that require a bioink, its composition shall be tuned in order to mimic the physiological environment of each individual tissue. Optimization of bioprinting procedures and bioink composition will allow to extend this technology to challenging cell types, such as hPSC-derived neurons, to build improved constructs for basic science and applied research. Despite 3D bioprinting methodologies have been applied only very recently to hPSCs, existing literature suggests that 3D bioprinted constructs may represent significantly improved models of human tissues, in terms of cytological and functional features (Ma et al., 2016). An increase of maturation markers was also reported in 3D bioprinted neural constructs (Gu et al., 2016, 2017). However, it is unclear if this was mirrored by the acquisition of more mature functional properties. Moreover, the possibility of bioprinting neurons and neuroglia, besides neural progenitors, remains unexplored. Extending this approach to the variety of neuronal and glial cell types that can be derived from hPSCs would offer new interesting opportunities in the field.

In conclusion, advancement in 3D bioprinting techniques and materials have opened the possibility to apply these methods to human pluripotent stem cells, both hESCs and hiPSCs. Compared to the virtually unlimited possibility of generating human tissues offered by these cells, at present relatively few examples exist of hPSC-derived bioprinted constructs. This number will conceivably increase in the near future, possibly including bioprinted human neural tissue models.

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