REVIEW ARTICLE

Applications of 3D Bioprinted-Induced Pluripotent Stem Cells in Healthcare

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Abstract: Induced pluripotent stem cell (iPSC) technology and advancements in three-dimensional (3D) bioprinting technology enable scientists to reprogram somatic cells to iPSCs and 3D print iPSC-derived organ constructs with native tissue architecture and function. iPSCs and iPSC-derived cells suspended in hydrogels (bioinks) allow to print tissues and organs for downstream medical applications. The bioprinted human tissues and organs are extremely valuable in regenerative medicine as bioprinting of autologous iPSC-derived organs eliminates the risk of immune rejection with organ transplants. Disease modeling and drug screening in bioprinted human tissues will give more precise information on disease mechanisms, drug efficacy, and drug toxicity than experimenting on animal models. Bioprinted iPSC-derived cancer tissues will aid in the study of early cancer development and precision oncology to discover patient-specific drugs. In this review, we present a brief summary of the combined use of two powerful technologies, iPSC technology, and 3D bioprinting in health-care applications.

Keywords: Induced pluripotent stem cells, Three-dimensional bioprinting, Regenerative medicine, Disease modeling, Cancer iPSCs, Drug screening.

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***Received*:** March 05, 2020; ***Accepted*:** June 24, 2020; ***Published Online*:** July 30, 2020

Citation: Soman SS, Vijayavenkataraman S, 2020, Applications of 3D Bioprinted Induced Pluripotent Stem Cells in Healthcare, *Int J Bioprint*, 6(4): 280. DOI: 10.18063/ijb.v6i4.280.

1 Introduction

The advent of induced pluripotent stem cell (iPSC) technology in 2006 paved the way for paradigm shifting changes in regenerative medicine, disease modeling, and drug discovery applications. The technology facilitates to de-differentiate an adult cell to its pluripotent stem cell state and then differentiate into defined cell lineages. iPSCs are phenotypically indistinguishable from embryonic stem cells and they can differentiate into specialized cells of the body in cell culture and in animal models. Initially, human iPSCs were derived using transduction of genes coding for four embryonic transcriptional regulators; Oct4, Sox2, Klf4, and c-Myc (OSKM), popularly known as the Yamanaka factors[1] or Oct4, Sox2, Lin28, and Nanog (OSLN)[2]. Each of the Yamanaka factor serve specific purposes, *Sox2* interacts with *Oct3/4* to control gene expression. This interaction is important in maintaining pluripotency[3]. *C-Myc* plays an important role in controlling growth and differentiation of cells[4], whereas *klf4* is crucial for cell division and maintenance of pluripotency[5]. Later, different combinations of at least 24 embryonic transcription factors were identified to induce stemness in adult cells[6]. The Yamanaka factors are highly conserved and sufficient to induce pluripotency across species.

Reprogramming of somatic cells is orchestrated by cooperative binding of pioneer

factors (Oct4, Sox2, and Klf4)[7], followed by epigenetic remodeling of entire genome and two waves of transcriptional events[8,9]. Each cell type in the body require different combinations of transcriptional factors to induce the stemness where Oct4 is considered as an indispensable core pluripotency gene in the reprogramming process[10]. Exogenous supply of Oct4 alone could convert adult neural stem cells into iPSCs. Recent work by An *et al.* showed that Sox2 and Klf4 were enough to prepare iPSCs from various types of somatic cells[7]. Small molecules that inhibit DNA or histone modifications were also used for generating iPSCs more efficiently along with the use of reprogramming transcription factors. The hematopoietic stem cells can be de-differentiated into iPSCs much more efficiently compared to the highly specialized cells such as B and T lymphocytes[11,12].

Fibroblasts are the most popular cell type used to generate iPSCs. However, well-differentiated adult cells such as keratinocytes, neural cells, fat cells, melanocytes, amniotic fluid cells, pancreatic beta cells, and peripheral blood derived cells had also been successfully reprogrammed to pluripotent stem cells. The capacity to induce pluripotency to somatic cells helps to generate pluripotent patient-specific cell lines that can help model human diseases and can aid in the reconstruction of damaged tissues and organs. The "disease in a dish" models derived from IPSCs provide insights into disease pathogenesis and can serve as a novel tool for drug evaluation in precision medicine field[13-15]. Human iPSCs reinforced with biocompatible scaffold materials are valuable in three-dimensional (3D) bioprinting applications[16]. Current bioprinting techniques allow to print undifferentiated iPSCs and iPSC-derived cells mixed with a suitable bioink[17,18]. Popular bioprinting techniques used to print iPSCs are extrusion, stereolithography (SLA), laser-assisted, and drop-on-demand bioprinting[19-22]. A single biomaterial or a mixture of several biomaterials in the bioink are used to suspend the desired cells for bioprinting[23,24]. The bioinks should be non-toxic, biocompatible and should provide structural support for the printed cells. The commonly used bioinks for printing iPSC derived cells are hydrogels derived from alginate, carboxymethyl chitosan, agarose, nano-fibrillated cellulose, hydroxypropyl chitin, gelatin methacryloyl (GelMA), and Matrigel. Most of these hydrogels need a crosslinker to give the final structure of the intended tissue constructs. Calcium chloride, ultraviolet (UV) radiation, photo crosslinking, and altered temperatures are used for crosslinking the bioink molecules[25-27].

Here, we review the applications of the 3D bioprinted iPSCs or iPSC-derived cellular products in healthcare, especially in regenerative medicine, disease modeling, and drug testing (**Figure 1**). The methods of reprograming of iPSCs were described. Glimpses of the technological advancement in organ bioprinting were discussed. The advantages, limitations, and future directions of using iPSCs in clinics were outlined.

2 Human iPSC reprogramming methods

For clinical application and disease modeling, the reprogramming method of choice should have adequate efficiency to produce iPSCs from less abundant samples[28]. Production of iPSCs using a combination of reprogramming methods can augment the efficiency of iPSCs generation even from the most difficult type of adult cells[29]. More than 10 years of extensive research on iPSC technology lead to the establishment of novel strategies for the production of iPSCs including the use of right cell type for reprogramming, use of non-integrative gene introduction methods, overexpression of gene enhancers of transcription factors, and the use of small molecules[30,31] (**Figure 2**).

2.1 Integrating viral vectors

Initial iPSC experiments used lentivirus and retrovirus vectors to deliver Yamanaka factors in adult fibroblasts[2,32]. These retroviral-vectors possess the risk of creating mutagenesis by integrating to the host cell genetic material[33]. Moreover, the reprogramming procedure is tedious, also, it can cause chromosomal instability and potential threat of tumorigenesis from the

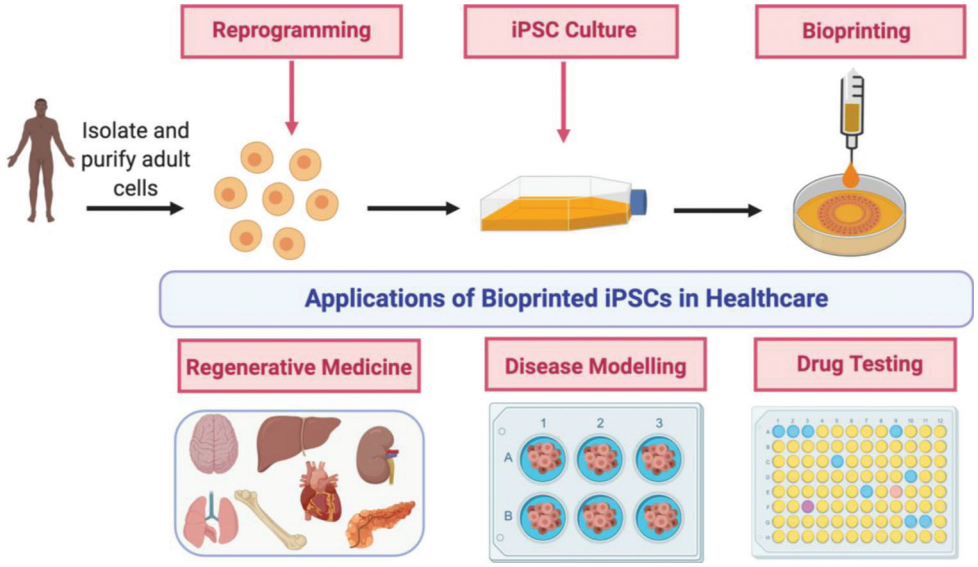

Figure 1. Bioprinting of induced pluripotent stem cell (Ipsc)-derived tissues for regenerative medicine, disease modeling, and drug testing. Adult somatic cells are collected from a donor or a patient; the cells are reprogrammed to iPSCs and differentiated to specialized cells.

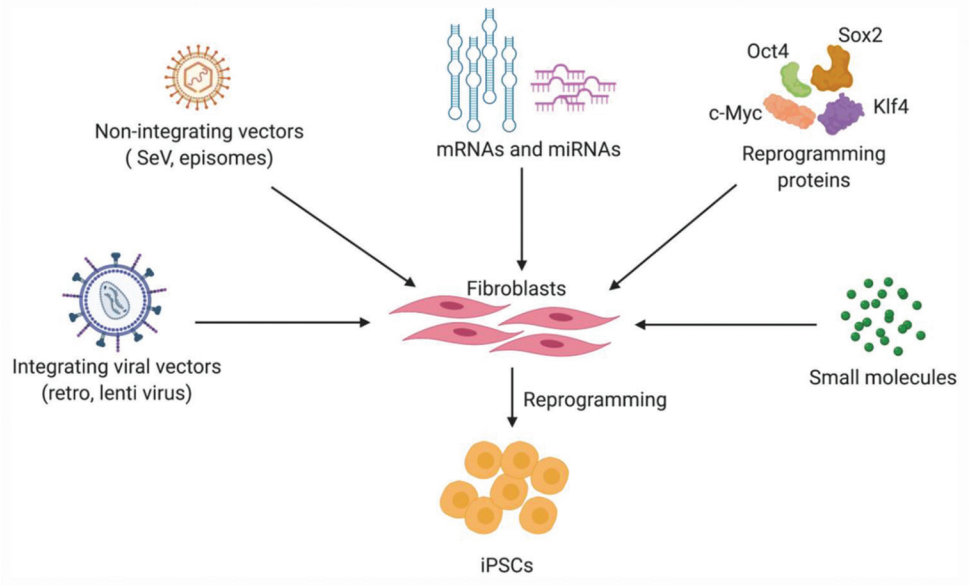

Figure 2. Reprogramming methods used to generate induced pluripotent stem cell.

viral vectors[1,2,31-34]. It is essential to do the quality control of the iPSC lines created using viral vectors using whole-genome single nucleotide polymorphism array and karyotyping to verify the genomic integrity.

2.2 Non-integrating vectors

To reduce the risk of unwanted genetic perturbations, there was introduction of non-integrating methods for transcription factor delivery. Recent methods of reprogramming use Sendai Virus (SeV) particles and episomes. SeV particles are used to transduce the reprogramming genes: Oct4, Sox2, Klf4, and c-Myc[8]. SeV reprogramming offers the absence of viral sequences in the host cell lines and is an efficient method to induce pluripotency[35]. In 2009, Yu *et al*. reported the episomal reprogramming in human cells where Epstein-Barr virus–derived

sequences were used for the non-integrated expression of the transcription genes that enable episomal plasmid DNA replication in dividing cells[36]. Polycistronic expression plasmids generates transgene-free and vector-free iPSCs with limited genomic integration, but this method requires multiple transfections[37,38].

2.3 mRNAs and miRNAs

Human primary fibroblasts were reprogramed by introducing synthetic modified mRNAs coding for reprogramming proteins. MicroRNAs like miRNA-367/302s are used to reprogram human primary fibroblasts into iPSCs[39]. The mRNA transfection is foot print free reprogramming. The capped mRNAs coding for a 6-factor modified-mRNA referred to as 5fM_3O mod-mRNAs was used in fibroblasts to make iPSCs[40]. Yamanaka factors and the miRNA-367/302s act synergistically to increase the efficiency of transfection[41]. It has been reported that microRNAs such as miR-294, miR-291-3, and miR-295 can replace *c*-myc transcription factor and help to generate homogeneous populations of iPSC colonies[42]. Downregulation of let-7 miRNA upregulates the expression of target genes of *c-myc* and *Lin-28* to promote cell reprogramming[43,44].

2.4 Reprogramming proteins

This method allows the direct introduction of the recombinantly expressed reprogramming factors to cells[49]. This method mitigates the risks associated with the introduction of viral and external DNA and harmful chemicals into the cells[46]. The reprogramming proteins Oct4, Sox2, Klf4, and c-Myc were successfully delivered into adult somatic cells with the help of cell penetrating peptides (CPP). The cationic amino acid rich CPPs are capable of penetrating the cell membrane barrier and deliver the exogene-free reprogramming proteins directly inside the cells[47]. This method enables the production of foot print-free iPSCs.

2.5 Small molecules

Reprogramming can be achieved using small molecules by epigenetic modifications[48]. Small molecules used for reprogramming fall under the category of epigenetic events regulators, mesenchymal-epithelial transition inhibitors, metabolic pathway modulators, wingless and integration site growth factor (WNT) signal pathway modulators, regulators of cell death, and senescence pathways[48]. These small molecules alone or in combination can substitute exogenous transcription factors. Using valproic acid, a Histone deacetylase inhibitor improved the reprogramming efficiency to 100-fold compared to the transcription factor mediated reprogramming method[49]. Another histone methyltransferase inhibitor compound, BIX-01294 activated calcium channels in the cell membrane, and improve reprogramming efficiency by increasing the expression of October 4 and Klf4[50,51]. In 2013, Hou *et al*. replaced all transcription factors and made chemical induced iPSCs of mouse embryo fibroblasts using the small molecules VC6TFZ and 2i but the reprogramming was incomplete[52]. Optimizing the use of small molecules to enhance reprogramming will definitely help to generate safer and higher quality iPSCs for clinical use without the risk of genomic integration and tumor induction.

The advantages and limitations of these reprogramming methods are summarized in **Table 1**.

3 Sources of iPSCs for bioprinting and cell differentiation strategies of iPSCs to different cell lineages

The iPSC technology allows the use of autologous cells derived from the patients to be used in regenerative medicine. The iPSC cell lines have been derived from a variety of cells namely neuronal progenitor cells, keratinocytes, hepatocytes, B cells, fibroblasts, hepatocytes, gastric epithelial cells, muscles, adipocytes, and adrenal glandular cells. The reprograming efficiency varies among the type of cells used depending on the developmental origins and the epigenetic status. Multiple studies showed that the efficiency of reprogramming of keratinocytes is better than fibroblasts[53-55]. The difference in the efficiency is

Table 1. Advantages and limitations of the reprogramming methods.

Delivery method	Advantages	Limitations	References
Integrated viral vectors	High efficiency, validated in many cell types	Create insertional mutations, tumor induction	[1,2,31-34]
Integration free viral vectors. For example, Sendai virus, Episomes	Completely free of vector and transgene sequences and can use in clinical applications, GMP compatible	Low efficiency of reprogramming, reprogramming efficiency varies with cell types	[8,35-38]
mRNAs and miRNAs	Faster, high efficiency, absence of integration, need low input of starting cells	Multiple transfection required, may elicit immune response, not evaluated in many cell types	[40-44]
Reprogramming Proteins	Foot print-free, cGMP compliant	Not evaluated in many cell types, expensive	[45-47]
Small molecules	Foot print-free, cGMP compliant, economical	Incomplete reprogramming, reprogramming efficiency varies from cell to cell	[48-52]

attributed to the high-level endogenous expression of c-Myc and Klf4 in keratinocytes compared to other cell types. For therapeutic scenarios such as internal organ reconstruction (e.g., liver, and peripheral nerve), an ideal starting material to create iPSCs should be peripheral tissue. Peripheral tissue samples such as keratinocytes collected from patients can be reprogrammed to produce iPSCs and these iPSCs can serve as a valuable source for the cellular component in the tissue bioprinting[56]. Peripheral blood cells may represent a good source to derive iPSCs because taking blood samples from patients are a less invasive clinical procedure. Several research groups standardized the protocols for making iPSC lines from blood cells[57,58]. The iPSC-derived cells such as neurons, hepatocytes, osteoblasts, myocytes, skin cells, and pancreatic beta cells are in great demand in clinics for regenerative medicine applications[59-61]. Small molecules and growth factors such as recombinant proteins are used in the iPSC cultures to differentiate specific lineage cells from iPSCs[62,63].

4 Application of bioprinted iPSCs in healthcare

3D bioprinting using iPSCs hold high potential for several applications in the health-care sector. Current bioprinting techniques allow to print undifferentiated iPSCs and differentiated iPSC-derived cells embedded in a suitable bioink. Development of novel bioinks, iPSC-derived cells, and the technological advancement in devising new generation 3D bioprinters has created a whole new field of medical bioprinting that hold great promise for artificial tissue/organ printing for regenerative medicine, disease modeling, and drug testing[64]. Here, in section 4, we describe about the commonly used bioprinting techniques to print iPSCs and the application of bioprinting in health-care field.

4.1 Commonly used iPSC 3D bioprinting technologies

Bioprinting iPSCs could avoid the ethical and immunological bottle necks of organ printing. iPSCs and iPSC derived cells for developing into cartilage, bone, skin, heart, liver, and neural tissues have been successfully printed using 3D bioprinting technology. iPSC-derived chondrocytes along with irradiated chondrocytes were bioprinted to cartilage tissues using mixture of alginate and nanocellulose bioink. RegenHu 3D discovery bioprinter was used to print the chondrocytes. Extrusion bioprinting is the most commonly used method to print iPSCs and organs, followed by SLA, laser-assisted, drop-on demand, inkject, and microvalve based methods. The extrusion method applies a pneumatic (air) or mechanical (screw or piston) force to extrude iPSCs or its derivatives embedded in hydrogel bioinks through a nozzle orifice using seamless direct printing[65]. The printing is carried out

in a spatially controlled layer-by-layer fashion precisely to biofabricate 3D tissue constructs. Extrusion printing permits printing of cell-dense high viscous hydrogels, but cells may experience high shear force stress during the printing process. All three germ layers[66], neural tissues[67], cortical neural constructs[68], chondrocytes[69], cardiac tissue,[70] and peripheral blood mononuclear cells have been bioprinted from iPSC or iPSC derived cells using extrusion method.

SLA and digital light projection (DLP) are popular nozzle-free bioprinting techniques work through photopolymerization. The liquid resin is solidified by UV laser beam in SLA. The DLP uses visible light for polymerization of the resin[71,72]. There are two types of SLA and DLP, i.e., bottom-up and top-down approach. The layer of resin on a support platform is cured by a light from above in bottom-up biofabrication, while the light source is located under a transparent platform in the top-down fabrication. iPSC-derived 3D liver models which mimic the native liver module architecture were printed using this technique[73]. The flipside of SLA is that, it can be detrimental to the living cells by damaging the genetic materials due to the use of UV. Moreover, the use of photo-initiator resins may be cytotoxic to the cells[74]. Vat polymerization (VP) based bioprinting is a novel and accurate bioprinting method tissue engineering applications[75]. Various types of photo-initiators are used for the cross-linking of the printed tissues in the VP based bioprinting. VP use light sensitive hydrogels such as polyethylene glycol–diacrylate (PEGDA) and gelatin-methylacryloyl (GelMA). Label-free diamagnetophoretic printing is another method for microtissue printing uses intrinsic diamagnetic forces to control positioning of cells in a paramagnetic medium. Magnetic bioprinting is a contactless technique which does not use nozzles and therefore promise less contamination of cell suspension. Whole blood cells were printed using this technique[76], this technique may be efficient to adopt to print iPSCs as there is fewer chemical manipulations that are involved.

Droplet-based bioprinting is simple, fast, and precisely controlled bioprinting method to deposit composites of cells, growth factors, biomolecules, drugs, and scaffolds. Droplet-based bioprinting is derived from inkjet printing technology. It has been noted as a prominent technique widely used in regenerative medicine to print cells due to its flexibility. The droplet-based bioprinting can be subdivided into inkjet, acoustic, and micro-valve bioprinting modalities. The drawback of using this technology is that the rage of biomaterials compatible for this method of bioprinting is limited[77]. A valve-based bioprinting method is used to print iPSCs differentiated post-printing into hepatocyte-like cells (HLC cells). A 40-layer thick alginate bioink containing HLC cells showed typical liver tissue structure and the construct secreted hepatic albumin throughout the differentiation protocol. The work proved that the valve-based printing process is safe to print human iPSCs by maintaining pluripotency and differentiation[19].

The laser-assisted bioprinting uses pulsed laser beam with a focusing device. It consists of an energy-absorbing layer coated with further layers of cell-encapsulated hydrogel. It is a nozzle-free bioprinting method that excludes clogging during printing[78]. Human iPSCs combined with bioinks were bioprinted with laser-assisted bioprinting method and the cells were evaluated for their efficacy, pluripotency, and differentiation capacity. The hyaluronic acid-based bioinks are ideal for laser-assisted bioprinting[16,79]. While designing a tissue structure for bioprinting, factors such as shape, size, resolution, scaffold materials, iPSCs, or iPSC derived cellular components, and post-processing tissue dynamics are to be considered. Bioprinting can be carried out as direct printing, crosslinking during the bioprinting, post-printing crosslinking, and hybrid methods, where more than one technique is used for printing the final tissue construct. The factors appear to affect cell survival after bioprinting are shear stress, laser exposure, duration, temperature, humidity, mechanical pressure, and vibration of the printing process. The selection of the printing techniques depends on the end use of the printed tissues, such as organ/tissue transplantation, disease modeling, or drug evaluation. Among, all the methods mentioned, extrusion bioprinting is the most

commonly used technique in iPSC bioprinting[80,81] (**Table 2**). Extrusion method causes less damage to the cellular components while printing, as it uses adjustable mechanical forces with no harsh treatments for the deposition of the bioink to the platform.

4.2 Regenerative medicine

Autologous iPSCs derived from individuals provide unlimited source of cells for tissue regeneration. The unspecialized iPSCs can differentiate and develop to organoids/spheroids with specific characteristics of organs *in vivo*[74,82-84]. These mini-organoids can serve as building blocks for bioprinting of whole organs. Bioengineers and surgeons are looking for novel methods to synthesize artificial skin substitutes that is readily available and easily implantable in burn injury patients[85,86]. Scaffold-free cellular spheroids obtained from a coculture of human iPSC-derived cardiomyocytes, fibroblasts, and endothelial cells were 3D printed and these cardiac cellular patches were tested successfully in rat models of myocardial infarction[87]. Bioprinted organ substitutes such as pancreas, ovary, liver, kidney, and nervous tissues also will be in high demand in the near future. **Figure 3** shows the workflow of 3D bioprinting of peripheral nerve tissue[88,89] for the treatment of peripheral nerve injury.

Human iPSCs are capable of differentiation into many types of specialized cells and have high value in clinical use. These cells require specific cell culture media to keep their pluripotent characteristics intact. The isolation, expansion, and maintenance of human iPSCs intended for clinical use should be cultured in xeno-free conditions in compliance with the good manufacturing practice to avoid hypersensitivity reactions after transplantation in patients[90,91]. However, many conventional protocols of iPSC culture require to culture in feeder cells. The feeder cells are usually derived from mouse embryonic fibroblasts (MEFs). The cells are cultured on feeder cells to reduce the genetic instability of the cultured cells[89]. Culturing in MEF feeder cells or the usage of matrix coating substance (e.g., gelatin or Matrigel) made of animal components make the iPSCs xeno-positive. Recent introduction of synthetic polymers enables to maintain the iPSC cultures in xeno-free environment[92].

Yamanaka factor introduction techniques use different type of retroviral or plasmid vectors to integrate to the genome of the cell to make it pluripotent. For making clinical grade iPSCs and

Table 2. Summary of iPSC-based Bioprinting works

Printing technique	Cell source	Cells/tissues printed	Bioink used	Reference
Extrusion	iPSCs, BJFF iPSCs	Cardiac	Collagen I, Matrigel, Gelatin	[70]
	Human iPSCs	Chondrocytes	Nano-fibrillated cellulose in alginate	[69]
	Fibroblasts derived human iPSCs	Germ layers	Geltrex	[66]
	Human iPSCs ((WT I line)	Neural construct	Matrigel/alginate mixture	[68]
	Human iPSCs	Neural tissues	Alginate, carboxymethyl-chitosan, agarose	[67]
	Human peripheral blood mononuclear cells derived iPSCs	Pluripotent cells	Hydroxypropyl chitin, Matrigel	[79]
Stereolithography	Human iPSCs	Hepatic progenitor cells	Gelatin methacrylate (GelMA), Glycidal methacrylate-hyaluronic acid (GMHA)	[73]
Laser-assisted	Human iPSCs from cord blood	Germ layers	Matrigel, Collagen type I, Alginate, Hyaluronic acid	[16]
Microvalve-based	Human iPSCs	Hepatocyte-like cells (HLCs)	Geltrex	[19]

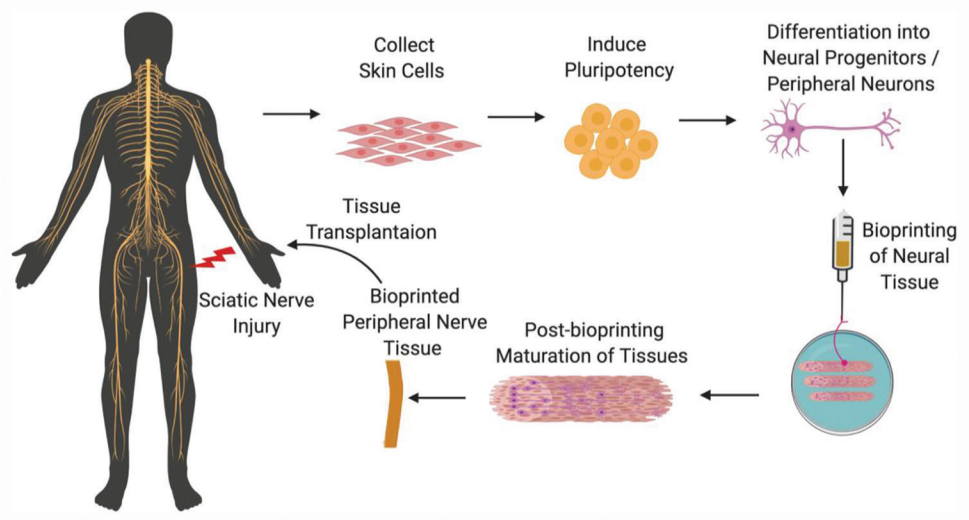

Figure 3. Workflow of three-dimensional bioprinting of peripheral nerve tissue for treatment of peripheral nerve injury. Sciatica is the pain due to the injury to the largest nerve in the body. Extreme case of sciatica needs surgical intervention and if possible, regenerative therapy. The autologous peripheral tissues can be collected from the patient, reprogram to induced pluripotent stem cells and differentiate to peripheral nerve tissue progenitors or peripheral neurons, then bioprint using a suitable scaffold material, allow the maturation of the bioprinted tissue in a bioreactor and transplant to the patient to repair and regenerate the injured nervous tissue.

its products, the cells should be free from any genetic integration of foreign DNA materials in the iPSC genome. mRNA-based, episomal, and recombinant protein-based introduction of transcription factors eliminate the risk of genomic integrations or aberrations in the iPSCs, which is an important step toward using the iPSCs for cell-based therapies in patients[93-95].

4.2.1 Type 1 diabetes mellitus (T1DM) treatment by the replacement of pancreatic beta cells

T1DM is known to be associated with the immune-mediated destruction of insulin producing pancreatic β-cells[96,97]. Effective treatment of T1DM is a long pending requirement in diabetes care. Islet cell transplantation is a traditional method of managing T1DM, but the transplanted islets are rejected by the host immune system. Bioprinted islets encapsulated in a suitable biocompatible material have been emerged as a treatment method to tackle this immune rejection. Biofabrication of 3D constructs of patient-derived iPSCs differentiated to insulin-producing pancreatic islet cells can potentially be an allogeneic source of cells for T1DM treatment. In 2019, Kim *et al.* successfully developed a pancreatic tissue-derived (pdECM)-bioink to provide the pancreatic tissue-specific microenvironment to bioprint the human iPSC-derived pancreatic islet cells. The study used decellularized porcine pancreas to provide the extra cellular matrix support for the growth of the cells[98]. Biotechnology companies such as Cellheal and Celprogen are working toward to bioprint the functional pancreatic tissues for diabetic treatment[99].

4.2.2 Reproductive system disorders

Ovarian failure is a major cause of infertility worldwide[100,101]. Functional 3D printed ovarian tissues would be a boon for infertility treatment. In 2017, Laronda *et al.* reported that the 3D bioprinted ovary using porous gelatin scaffold material could support the growth and maturation of printed ovarian follicles in laboratory conditions. The lab-grown ovary is tested in a sterilized mouse model and found to be functional. The bioprosthetic ovary used scaffold materials such as gelatin and gelatin methacrylate for bioprinting[102]. The structural features of the scaffold material such as pore

size, pore geometry, and the surface contact area are the deciding factors for successful growth of functional ovarian tissue. Stem cells isolated from the patient's own ovarian tissue can serve as the starting material to bioprint the functional ovary.

4.2.3 Thyroid gland replacement in thyrectomized patients

Bioprinting of a functional vascularized mouse thyroid gland construct from embryonic tissue spheroids was reported by Bulenova *et al.* in 2017[103]. Self-assembling thyroid spheres, thyrocytes, and endothelial cells suspended in collagen gel were used for bioprinting the thyroid gland. The bioprinted construct was implanted in a hypothyroid mouse and it could normalize the blood thyroxine levels and body temperature in the tested mice. Bioprinting of functional mouse thyroid gland tissue represents a major advance in bioprinting technology and organ regeneration research. In March 2019, NASA announced a plan for bioprinting thyroid gland in international space station to study the effect of microgravity on organs[104].

4.3 Bioprinted iPSCs in disease modeling

The most advantageous aspect of using induced pluripotent cells in clinics is the ability of reprogramming of autologous cells taken directly from patients**.** At present, the majority of disease modeling studies makes use of the traditional 2D cultures. Any monogenic or polygenic disease conditions can be re-created in a cell culture system[83]. While 2D cultures are good to understand the molecular level interactions, they possess several limitations including lack of heterogenic cell environment and the cell to cell communication cues[105]. The disease progression hugely depends on the extracellular matrix (ECM) mechanics and the cell to cell interactions[106]. Cellular phenotypes and the non-cell autonomous disease pathogenesis require mimicking the disease conditions in a more realistic 3D environment. 3D disease models would help in understanding the disease mechanism in detail in the early stages of the disease[107,108]. A few examples of disease modeling in cardiac, neurodegenerative and neurodevelopmental diseases and oncology are briefly discussed in the following sections.

4.3.1 Cardiac diseases

Cardiovascular diseases remain the leading cause of death in the developed world, accounting for more than 30% of all deaths. Collection of cardiac tissue from patients with disease causing mutations for genetic studies and functional analyses is a highly invasive procedure. iPSCs derived from the peripheral tissues of patients with disease specific mutations are a valuable tool to study the cardiac pathophysiology and drug development. Cardiac tissues were biofabricated using hydrogels and supporting cells such as cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts[109,110]. The cells were cocultured and engineered to resemble their natural physiological microenvironment and recapitulate coordinated contractile and electrophysiological interactions with the ECM and heterogeneous cell types that make up the myocardial tissue environment[111]. The iPSC-derived cardiac cells were successfully used to model cardiac diseases such as dilated cardiomyopathy and myocardial infarction[112]. These disease models help identifying the cellular phenotypes critical to cardiac pathology[113,114]. The microfluidic organ-on-chip methods were also developed to evaluate the vascular perfusion in cardiac tissue. The tissue composition and architecture of the *in vitro* 3D microdevice can be precisely defined using microfabrication methods[115-117]. The iPSC and embryonic stem cell derived organ-on-chip systems are being used for modeling a wide range of diseases, including dilated cardiomyopathy, kidney glomerular injury, and wound healing[118,119].

4.3.2 Neurodegenerative and neurodevelopmental diseases

4.3.2.1 Alzheimer's disease (AD)

AD is a progressive neurodegenerative disorder characterized by loss of cognition and disruption of basic functions, such as swallowing, walking,

attention, and memory[120]. All major nerve cell types can be differentiated from iPSCs and can be cultured in complex conditions, which mimic the AD conditions. Precise Genome editing techniques can be used to introduce or correct AD-linked mutations to examine phenotypes in isogenic backgrounds cells[14]. It has become increasingly clear in recent years that multiple different brain cell types can contribute to AD progression[121]. Thus, examining their interactions and impacts on each other are of critical importance. The iPSCs can be differentiated into neural crest or neural progenitor cells, which can subsequently be patterned to different neuron subtypes including glutamatergic, GABAergic, cholinergic, and dopaminergic neurons[122-124]. 3D bioprinted AD models will facilitate the development of effective therapeutics to combat AD-induced dementia. Moreover, bioprinted AD tissue models can serve as a more humanized model system for AD drug testing, as many drugs tried in experimental animals failed in clinical trials due to species variability[125,126].

4.3.2.2 Parkinson's disease (PD)

PD is the second most common neurodegenerative disorder[127]. Studies using iPSC-derived dopaminergic neurons from patients with monogenic and sporadic PD have successfully illustrated key features of PD pathophysiology, including impaired mitochondrial function, increased oxidative stress, and accumulation of α-synuclein protein, namely, Lewy bodies[128]. Using iPSC-derived dopaminergic neurons from PD patients with mutations in the disease causing genes, many investigators have drawn mechanistic insights on how mutations of these genes are linked to PD. Coculturing glial cells and neurons both derived from iPSCs of PD patients should therefore be another platform to advance insights into the multifactorial pathogenesis of PD. Bioprinted dopaminergic neurons can be treated with neurotoxins such as 6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, paraquat, and rotenone to induce PD like syndrome to study PD pathogenesis[129-131].

4.3.2.3 Amyloid lateral sclerosis (ALS)

ALS is the most prevalent motor neuron (MN) disease characterized by the progressive loss of the upper and lower MNs, leading to muscular atrophy, paralysis, and death within 5 years after the first diagnosis[130]. The iPSC-derived disease models of ALS showed increased oxidative stress and DNA damage in neurons. ALS specific mutations and altered transcriptome profile were also noticed in the iPSC models. Osborne *et al.* reported the effectiveness of the small molecule vardenafil, in regaining the resilience of MNs by regulating the insulin-like growth factor-II signaling in an iPSC-derived ALS model[132]. Researchers created 3D bioprinted ALS disease model using iPSC-derived MNs from a patient with *TDP-43* gene mutation. *TDP-43* gene mutation causes sporadic ALS. Bioprinted iPSC derived autologous tissue models of ALS disease serve as a valuable tool for studying the disease pathology as well as aid in the screening of personalized drugs against the disease[133].

4.3.3 Bioprinted iPSCs in oncology

The iPSCs derived from cancer tissues present a range of new opportunities for the study of human cancer. If human cancer cells were converted to pluripotency and then allowed to differentiate back into specific cancer tissue, they might shed light on the early stages of cancer[134].

Although 3D printing was developed decades ago, recent times witnessing a huge jump in adapting this versatile technology to the field of cancer modeling by fabricating sophisticated biological structures typical to cancer tissues. The cancer cells could be isolated from surgically removed cancer tissues. The isolated tumor cells are reprogrammed to pluripotent state by introducing the four transcription factors Oct4, Sox2, Klf4, and c-myc using a suitable method. The colonies are grown for a period of 2 – 4 weeks and cells with pluripotent stem cell morphology and molecular characters are expanded to create specific cancer iPSC lines[135]. These lines are differentiated into the tumor cell type of origin. The iPSC-derived differentiated cells could be then bioprinted into tumor tissues which

mimic the tumor microenvironment (TME). The commonly used cancer bioprinting methods are: Inkject printing, extrusion-based printing, lase-assisted printing, and SLA. The tissues can be 3D bioprinted in the format of spheroids, organoids, coculture with other tumor TME cells, and organ-on chip. Improvements in the 3D bioprinting technology enable to distribute the cancer derived iPSCs in a 3D space with high precision and reproducibility. The 3D bioprinted tissues can be used as cancer tissue-on-chip models or transplanted into animal models to study different stages of cancer pathogenesis.

The most important hurdle in establishing iPSC-derived cancer models are the variation of intrinsic transcription factors in the cancer cells. This variation can affect the reprogramming efficiency of tumor cells. Many published studies showed that cancer cells are generally difficult to reprogram than normal cells[136]. The differentiation of cancer iPSCs to its initial tumor cell of origin also appears tedious and inconsistent. The success rate of establishing a cancer iPSC depends on the type cancer. So far, the successful reprogramming of myeloid tumors is established[137-139]. Establishing protocols for generating cancer iPSCs can help to model cancer progression, to understand the complex cancer genetics, and contribution of TME in cancer progression, anti-cancer drug development, and precision oncology (**Figure 4**).

4.4 Bioprinted iPSCs for drug and cosmetic testing

4.4.1 Bioprinted iPSCs for drug testing

More than 90% of drug molecules under different phase of clinical trials fail to reach market because of unanticipated toxicity to vital organs or lack of efficacy. This failure rate is partly attributed to the use of overly simplistic 2D cell culture-based assays[140]. The spectrum of activity of most of the drug molecules varied across the species, so animal testing has limited predictive value[107]. 3D bioprinting and iPSC technology enable printing of

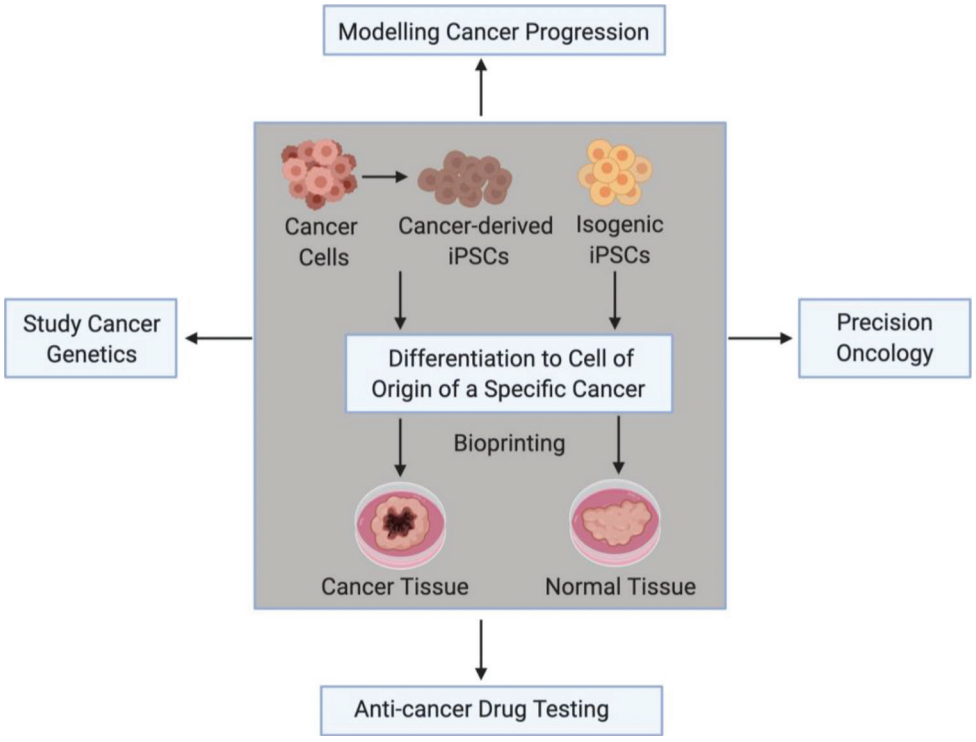

Figure 4. Bioprinted cancer tissue with induced pluripotent stem cell (iPSC)-derived cells: Establishing protocols for generating cancer iPSCs can help to model cancer progression, to understand the complex cancer genetics, and contribution of tumor microenvironment in cancer progression, anti-cancer drug development, and precision oncology.

any tissue in its native architecture by preserving the complexity of cellular pathways, cell-cell interactions, and cellular-microenvironment interactions. The tissue microenvironment is critical for the understanding of drug acting mechanisms in humans[141]. The use of 3D bioprinted tissue is predicted to be an integral part of future drug discovery research for improved *in vitro* assays with better predictive value.

4.4.2 Cosmetics testing

The European Union Cosmetics Directive was introduced in 1976 to enforce high safety standards for cosmetics across the EU member states. In 2009, the EU commission has introduced new directives to phasing out animal testing for cosmetic products. The directive bans the testing of the cosmetic products and cosmetic ingredients on animals. The law even prohibits the marketing of finished cosmetic products and ingredients tested on animals in the European Union[142]. Hence, the cosmetic industry is looking for methods to replace the animal testing. Bioprinted artificial skin tissue can substitute the use of animals for cosmetics testing (**Figure 5**). At present, the human skin models required for cosmetic testing are manufactured by layering fibroblasts in a collagen hydrogel, then adding keratinocytes on top. The cells are allowed to differentiate and mature into the different epidermal layers. This type of artificial skin models is simple and would not exactly reflect the complexity of the native skin. Advanced bioprinting technology and iPSCs as cell source allow fabrication of more realistic artificial skin models for drug testing as well as for regenerative medicine[143]. This would help develop skin models with different skin types with respect to race (Asian, Caucasian, etc.), character (dry, oily, etc.), or other specific skin types depending on the intended use of the cosmetic as these models incorporate iPSC-derived cells. Collagen-based skin construct reinforced with biocompatible materials such as polycaprolactone (PCL) mesh prevented the contraction of collagen during tissue maturation and enable manufacturing of biomimetic human skin models[144,145]. Other biomaterials such as polylactic acid, Pluronic, alginate, chitosan, hyaluronic acid, fibrin, and

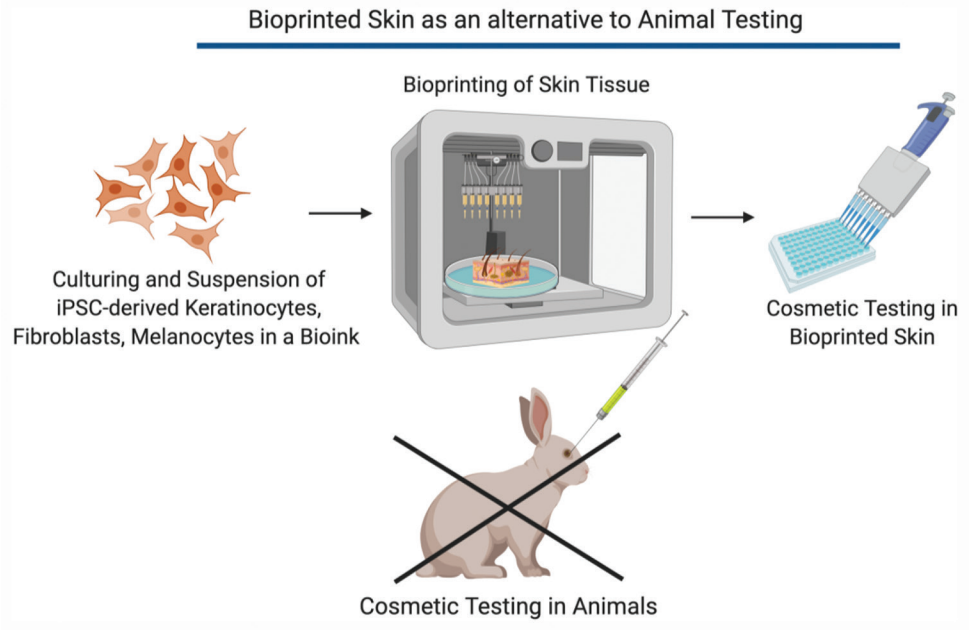

Figure 5. Bioprinting of human skin tissue models for cosmetic testing to replace the animal testing procedures. Use of induced pluripotent stem cell-derived skin cells would help develop skin models with different skin types (Asian, Caucasian, etc.), character (dry, oily, etc.), or other specific skin types depending on the intended use of the cosmetic.

gelatin, or a combination of PEGDA and GelMA were used as the scaffold material for printing skin models[145-147].

5 Bioinks used in iPSC bioprinting

Native tissue has complex architecture consisting of different cell types, ECM materials, growth factors, and many signaling molecules. ECM is organized in a highly delicate manner in a tissue to serve the tissue specific functions such as shape, consistency, mechanical strength, and molecular miscibility. Bioinks are cell laden hydrogels containing ECM components of the tissue to be printed. The materials used in the bioinks should be biocompatible without eliciting any undesirable response inside the body, should allow seamless printing, and should have tissue compatible rheological properties[56]. The bioink components can be natural, synthetic, different types of cells, and soluble growth factors specific for the cell types used. Natural polymers such as collagen, gelatin, fibronectin, laminin, and silk fibroin have been widely used in bioinks to augment cell attachment and migration in the matrix material[148] Polysaccharides such as alginate, agarose, and chitosan are also widely using in bioinks. Alginate is obtained from a type of brown algae and is widely used in 3D bioprinting applications due to its biocompatibility, promotion of cell proliferation, low price, and the ability of crosslinking in calcium ion solutions. However, alginate lacks sufficient mechanical stiffness for 3D bioprinting. Agarose is another biocompatible polysaccharide but it liquifies above physiological temperatures. Chitin and chitosan obtained from crustaceous animal are widely used polysaccharide component of bioinks, but it is slow in solidification. The methacrylated form of gelatin (GelMA) is a popular bioink component to print iPSCs which possess easily tunable physiochemical properties to use in bioprinters[149].

Still, many of these polysaccharides are too fragile and lack sufficient mechanical strength to retain in the transplant tissue site and often suffer from low mechanical properties, and thus, other materials have been combined as additive elements in the bioinks[150]. PCL and Poly (propylene fumarate) are polymers utilized in bioprinting due to its superior viscoelastic and rheological properties, biodegradability, and biocompatibility compared to the natural compounds. Synthetic hydrogels such as PEGDA are used as resins in 3D bioprinting, where cells can be entrapped[147]. The major limitation of these kind of hydrogels is that the bioprinted structure tends to collapse because of low viscosity and low mechanical strength[151]. New generation bioprinters have enabled us to print combinatorial bioinks with spatial and nanoscale resolution in seamless swift ways, aiming to reproduce the complex architecture of the native tissues. There are different types of bioinks available depends up on the tissue structure. One example is that researchers bioprinted hepatic tissue constructs using iPSC derived hepatocytes, endothelial cells, and mesenchymal cells resuspended in two different bioinks; GelMA with stiffness similar to healthy liver tissues, and a mix of glycidyl methacrylate-hyaluronic acid/GelMA which supported vascularization[73,152]. New generation polymers responsive to light, thermal, magnetic, humidity, and pH stimuli would allow the 3D bioprinting to leap to the next level.

6 Challenges associated with the use of reprogrammed iPSCs and bioprinting:

The combinatorial application of 3D bioprinting and iPSC technologies would have a major impact on regenerative medicine research. However, how much have we achieved to take this technique to clinics and how far we have to go? Many obstacles still remain regarding the production of safer iPSCs that are to be resolved to take full advantage of this technology for therapeutic purpose[67]. One of the most important problems is the use of retroviral and lentiviral vectors to introduce the transcription factor genes into somatic cells for cell reprogramming, which can cause mutagenesis and tumor induction in the host cell. The iPSCs derived using viral vectors may be still suitable for the study of disease mechanisms or for drug testing but they lack the suitability for clinical

bioprinting. The use of non-integrative methods and small molecules to activate the pluripotency program in somatic cells represents the safest approach to produce clinical grade iPSCs cells. High throughput screening to identify small molecules for cell reprogramming is ongoing in many laboratories, with a goal to establish iPSCs free of any exogenously introduced DNA fragments. Incompletely differentiated cells evoked immune response in transplanted animals[153]. Therefore, obtaining completely differentiated cells for therapeutic purpose are of prime importance. Futuristic technology should be focused on establishing safe strategies for genetic modification of iPSCs, devise efficient methods for differentiation and purification of iPSCs into required cell types *in vitro* for transplantation.

After production of iPSCs, there are limitations in the bioprinting process itself and associated challenges in the preparation of optimized bioinks suitable for each cell type. 3D bioprinting has the advantage of reconstructing complex structures from computed tomography or magnetic resonance imaging images and producing accurate structures from predetermined digital designs such as computer-aided design models. The support scaffold materials with suitable mechanical and biological properties can be designed and printed using advanced 3D printers. Directed differentiation of printed iPSCs to different cell lineages is required for organ printing. When we use undifferentiated iPSCs for bioprinting, the printing parameters should be adjusted to avoid any mechanical damage to the cells, as iPSCs are highly sensitive cells. Depending on the type of the bioprinting method used, the cells are exposed to high shear forces, radiation-induced damage, and electric or thermal stresses during the printing process[154,155].

Vascularization and innervation of the bioprinted tissue are a challenge to achieve. Bioprinted iPSC constructs are unable to form long-term viable and vascularized tissue. To resolve this problem, researchers at Wyss institute recently developed a method called sacrificial writing into functional tissue (SWIFT), which is a multistep biomanufacturing process that involves creating organ building blocks composed of hundreds of thousands of iPSCs and then rapidly 3D bioprinting vasculature into those building blocks[70]. The SWIFT method could create a perfusable cardiac tissue that fuses and beats synchronously for more than a week, taking the field of bioprinting vascularized functional tissues using iPSCs to the next level.

7 Future perspectives for iPSCs in bioprinting

Despite the challenges associated with the use of reprogrammed iPSCs and limitations of bioprinting, the potential of bioprinting iPSC-derived tissue is tremendous in the health-care field. Resolution of these challenges will have significant implications in the understanding of human diseases and will have major effects on the treatment of these diseases. Future perspectives of bioprinting iPSCs should focus on:

- Establishing xeno-free and footprint-free clinical-grade iPSC reprogramming protocols: The use of non-integrative methods and small molecules should be further explored. High throughput screening to identify small molecules for cell reprogramming to establish iPSCs free of any exogenously introduced DNA fragments would be potential area to focus on.
- Development of tissue-specific bioinks for bioprinting: New bioinks with tunable mechanical and rheological properties that mimic the native tissue ECM is to be developed and a deeper understanding of cell-bioink interactions must be sought as the mechanobiology and the molecular pathways would have a major effect on the differentiation of the bioprinted iPSCs.
- Improved bioprinting strategies to mitigate harmful effects on cells: Since iPSCs are sensitive cells (not as sturdy as cancer cell lines), the mechanical, thermal, or chemical stressors induced by the bioprinting process might result in cell-phenotype changes and functionality. Strategies to mitigate the exposure of cells to these process-induced stressors must be developed.
- Integrated bioreactor systems for tissue maturation: Bioprinting of functional tissues with iPSC-derived cells would be successful only if they can be matured and maintained over a long-term in physiologically-relevant

environments. Hence, development of suitable post-processing strategies such as integrated perfusion bioreactor systems becomes necessary.

- Pathway for clinical translation: A coordinated effort between the clinicians, scientists, and bioengineers in solving the technological limitations and support from the government and policy-makers would go a long way in establishing a pathway for clinical translation of bioprinted iPSC-derived tissues for regenerative medicine, disease modeling and drug testing.

Conflicts of interest

The authors declare no conflict of interest.

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