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A Concise Review on Induced Pluripotent Stem Cell-Derived Cardiomyocytes for Personalized Regenerative Medicine

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Abstract

The induced pluripotent stem cells (iPSCs) are derived from somatic cells by using reprogramming factors such as Oct4, Sox2, Klf4, and c-Myc (OSKM) or Oct4, Sox2, Nanog and Lin28 (OSNL). They resemble embryonic stem cells (ESCs) and have the ability to differentiate into cell lineage of all three germ-layer, including cardiomyocytes (CMs). The CMs can be generated from iPSCs by inducing embryoid bodies (EBs) formation and treatment with activin A, bone morphogenic protein 4 (BMP4), and inhibitors of Wnt signaling. However, these iPSC-derived CMs are a heterogeneous population of cells and require purification and maturation to mimic the in vivo CMs. The matured CMs can be used for various therapeutic purposes in regenerative medicine by cardiomyoplasty or through the development of tissue-engineered cardiac patches. In recent years, significant advancements have been made in the isolation of iPSC and their differentiation, purification, and maturation into clinically usable CMs. Newer small molecules have also been identified to substitute the reprogramming factors for iPSC generation as well as for direct differentiation of somatic cells into CMs without an intermediary pluripotent state. This review provides a concise update on the generation of iPSC-derived CMs and their application in personalized cardiac regenerative medicine. It also discusses the current limitations and challenges in the application of iPSC-derived CMs.

Keywords Cardiomyocytes · Cardiac tissue engineering · Direct reprogramming · Pluripotent stem cells · iPSC · Regenerative medicine

Introduction

Induced pluripotent stem cells (iPSCs) are laboratory-derived stem cells that can be reprogrammed from somatic cells by introducing reprogramming factors using viral or non-viral vectors. Colonies of iPSCs morphologically, molecularly and phenotypically resemble embryonic stem cells (ESCs). They possess self-renewal and pluripotency properties to differentiate into cells of all three germ layers [1–3]. Therefore, they are considered a valuable tool for in vivo and in vitro study of human development and related diseases. It additionally provides an advantage of being patient-specific autologous cell source to avoid immune rejection and does not pose ethical issues as the cells can be harvested from the willing adult.

The breakthrough research of iPSC isolation was performed in the year 2006 by Nobel-prize winner Dr. Shinya Yamanaka at Kyoto University using retroviral vectors for introducing Oct4, Sox2, Klf4 and c-Myc, together called as Yamanaka factor or OSKM factor, into mouse fibroblast [4, 5]. A year later, human iPSCs were reported by two independent group of scientist from Japan, by introducing OSKM factors [5], and United States, by introducing Oct4, Nanog, Lin28 and Sox2 (called OSNL factor), in human somatic cell [6]. Since then a number of modified methods based on viral or non-viral delivery of reprogramming factors into the target cells are under investigation and have shown promising results. A number of efforts are also being made to increase the

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reprogramming efficiency and derive the iPSCs from various sources in both human and animals.

The reprogramming factors have been delivered into somatic cells by various viral vectors such as lentiviruses [7], adenoviruses [8], retroviruses [5], sendai virus [9] and non-viral methods such as synthetic bacterial plasmids [10], recombinant proteins [11], RNA [12], self-replicating RNAs [13] and miRNA [14], piggyBac transposons [15], CRISPR-Cas9 [16, 17] and Cre-lox system [18, 19] (Table 1). Initial research used retroviral and lentiviral vectors for deriving the iPSCs but such cells were not suitable for therapeutic applications because the exogenous genes can permanently integrate into the genomic DNA. Thus, non-integrating adenoviral or sendai viral vectors, transposons, plasmid vectors and DNA-free methods using nanoparticle or carbon nano-tube were investigated and become popular choice due to their reduced risk of getting integrated. The reprogramming efficiency of non-integrating methods was much lower (0.001–0.1%) than those of integrating vectors (0.1–1%) [56, 57]. A number of additional genes such as Glis 1, Utf1, hTERT [58, 59], small molecules such as GSK3-β inhibitors (e.g. CHIR99021, BIO, IQ-1), MEK/ERK inhibitor (e.g. PD032501, Pluripotin or SC1), adenylyl cyclase activator (e.g. Forskolin), TGFβ inhibitor (e.g. A-83-01, SB43152), Vitamin C, ROCK inhibitor (e.g. Thiazovivin,Y-27632), PI3K/Akt activator (e.g. PS48) and epigenetic modifiers such as DNMT inhibitors (e.g. 5-Azacytidine), HDAC inhibitors (e.g. Trichostatic A, Valproic acid), G9a inhibitor (e.g. BIX0129) were found to further improve the reprogramming of somatic cells into iPSCs [60]. Many scientists now prefer to use sendai viral vectors due to their non-integrating nature, ability to infect wide range of cells and high transfection efficiency for a footprint-free reprogramming [61].

The iPSCs have now been successfully generated from both normal and diseased tissues, which can be used for therapeutic purpose as well as for understanding the pathophysiology of diseases and the mechanism of underlying genetic mutations. The isolation of iPSCs has been successful from human patients suffering from various diseases including Schizophrenia [62], Rubinstein-Taybi Syndrome 1 [63], Joubert Syndrome [64], Amyotrophic Lateral Sclerosis [65], Neurofibromatosis type 1 [66], Non-ketotic Hyperglycinemia [67], Hutchinson-Gilford Progeria Syndrome [68], Meckel-Gruber Syndrome [69], Autism Spectrum Disorder [70, 71], Bardet-Biedl Syndrome [69], Retinitis Pigmentosa [72], Parkinson's Disease [73, 74], Leukoencephalopathy [75], Pendred Syndrome [76], Cone-Rod Dystrophy [77] etc. to list a few. A variety of cell sources including cord blood [48] and peripheral blood mononuclear cell (PBMC) have also been successful [70, 78]. The factors have also been expressed into somatic cells such as myogenic cells, keratinocytes, cardiac fibroblasts, melanocytes, neuronal cells and chondrocytes of mice, rats, rabbits, dogs, pigs, cattle, monkeys and other animals to derived cell-specific iPSCs under in vitro condition (Table 1). Even the most terminally differentiated cells such as T cells [61], B cells [79], myocytes [80] have been used for the isolation of iPSCs. In addition, different imaging techniques such as bioluminescence, magnetic resonance, fluorescence imaging, and positron emission tomography have also been developed for in vivo tracking of transplanted stem cells [81] for iPSCs-based research and therapy in biomedical field.

Large scale xeno-free culture of iPSCs is an essential requirement for producing clinically usable cells with good manufacturing practices (GMP). A number of scalable culture systems are thus being investigated and proven to be useful for in vitro culture of iPSCs. The iPSCs expansion has been performed in spinner-flasks using a xeno-free culture medium such as Essential 8 Medium, incorporated with defined matrices such as recombinant vitronectin [82] or using microcarrier-based systems [83, 84]. Newer systems such as single-use Vertical-Wheel™ bioreactors [85, 86] have been developed for cost-effective long term expansion and differentiation of human iPSC either on microcarriers or as suspension aggregates. Moreover, bioreactors allow for more advanced feeding strategies. For instance, perfusion, where spent medium is continuously being replaced with fresh medium, was shown to improve the iPSC expansion when compared to a more traditional repeated batch feeding [87, 88]. The details of various bioreactors for expansion and differentiation have been reviewed elsewhere [89, 90].

Reprogramming Factors and Small Molecules for Generation of iPSCs

The generation of iPSCs consists of several steps, each requiring advanced skills and sophisticated laboratory facilities. A key to the success of iPSCs generation is the introduction of essential reprogramming factors into the somatic cells. Fibroblasts cells were earlier reprogrammed to muscles cells by introduction of cDNA into mouse fibroblasts [91] but major revolution was brought up by Yamanaka's group in 2006. The Yamanaka' group initially started with 24 factors for reprogramming of mouse fibroblasts but finally found four factors to be essential and sufficient in reprogramming the somatic cells into pluripotent cells. The octamer binding transcription factor (Oct4; also called Pou5f1) plays a vital role in embryonic development and is the master regulator of stem cell pluripotency. Sex determining region Y box2 (Sox2) is a transcription factor that regulates the embryonic development, cell fate and self-renewal of stem cells through the regulation of Oct4 expression [92]. On the other hand, Kruppel like factor 4 (Klf4) has both transcriptional activation and repression domains and is a member of zinc finger transcription factors that is involved in cell differentiation, proliferation and survival. The Klf4 is believed prevent apoptosis and activate Sox2 for promoting self-renewal of iPSCs [93]. The c-

Table 1 Generation of the iPSCs from various types of cells

S. No.	Types of cell	Species	Method used	Differentiation	References
1	PBMC	Human	CRISPR/Cas9	Hepatocyte	[20]
2	PBMC	Human	Sendai viral vector for OSKM factor	Neuronal cells, germ layers	[21]
3	MEF	Human	Elastin-like polypeptide transfection	Three germ layers	[22]
4	PBMC	Human	Sendai reprogramming kit	Three germ layers	[23]
5	Dermal fibroblast	Human	Sendai reprogramming kit	MSCs	[24]
6	Dendritic cells	Mouse	Sendai reprogramming kit with KOS	Embryoid body	[25]
7	Urine cells	Human	Episomal vector	Kidney organoids	[26]
8	Hematopoietic cells	Human	Measles virus vector	Embryoid body	[27]
9	AFDSC	Human	Sendai reprogramming kit	CMs	[28]
10	PBMC	Human	Episomal vector	Embryoid body	[29]
11	Kidney cells	Human	CRISPR/Cas9	Embryoid body	[30]
12	Skin fibroblast	Human	Episomal plasmid with OSKML factor	Teratoma formation	[31]
13	PBMC	Human	Sendai reprogramming kit	Embryoid body	[32]
14	Erythroblast	Human	CRISPR	–	[16]
15	HEK293 and Fibroblasts	Human	CRISPR	Three germ layers	[17]
16	Amniotic fluid stem cells	Human	Episomal plasmid	Teratoma formation	[33]
17	Fibroblasts	Mouse	CHIR, Forskolin, 616,452 and DZNep	Teratoma formation, Chimera construction	[34]
18	Urine-derived cells	Human	cyclic pifithrin-a, A-83-01, CHIR99021, thiazovivin, sodium butyrate and PD0325901	Teratoma formation	[35]
19	Extra embryonic endoderm like cells	–	AM580, EPZ004777, SGC0946, and 5-Aza	Teratoma formation	[36]
20	Fibroblast	Human	OSKM followed by TAT-Cre treatment	CMs	[19]
21	MEF	Mouse	Lentiviral system followed by Cre-lox/HSV-tk/Gan technology	Three germ layers	[18]
22	Somatic cell	Mouse	Mbd3/NuRD with OSKM factors	Teratoma formation	[37]
23	Somatic cell	Mouse	Sox2, Klf4 and c-Myc with Forskolin, 2-methyl-5-hydroxytryptamine and D4476	Teratoma formation	[38]
24	MEF	Mouse	Sox2 protein	Germ layers	[39]
25	Fibroblasts	Mice	Oct 4 with valproic acid, tranylcypromine, CHIR99021	Embryoid body, Neuronal cells	[40]
26	Fibroblasts	Mouse	miR302/367	–	[41]
27	Renal tubular cells from urine	Human	OSKM factor	–	[8]
28	Fibroblasts	Human	PD0325901, CHIR99021, A-83-01, HA-100 and LIF	Embryoid body	[42]
29	MEF	Mouse	Oct4, Klf4, c-Myc with iPYrazine	Three germs layers, Teratomas	[43]
30	Human foreskin fibroblasts	Human	pDNA for reprogramming factors	Cardiac myocyte	[44]
31	Different types of fibroblasts	Human	Synthetic modified mRNA	Myogenic cells	[12]
32	Skin fibroblasts	Monkey	Oct4, Sox2, and Klf4	Neuronal cell types	[45]
33	Adult fibroblasts	Mouse	Proteins based reprogramming factors	Three germs layers	[46]
34	MEF	Mouse	Nr5a2shRNA	–	[47]
35	Human cord blood	Human	OSKM factors	Germ layers, CMs	[48]
36	$CD34^+$ cells	MPDs	OSKM factors	Hematopoietic progenitor cells	[49]
37	Fibroblasts	Murine	Oct4, Sox2, c-Myc with Kenpaullone	–	[50]
38	MEF	Mouse	Esrrb shRNA	–	[51]
39	Pancreatic β cells	Mouse	OSKM factor	–	[52]
40	Fibroblast	Mouse/ Human	OSK factor	–	[53]
41	Neural progenitor cells	Mouse	Klf4/Sox2/c-Myc and BIX01294	–	[54]

Table 1 (continued)

S. No.	Types of cell	Species	Method used	Differentiation	References
42	MEF	Mouse	cDNAs for OSKM factors	–	[55]
43	MEF or adult fibroblasts	Mouse	OSKM factors	Three germ layers	[4]

MEF Mouse embryonic fibroblast, *MPDs* Human with Myeloproliferative disorders, CMs-cardiomyocytes, *ESC* Embryonic stem cell, *PBMC* Peripheral blood mononuclear cells, *AFDSC* Amniotic fluid derived stem cells

myelocytomatosis (c-Myc) belongs to the family of proto-oncogenes that controls the cell cycle and cell proliferation [94]. It recruits chromatin-modifying proteins to facilitate open chromatin structure through histone acetylation and, increases the expression of Oct4- and Sox2-regulated genes. The use of c-Myc for iPSCs was associated with tumorigenesis and it was later shown that it can be replaced with transformation-deficient L-Myc [95]. A modified protocol was also developed that did not require the use of c-Myc for iPSCs generation [53].

In human, Nanog and Lin28 were shown to be a good substitute for Klf4 and c-Myc. Nanog is a homeoprotein and is capable of maintaining pluripotency through Oct4 and is independent of Sox2 and LIF/Stat3 [96]. Lin28 is a RNA binding protein, which repress Let-7 miRNA influences translation machinery to regulate self-renewal of iPSCs [97]. A number of other genes such as Glis 1, H1foo, Utf1, hTERT in combination with one or more of OSKM or OSNL factors have also been tested and found to be useful in derivation of iPSCs in both human and mice [58, 59, 98]. In other studies, cell deficient in one or more components of Arf-Trp53 pathway [99], p53-p21 pathway [100], NuRD complex [37] were also found be to better amenable to reprogramming by OSKM or OSNL factors. However, among various reprogramming factors, OSKM and OSNL remain to be the most commonly used reprogramming factors in rodent and human, respectively. The OSKM with/without other factors have also been used in other animal species such as pig and cattle with variable degree of success.

A number of small molecules have also been investigated to substitute for the reprogramming factors. Small molecules are small molecular weight chemical compounds that can easily penetrate through the cell membrane and thus, does not require complicated procedure of vector construction or viral/ non-viral vectors. The dosage and duration of treatment with small molecules can also be easily controlled and they can be easily removed after initiation the reprogramming cascade. Thus, they offer an attractive system for generation of iPSCs. A number of small molecules such as GSK3-β inhibitors (e.g. CHIR99021, BIO, IQ-1), MEK/ERK inhibitors (e.g. PD032501, Pluripotin or SC1, PD0325901), adenylyl cyclase activators (e.g. Forskolin), TGFβ inhibitors (e.g. A-83-01, SB43152, SB-431542, LY-364947), Tyrosin kinase inhibitors (e.g. iPYrazine), Scr kinase inhibitors (e.g. Dasatinib, PP1), ALK inhibitors (e.g. SB-431542, RepSox or E-616452, A-83-01), ROCK inhibitors (e.g. Thiazovivin,Y-27632, HA-100), PI3K/Akt activators (e.g. PS48), RAR agonists (e.g. CD437, AM580), mTOR inhibitors (e.g. Rapamycin, PP242), Sirtuin activators (e.g. Resveratrol, Fisetin) and epigenetic modifiers such as DNMT inhibitors (e.g. 5-Azacytidine, RG-108, Decitabine), HDAC inhibitors (e.g. Trichostatic A, Valproic acid, Sodium Butyrate, SAHA), Histone methyltransferase G9a inhibitors (e.g. BIX0129), global histone methylation inhibitor (e.g. 3-deazaneplanocin A or DZNep), Lysine-specific demethylase1 inhibitors (e.g. Parnate), histone H3K79 methyltransferase inhibitors (e.g. EPZ004777, SGC0946) were found to be useful in induction, promotion or acceleration of reprogramming or in enhancing the self-renewal and survival rate of iPSCs. Li et al. [40] found that iPSCs could be generated using Oct4 only when combined with a HDAC inhibitor (valproic acid), a histone H3K4 demethylation inhibitor (tranylcypromine), a GSK3-β inhibitor (CHIR99021) and a TGFβ inhibitor (616452). Later, the same group had identified three other small molecules viz. Forskolin (adenylyl cyclase activator), 2-methyl-5-hydroxytryptamine, and D4476, which could replace the requirement of Oct4. Thus, the seven small molecules together can reprogram the somatic cells into iPSCs without the need of OSNL or OSKM factors [38]. In other studies, combination of PD0325901, CHIR99021, A-83-01, HA-100 and LIF [42] or AM580, EPZ004777, SGC0946, and 5-Aza [36], or CHIR, Forskolin, 616,452 and DZNep [34] or cyclic pifithrin-a, A-83-01, CHIR99021, thiazovivin, sodium butyrate and PD0325901 [35] were found to be sufficient for generation of iPSCs without the need of reprogramming factors.

The small molecules can also be used in combination with OSNL or OSKM factors to improve the efficiency of reprogramming. A number of small molecules such as Curcumin, Vitamin C, LY294002, Spermidine, Dexamethasone, Quercetin, N-oxaloylglycine, 2,4-dinitrophenol, 2-Hydroxyglutaric acid, Nicotinic acid, Fructose 6-phosphate, Anisomysin, Lithium Chloride, PKC inhibitors, DNMT inhibitors, HDAC inhibitors, GSK3-β inhibitors, MEK/ERK inhibitors, G9a inhibitors were found to enhance the efficiency of reprogramming or promoting the completion

of reprogramming process. In fact, some small molecules could be used to replace the need of one or more reprogramming factors. For example, BIX01294 could replace Oct4 in reprogramming the neural stem cells to iPSCs [54]. Similarly combination of Forskolin, 2-methyl-5-hydroxytryptamine and D4476 could replace the requirement of Oct4 for iPSC generation [38]. In other studies, Kenpaullone, could replace the need of Klf4 [50] whereas 616,452 [101], iPYrazine [43], Dasatinib [101], PPI [43], SB-431542 and LY-364947 [102] could replace the need of Sox2 for iPSC generation. The use of small molecules also reduced the duration of reprogramming from 40 days to 16 days as it did not require several days for selection of cells and construction of vectors. Furthermore, it could overcome the possibility of tumerogenesis associated with viral vector methods of iPSCs generation. However, the efficiency of reprogramming and the ability of iPSCs generation using small molecules varied with cell types and cell lines and, required optimization in selecting the type of small molecule and their treatment regimen [103]. Identification of newer molecules and elucidation of their mechanism of action are active areas of research. A detailed review on various small molecules for generation of iPSC can be found elsewhere [104].

Applications of iPSCs

The iPSCs have found application in various therapeutic areas, including orthopaedics, dental medicine, treatment of wound and injuries, cardiology, neurology, immunology, inflammatory diseases, ontology and metabolic diseases. A number of studies have demonstrated the use of human iPSCs to create functional cells, which upon transplantation into rodent models, could restore the functionality of the affected organs. For example, it has been shown that functional retinal pigment epithelium cells could be generated from human iPSCs. When these iPSCs-derived retinal epithelia were transplanted into the eye of disease rodent model or in human, the transplanted cells resulted in long-term preservation of vision (Li et al., 2012). Similar results were also observed in human clinical trials wherein transplantation of iPSC-derived retinal pigment epithelium cells improved the vision of patients affected with macular degeneration [105]. Human trials have also shown success with transplantation of iPSC-derived neurons in patients affected with Parkinson's Disease [106] and other clinical trials are ongoing for spinal cord injury and heart failure patients [107]. In case of genetic diseases, it is also possible to correct the genetic defects or mutations in the iPSCs before their targeted differentiation and transplantation. Hanna et al. [108] showed that fibroblast from mouse model of sickle cell anemia could be reprogrammed to iPSCs and corrected for the mutation in the hemoglobin allele by gene targeting. The in vitro differentiation of iPSCs into hemotopietic progenitors and subsequent transplantation into the bone marrow of the diseased mouse (irradiated by gamma irradiation to deplete endogenous proliferating cells in the bone marrow) showed that ~70% of peripheral blood cells were iPSCs-derived. The correction of sickle cell anemia in this study laid the foundation for treatment of genetic diseases by iPSCs-based therapy. Consequently, iPSCs-based therapy has attracted great commercial interest. The current stem cell therapy has an estimated market size of ~$750 million and is expected to increase to ~$11,000 million by year 2030 at a projected compound annual growth rate (CAGR) of ~28%. Countries such as USA, Canada, Germany, United Kingdom, France, Italy, Australia, China, South Korea are leading in iPSCs research whereas it is slowly picking up in India, Middle East, Africa and Latin America. Companies such as Astellas Institute for Regenerative Medicine, CellSeed Inc., California's Stem Cell Agency, Fujifilm Cellular Dynamics Inc., PBS Biotech Inc., Regenerative Patch Technologies LLC, Reliance Life Sciences Private Limited, Stempeutics Research Private Limited, ViaCyte Inc., Vericel Corporation, WiCell Research Institute etc. are actively involved in the stem cell research and many of them are aiming to capture the global market of iPSCs-based therapeutics worldwide (Box 1). With growing market, various regulatory agencies of different countries such as USA (Food and Drug Administration's Center for Biologics Evaluation and Research), Canada (Health Canada), Japan (Pharmaceuticals and Medical Devices Agency), South Korea (Korea Food and Drug Administration), China (National Medicinal Products Administration), India (Central Drugs Standard Control Organization) have classified the iPSCs-based therapy as drugs and framed regulatory policies. However, many developing is yet to have a comprehensive regulation on stem cell research and their therapeutic application.

Box 1 Market segment of stem cell therapeutics. Including iPSCs

Major Therapeutic Applications	Leading Countries
Cardiology	Australia
Dental Medicine	Canada
Immunology	China
Inflammatory Diseases	France
Metabolic Diseases	Germany
Neurology	Italy
Oncology	Japan
Orthopaedics	South Korea
Wound and Injuries	UK
	USA
	Emerging (India, Middle East, Africa, Latin America)

Important Companies	
AlloSource	Medipost Company Limited
Anterogen Company Limited	Mesoblast Limited
Astellas Institute for Regenerative Medicine	Nu Vasive Inc.
Athersys Inc.	Orthofix Inc.
Asterias Biotherapeutics Inc.	Osiris Therapeutics Inc.
California's Stem Cell Agency	PBS Biotech Inc.
CellSeed Inc.	Regenexx
Chiesi Farmaceutici SPA	Regenerative Patch Technologies LLC
Chorestem Inc.	Realiance Life Sciences Private Limited
Coreill Institute	Stempeutics Research Private Limited
Fujifilm Cellular Dynamics International	Takeda Pharmaceutical Company Limited
JCR Pharmaceuticals Company Limited	US Stem Cell Inc.
Lineage Cell Therapeutics	Vericel Corporation
	ViaCyte Inc.

The iPSCs has also been used to model various genetic and metabolic diseases such as Schizophrenia, Hyperglycinemia, Retinitis Pigmentosa, Parkinson's Disease, Autism Spectrum Disorder, Amyotrophic Lateral Sclerosis etc. The specific cell types can be derived from disease-affected patients by directed differentiation of donor's iPSC and manipulated in vitro to study the underlying pathophysiology and signaling mechanism involved in the causation of the disease. For example, Akkouh et al. [62] derived the iPSCs from human Schizophrenia patients and differentiated them into astrocytes, which could be used for studying the involvement of astroglia-CCL20-CCR6-T(reg) axis in the pathophysiology of Schizophrenia. On the other hand, Atchison et al. [68] could isolate iPSCs from human patients, suffering from Hutchinson-Gilford Progeria Syndrome, and differentiate them into smooth muscle and endothelial cells for developing blood vessels by tissue engineering. Thus, the iPSCs approach provides a good substitute for the animal models by eliminating idiosyncrasy and minimizing the variation in results due to cross-species differences or due to difference in cell signaling mechanism in disease causation. The use of human iPSCs can also reduce the number of animals required for experimentation and improve the success of clinical trials.

The iPSCs from normal individual can also been differentiated into homogeneous population of difficult-to-obtain cell types for studying the etiology and treatment of diseases. Ali et al. [78] generated human iPSCs from PBMC and differentiated them into lens-like lentoid bodies. Akita et al. [109] differentiated mouse iPSCs into cardiomyocytes (CMs) and used them as model to study the usefulness of Lubiprostone, a chloride channel opener, in the treatment of cardiac diseases. Anastasaki et al. [66] developed iPSCs from Neurofibromatosis type 1 patients to identify differential effects of NF1 mutations on central nervous system. In other studies, fibroblast cells (from human foreskin, Skin and Dermal fibroblast) have been successfully converted into iPSCs and further differentiated into insulin producing cells (IPCs) for the treatment of diabetes [110–113]. Thus, it is expected that discoveries made using iPSCs will change the era of drug development or other therapeutic interventions. Considering all these factors, iPSCs are being explored by scientist as possible time-, resources- and cost- saving alternatives. The iPSCs are considered as multi-purpose tool for biomedical research and the technology is continually evolving in parallel with the development of disease modeling and drug development studies.

Application of iPSCs in Cardiac Regenerative Medicine

Cardiovascular diseases (CVDs) due to Myocardial Infarction (MI), Myocardial Ischemia, Atherosclerosis, Arrhythmia, Cardiomyopathies, Heart valve disease, and Long–QT syndromes (LQTS) are major cause of death all over the world. The World Health Organization (WHO) estimated that, ~17.5 million populations per year of all global death are due to CVDs and is estimated to rise up to ~23 million by 2030 [114]. Despite significant research in the treatment of CVDs, including heart surgery, heart transplant, ventricular assist device implantation etc., it remains a major therapeutic challenge. Since most CVDs are associated with damage to cardiac and vascular cell by apoptotic and necrotic processes [115], stem cell-based therapy is rapidly emerging as a new therapeutic approaches for the treatment of CVDs [116].

The iPSCs can serve as a basis for autologous cell-replacement therapy in CVDs. The easily accessible cells such as fibroblasts or PBMCs can be collected from the diseased patient and reprogrammed into iPSCs to produce CMs, endothelial cells, muscles and other heart cells by directed differentiation. The differentiated cells can then be used as therapeutic cells for transplantation by cardiomyoplasty or after development of tissue engineering constructs (Fig. 1). Since the somatic cells for iPSCs generation originates from the patient's own body, iPSCs-derived heart cells are less prone to immune rejection and ethical issues. Consequently, post-transplantation regimen for immunosuppressive drugs can be minimized. In case of genetic diseases, it is also possible to correct the genetic defects or mutations in the iPSCs before their targeted differentiation and transplantation.

The iPSCs-derived CMs can also be used to effectively mitigate the cardiac disease under in vitro condition and develop novel drugs or to understand the pathophysiology of the cardiovascular diseases [109]. The iPSC-derived CMs can be readily generated from patient-derived somatic cells and can be cultured and maintained indefinitely as homogeneous population of cells for drug testing or disease modeling (Table 2). For example, familial dilated cardiomyopathies, caused by mutation in TTN, TNNT2, LMNA, MYH7, MYH6, SCN5A

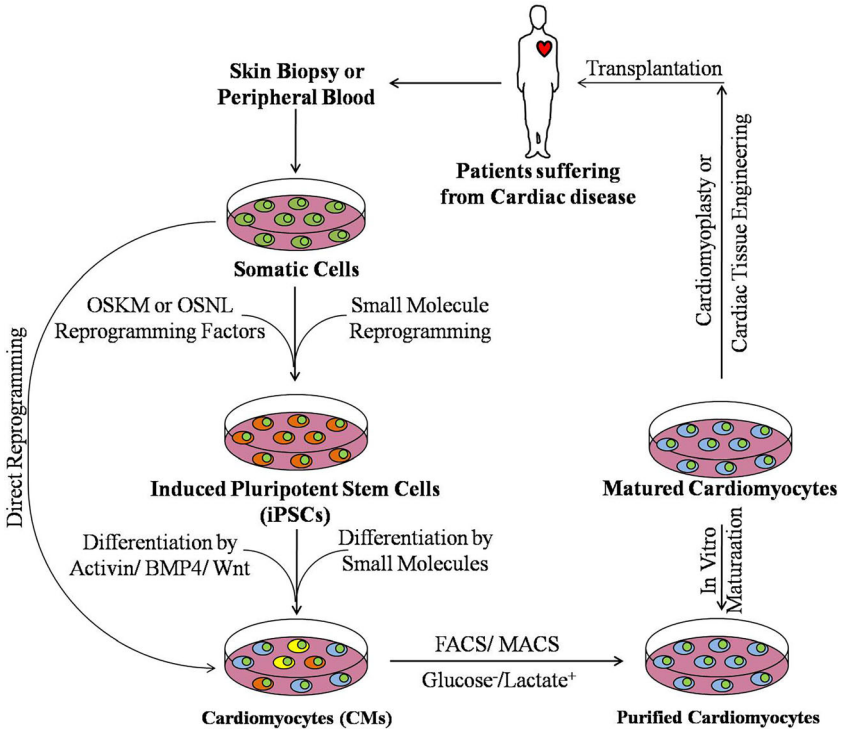

Fig. 1 An outline of steps involved in the application of iPSCs-derived cardiomyocytes in cardiac regenerative medicine

could be recapitulated in vitro in their morphological and functional phenotypes of dilated cardiomyopathies by iPSCs-derived CMs to serve as platform to understand the underline disease mechanism of drug screening [128]. Similarly, iPSC-derived CM was used to develop a disease model of LQTS caused by autosomal dominant disorder of at least 15 gene mutations [129, 130]. Use of iPSC-CMs in modeling cardiac diseases and drug screening are reviewed elsewhere [131, 132].

The therapeutic potential of iPSCs through their autologous in vivo transplantation in animal models of CVDs has been well documented in several studies [117, 133]. Autologous transplantation of iPSCs-derived CMs not only improved the contractile function of severely damaged myocardium in animal models of MI but also improved the cardiac bioenergy [118]. On the other hand, allogenic transplantation of animal iPSC-derived cells into immunodeficient mouse models of MI and hindlimb ischemia was also shown to have beneficial effect on cardiovascular function [134, 135]. The utility of human iPSCs in the treatment of CVDs has also been demonstrated using SCID mouse or immunosupressed non-rodent models, including primates (Table 2). These studies have clearly established that cardiac function could be improved by transplantation of iPSC-derived CMs in diseased heart. However, the mechanism of beneficial effects remains debatable. Some studies have suggested that the improvement in

Table 2 Application of iPSCs in different heart diseases/syndromes

Cells	Transplanted in	Application	References
iPSCs-derived CMs	Rat	Myocardial Infarction	[117]
iPSCs-derived CMs	Pig	Myocardial Infarction	[118]
iPSCs-derived hepatocytes	FH Model	Cholesterol metabolism	[119]
iPSCs-derived extracellular vesicles	Mice	Cardiac repair	[120]
iPSCs-derived CMs	Transgenic mice	Cardiomyopathies	[121]
iPSCs	–	Muscular Dystrophy	[122]
iPSCs-derived muscles cells	–	Blood Vessel Model	[123]
iPSCs-derived CMs	Rats	Biological pacemaker	[124]
iPSCs-derived CMs	Monkey	Infracted heart	[125]
iPSCs-derived CMs	Immunodeficient mice	Therapeutic Potential in Heart	[126]
iPSCs	Pig	Chronic Myocardial Ischemia	[127]

cardiac function may be due to paracrine effects through secretion of cytokines and not due to iPSC-CM per se [136]. A few studies have also used non-rodent large animals such as sheep and pigs but engraftment of human iPSC-derived CMs has been poor [137, 138]. Furthermore, transplantation in large animal models was also associated with ventricular arrhythmias, which was not frequently reported in rodents, perhaps due to their rapid heart rate [125, 139]. Interestingly, the intramyocardial engraftment could be improved by co-injection of MSCs [137] or injection through IGF-encapsulated microspheres [138]. More recently, Yoshiki Sawa and colleagues from Osaka University have received the approval for a clinical trial to graft iPSCs derived allogeneic sheets onto diseased hearts of three patients in a year and later can be trial on ten patients [140]. More than 50 iPSCs clinical trials are registered with ClinicalTrials.gov. Unfortunately, there are several hurdles to in vivo transplantation of iPSC-derived CMs for CVDs, which are reviewed elsewhere [141].

Generation of Cardiac Cells from Stem Cells

An ideal cell source for cardiac tissue engineering and regenerative medicine is the one which is easily available in sufficiently large numbers and is capable of differentiating into contractile, non-immunogenic and electro-physiologically compatible myocardium. Primary CMs are the first choice of cells but they have minimal proliferative capacity and are difficult to obtain in large numbers. Therefore, alternative cell sources such as cardiac stem cells (CSC), cardiac progenitor cells (CPC), ESC, iPSCs, hematopoietic stem cells (HSC), mesenchymal stem cells (MSC), male germ-line stem cells (GSC) etc. have been investigated to derive and provide large number of CMs required for regenerative medicine and cardiac tissue engineering (~10^5 cells/cm^3).

Cardiac stem cell (CSC) and progenitor cells (CPC) are found in the pharyngeal mesoderm, called as secondary heart field, and in neural crest of the fetal heart [142]. In adults, they may occur as small cluster of self-renewing, clonogenic cells interspersed between terminally differentiated CMs [143]. These cells are multipotent in nature and can differentiate into beating CMs upon treatment with oxytocin [144, 145]. Combined delivery of pericytes and CSCs was shown to improve the healing of mouse infracted heart through stimulation of vascular and muscular repair by secreting various growth factor and higher quantities of angiopoietins and microRNA-132 [146, 147]. CPCs have also been successfully used to generate beating thin films [148] or thick tissue by 3D printing technology [149]. However, CPCs exist in low numbers and are difficult to obtain as pure population of cells in sufficient numbers required for the regenerative medicine [150].

Embryonic stem cells (ESC) are isolated from early stage embryos and are considered as 'gold standard' for pluripotent stem cells. Authentic ESCs have been successfully isolated in both human and animals and are commercially available as cell lines for research purpose [151]. They are capable of differentiating into cells of all three germ layers including those of the heart. A number of studies have reported successful differentiation of ESCs into CMs by generation of embryoid bodies (EBs) and treatment with retinoic acid, ascorbic acid, fibroblast growth factor, nicotinamide, 5-azadeoxycytidine etc. [152, 153]. Pure population of ESC-CMs can be cultured in large scale in advanced bioreactor systems that can provide physical cues to mimics in vivo conditions for heart development [154]. Thus, they have become the most attractive cell source for cardiac tissue engineering. Several researchers have been successfully developed cardiac patches [155, 156] and 3D cardiac tissue construct using ESCs [157]. However, in clinical settings, use of ESC-derived CMs may pose potential risk of immunogenicity and uncontrolled differentiation into teratoma. There are also ethical issues associated with their derivation which limits their clinical application in human [158]. Use of patient specific ESCs, derived from parthenogenetic embryos or somatic cell nuclear transfer (SCNT), may obviate some of the issues of immunogenicity [159, 160]. Nevertheless, clinical application of ESCs and their derivatives are currently prohibited in almost all countries until above issues are fully resolved.

Adult stem cells (ASC) such as hematopoietic (HSC) and mesenchymal stem cells (MSC) have also been investigated as alternative to ESCs for generation of CMs. Clinical-grade HSC and MSC can be isolated from bone marrow by means of GMPs. MSCs can also be isolated from umbilical cord, Wharton's jelly, adipose tissue, amniotic fluid, chorionic villi, placenta, dental pulp etc. in adults and does not pose immunogenic or ethical problems. Clinical trials have shown that administration of MSCs to MI patients improved the ejection fraction and left ventricular volumes [161]. Several animal models have also demonstrated successful differentiation of MSCs into CMs in vivo [162–165] and therefore MSCs appears to be an attractive cell source for CMs. However, there is conflicting report on the ability of HSC and MSCs on their ability to differentiate into CMs. While some reported increased the expression of CMs markers and differentiation of MSCs into spontaneously beating cells by treatment of MSCs with 5-Azadeoxycytidine, a DNA demethylation agent [166–168], others reported no benefit of 5-Azadeoxycytidine treatment on differentiation of MSCs into CMs [169]. Other methods such as use of platelet rich plasma (PRP) [170], TSA [171], platelet-derived growth factors (PDGF) [172], serum source [173] and co-culture with CMs [174, 175] have also used either alone or in combination with 5-Azadeoxycytidine for differentiation of MSCs into CMs. It is believed that the improvement in cardiac function through HSC or MSCs cell transplantation is due to the secretion of soluble factors by these cells and activation of Wnt signalling pathways [176],

rather than transdifferentiation [177]. In our study, we found that MSCs, isolated from umbilical cord matrix by explant and enzymatic digestion method, differentiated into cardiac cells upon treatment with 5-Azacytidine. Upon treatment with 5-Azacytidine, cells enlarged, assumed elongated appearance and were connected with adjacent cells to become multinucleated [178]. After 10–15 days cells start forming small clusters and by 21 days, thick clump-like structure resembling cardiac myocytes were observed. The differentiated MSCs expressed α-actin, cardiac troponin, smooth muscles actin (SMA), myogenin, and smoothelin but spontaneous beating of cell was not observed [178].

Germ line stem cell (GSC)/Spermatogonial stem cell (SSC) in testis have also been viewed as a potential source of CMs [179, 180]. Under appropriate culture conditions, GSCs can acquire pluripotency to become germ-line pluripotent (GPSC) or multipotent adult germ-line stem cells (maGSC), which can differentiate into cells of all three germ layers including beating CMs [180–182]. The Flk^+ CMs derived from maGSCs showed similar gene expression pattern, responsiveness to isoproterenol and electrical properties as observed with CMs derived from ESCs [179]. Transplantation of maGSCs enhanced left ventricular wall thickness, ejection fraction, systolic velocity and angiogenesis in ischemic heart of mouse [183] and after four week of treatment small number of CMs was observed in ischemic area of mice. The mGSC-derived CMs expressed cardiac-markers such alpha cardiac actinin, conexin-43, cardiac troponin T, and tropomyosin similar to the ESCs derived CMs. The maGSCs can also differentiate into vascular endothelial and smooth muscles cells without tumour formation [184] and therefore, form a good alternative to ESCs for cardiac tissue engineering [185]. In our studies, mouse maGS cells could be successfully differentiated into beating CMs [186]. However, the concentration of SSCs in testis is very low and their isolation protocols in human has not been standardized. Thus, further studies are required to utilize the potential of GSC and SSCs in regenerative medicine and cardiac tissue engineering.

Generation of Cardiac Cells from iPSCs Differentiation of iPSCs into cardiac lineage requires step-wise approaches to mimic the cell signaling process during cardiac development in vivo. Under in vitro conditions, iPSCs can be differentiated into CMs through embryoid body (EB) formation and modulating the Activin/Nodal/TGFβ, Wnt/β-Catenin and BMP signaling pathways, similar to those used for directed differentiation of ESCs. In vitro culture of iPSCs in hanging drops using a serum-free medium leads to the formation of cavity-containing EBs, which can then be treated with differentiating agents such as activin A and bone morphogenetic protein 4 (BMP4) and trichostatin A (TSA) [187]. Cardiac differentiation can also be achieved in a 2D monolayer culture on ECM proteins (e.g. Matrigel™) or feeders cells such as endoderm-like cells (END-2) [188], fibroblasts [189] or OP9 stroma cells [190], which produces activin-A and BMP. Among several developments, inhibitors of Wnt signaling pathway were found to be most favorable for derivation of CMs in combination with activin A and BMP4. However, clinical-scale cardiac differentiation is only possible with scalable platforms such as bioreactors. In addition to allowing the production of large numbers of CMs, bioreactors provide additional advantages such as homogeneity of the vessel contents, ensured by dynamic culture condition, typically provided by a mechanical impeller, as well as monitoring and control of culture variables, such as pH and dissolved oxygen. Cardiac differentiation of iPSCs in suspension has already been described on a number of studies, either growing the cells attached to microcarriers [191] or as aggregates [192–194]. Suspension protocols based on temporal modulation of Wnt signaling can generate over 90% $cTNT^+$ cells [192]. This protocol can be further improved by activation or inhibition of other signaling pathways [193], or by integration with additional steps for CM purification (e.g. through metabolic selection with lactate) and recovery [191]. Halloin et al. [194] developed an integrated protocol based only on the use of low-cost, xeno-free media for both expansion (E8) and cardiac differentiation (CDM3), allowing for the production of about 1×10^6 iPSC-CMs/mL at a purity of at least 90% $cTNT^+$ cells. This is an important step towards GMP production of CMs at a more affordable cost. Alternatively, if the cells are to be used for drug screening or disease modelling, small-scale, high-throughput systems may be desirable, to perform simultaneous testing of various different conditions. Although, to the best of our knowledge, cardiac differentiation of iPSCs has yet to be described on microfluidic systems, these have already been shown to sustain CM culture [195, 196].

The efficiency of cardiac differentiation can further be improved by using small molecules such as inhibitors of TGFβ/activin/nodal signaling (e.g. SB431542; [197]) or p38MAP kinase (e.g. SB203580, [188]) or activation of Wnt/β-Catenin signaling (e.g. CHIR99021, [198]. Methods have also been developed to obtain CMs differentiation by using small molecules alone [199]. Hatani et al. [200] demonstrated an efficient methodology for differentiating iPSCs into CMs using EBs with the help of dorsomorphin and SB431542. Several studies have now shown that the CMs derived from human ESCs and iPSCs do not differ significantly with respect to sarcomeric organization, cardiac gene expression, responsiveness β-adrenergic stimulation, beat rate variability and power law behavior [2, 3, 152]. Consequently, a number of studies have used iPSCs as an alternative to ESCs for CTE [201]. Derivation and differentiation of iPSCs into CMs are shown in (Table 1). A detailed review on various protocols for in vitro differentiation of CMs from stem cells is available elsewhere [202].

The stem cell-derived CMs are occasionally a heterogeneous population of CMs, smooth muscle cells, fibroblasts and endothelial cells and may require an enrichment or purification step. In earlier studies, density gradient centrifugation, use of molecular markers introduced through genetic modification or fluorescence-activated cell sorting (FACS) of mitochondria-rich CMs, identified by intense staining of MitroTracer Red™ dye, were found to be useful in enrichment of stem cell-derived CMs. In later studies, FACS or magnetic assisted cell sorting (MACS) of CMs by using monoclonal antibodies against VCAM1 [203] and SIRPα [204](Dubois et al., 2011) were also found to be suitable for purification of iPSC-derived CMs. In another study, Miki et al. [205] showed that synthetic miRNA switches can also be used for purification of CMs. The microRNA (miR-1, miR-208a and miR-4991-5p) switches were shown to be capable of enriching stem cell-derived CMs. However, these strategies require expensive equipment or are tedious and not possible under clinical settings. Tohyama et al. [206] showed that culture of stem cell-derived CMs in glucose-depleted culture medium supplemented with lactate allowed the preferential growth of CMs over non-CM cells and hence could be a good selection medium for CMs. The same group also showed that glutamine was essential for the growth of undifferentiated stem cells and therefore, glucose- and glutamine-depleted medium supplemented with lactate may be more suitable for purification of CMs [207]. More recently, Zhang et al. [208] have generated human iPSCs with double reporter system, which can delineate different lineages of cardiac cells. However, which such systems are very useful for research purpose, they cannot be used for therapeutics. The FACS/MACS-based cell sorting and lactate-based purification methods are currently favored by most researchers.

The purified populations of stem cell-derived CMs may still be functionally heterogeneous. The CMs in atrium, ventricle and conduction system of heart have different electroconductive property and contractility. Thus, to be able to use for correcting ventricular defect, the iPSC-derived CMs must be terminally differentiated to ventricular type of CMs. Studies have shown that most differentiation protocol for stem cell-derived CMs are biased for ventricular type of CMs and can become atrial- or nodal-like CMs by treatment with retinoic acid [209] or manipulation of BMP and retinoic acid signaling [124], respectively.

Maturation of iPSC-Derived CM A crucial requirement for clinical application of iPSC-derived CMs is the level of maturation of the cells. The iPSC-derived CMs should mimic adult CMs in their electrical conductivity, contractility and response to environmental stimuli, such as neurotransmitters, hormones and pharmacological substances. These maturation events occur in vivo over a period of several years but have to be recapitulated in vitro over a few days or weeks.

There are several important differences between adult CMs and those obtained from iPSC differentiation in vitro, which typically resemble fetal CMs [210–212]. In particular, in terms of morphology, while adult CMs are large, rod-shaped, multinucleated/polyploid cells, iPSC-derived CMs are smaller, rounder and mononucleated. Structurally, iPSC-derived CMs show immature phenotype of myofibrillar organization, sarcomeric element and ill-developed intercalated discs [211, 213, 214]. The iPSC-derived CM presents disorganized sarcomere, in contrast to the highly organized sarcomeres in native CMs. In terms of metabolism, iPSC-derived CM typically rely on glycolysis for energy production, using glucose as the major energy source, whereas native CMs are known to rely on glycolysis during early development but shift to fatty acid β-oxidation upon maturation. Thus, iPSC-CMs seems to be immature in their metabolic profile [212, 215, 216]. Also, CMs with different levels of maturation have differences in terms of electrophysiological properties and calcium handling [211, 212]. On the contrary, The iPSCs- CMs may displayed atrial, nodal or ventricular like action potential [212] and the stimulated force required for beating of iPSC-derived CMs are in the range of 0.08–4 mN/mm2, which is much lower than those of adult CMs (40–80 mN/mm2). Thus, optimization of electrophyiological parameters is necessary for maturation of iPSCs-derived CMs and is experimentally and technically challenging.

Strategies to create a micro-environment capable of mimicking the in vivo CM maturation include delivery of growth factors [217, 218] and hormones[219], co-culturing with other cells [220, 221], providing ECM support [222, 223], aggregation and 3D culture [224], electrical stimulation [225] and long cultivation [217, 226]. In a recent study, Kolanowski et al. [227] developed a microfluidic system that provided topological cues for maturation of iPSCs-derived CMs by cyclic pulsatile hemodynamic forces. In another study, topological cues were provided by culturing the iPSCs-derived CMs on the surface of Polyethylene Terephthalate Textiles to improve the maturation [228]. Incremental improvements in iPSCs-derived CMs maturation may be possible by combining several of the above mentioned approaches [229].

However, the level of maturity required for regenerative medicine applications of iPSC-derived CMs is not yet completely defined. There is an interest in generating CMs which are able to recapitulate in vivo-like characteristics for engraftment, in order to facilitate host-graft cell coupling, to have appropriate contractile performance and to avoid side effects such as arrhythmias, which were reported in transplantation studies [125]. Conversely, since the proliferative ability of these cells decreases with their maturation, adult CMs have been shown not to be able to survive upon transplantation [230]. This suggests a partial but not excessive maturity to be ideal for iPSC-derived CMs. However, this presents another problem. Since CMs undergo a variety of changes

throughout their maturation, there still is not a clear metric to indicate the extent of this process [231]. As such, advances in the production of iPSC-derived CMs that can be usable in regenerative medicine will require not only objective methods of measuring maturity, but also, following establishment of this methods, studies on the ideal level of maturity for these cells.

Application of iPSC-Derived CMs for Cardiac Tissue Engineering

The iPSCs-derived CMs and other cardiac cells can be used to treat the CVDs either by cardiomyoplasty or through development of tissue engineered cardiac constructs. Studies have shown that direct intra-myocardial injection of allogenic iPSCs-derived CMs into the infracted heart of monkey could improve the cardiac function without immune rejection during the 12 weeks of study period [125]. In order the improve the functionality of the injected human iPSCs-CMs, trililneage injection with endothelial and smooth muscle cells has also been attempted along with transplantation of a fibrin patch loaded with insulin growth factor [232]. Such trilineage cell transplantation strategy was found to improve the integration of cells into the host myocardium and improve the ventricular function in pig models. However, a long-term follow up was not done. Most studies have shown that transplantation of iPSCs-CMs, with or without endothelial cells, fibroblast and smooth muscle cells, could integrate into the host myocardium but lead to cardiac arrhythmia upon long-term follow up. It has been suggested that the short-term beneficial effects of transplanted iPSCs-CMs may be due to release of cardio-protective, pro-angiogenic and anti-apoptotic factors by the transplanted cells [136]. Using luciferase and GFP expressing iPSCs-CMs, Ong et al. [233] showed that iPSCs-CMs have low retention rate upon transplantation into mice model and the improved cardiac function was due to their paracrine effects on neo-angiogenesis and reduced apoptosis. Thus, to improve the long-term survival and retention rates of the injected iPSCs-CMs, a number of bioengineering approaches such as encapsulation in biomaterials, cell-sheet engineering, seeding on scaffolds, 3D bioprinting etc. (Fig. 2) have been investigated.

Studies have shown that encapsulation of iPSCs-CMs into biocompatible hydrogels can improve their distribution and retention upon injection into the heart. Several hydrogel forming biomaterials such as fibrin, collagen, chitosan, alginate, agarose, hyaluronic acid, methylcellulose have previously been used for encapsulation of ESC-derived CMs for myocardial injection. Therefore, several researchers have also evaluated the encapsulation of iPSCs in hydrogels and their beneficial effects on ameliorating cardiac dysfunction. Injection of human iPSC-CMs, encapsulated in PEG hydrogel, into infarcted heart improved the muscle content and cardiac function of the rat, although no donor-derive cell was detected after 10 weeks of injection [234]. To improve the mixing of iPSCs-CMs and facilitate their injection into myocardium through a syringe, thermo-sensitive hydrogels have also been used. A hydrogel formed from copolymer of PEC-PCL and conjugated with a collagen-binding peptide remain in solution form at room temperature and can easily be mixed with the human iPSCs [235]. When these iPSCs-CMs loaded solutions were injected into infarcted myocardium of rat, it formed a hydrogel and improved cardiac structure and function. More recently, an injection device was also developed for homogeneous distribution of human iPSCs-CMs spheroids and their injection into myocardium [236]. Encapsulation of human iPSC-CM spheroids in gelatin hydrogels and their epicardial injection into pig heart using the newly developed device showed better distribution and retention of cells than direct injection of iPSCs-CMs.

The iPSCs-derived CMs can also be developed cardiac patches by cell-sheet engineering or using polymer molds. Multi-layered cardiac tissue sheets have been prepared by stacking multiple numbers of CMs sheets grown on culture dishes coated with temperature-responsive poly(N-isopropylacrylamide) [237]. Transplantation of such human iPSCs-CMs derived cell sheets into infracted heart restored the cardiac function for up to 8 weeks in pigs [238]. Similar results were also observed upon transplantation of human iPSCs-CMs derived cell sheets in mice [239]. Unfortunately, transplantation of these iPSCs-CMs derived cell sheets resulted in limited success and the cells either disappeared at a later stage [238] or lead to arrhythmia due to immaturity of the cells [239]. Furthermore, cell sheets were difficult to handle and suture into the myocardium during surgical intervention. In order to overcome these issues, thick 3D myocardial tissue were fabricated from human iPSCs by cell-sheet engineering followed by a polysurgery strategy [240]. The method could also be modified to produce tubular cardiac tissue from iPSCs-CMs by wrapping the triple-layered cell sheets around the inferior vena cava of nude rats [241]. Newer perfusion bioreactors have also been developed by the same group of researchers for in vitro culture of cell-sheet derived 3D tissue. The technology is patented and licensed to CellSeed Inc. for commercialization.

In another approach, cardiac patches have been developed by mixing iPSCs-derived CMs in a hydrogel solution containing fibrinogen, Matrigel™ and thrombin and molding them in a pluronic-coated PDMS molds [242]. The cardiac patches could be scaled to larger dimensions required for clinical transplantation and, upon transplantation into nude mice, could engraft and maintain their electrical function. In yet another approach, cardiac patches have been developed by entrapment of iPSCs-CMs on fibrin gels. These patches could be directly applied on the damaged heart tissue and were found to improve the cardiac function in rat [243] and guinea

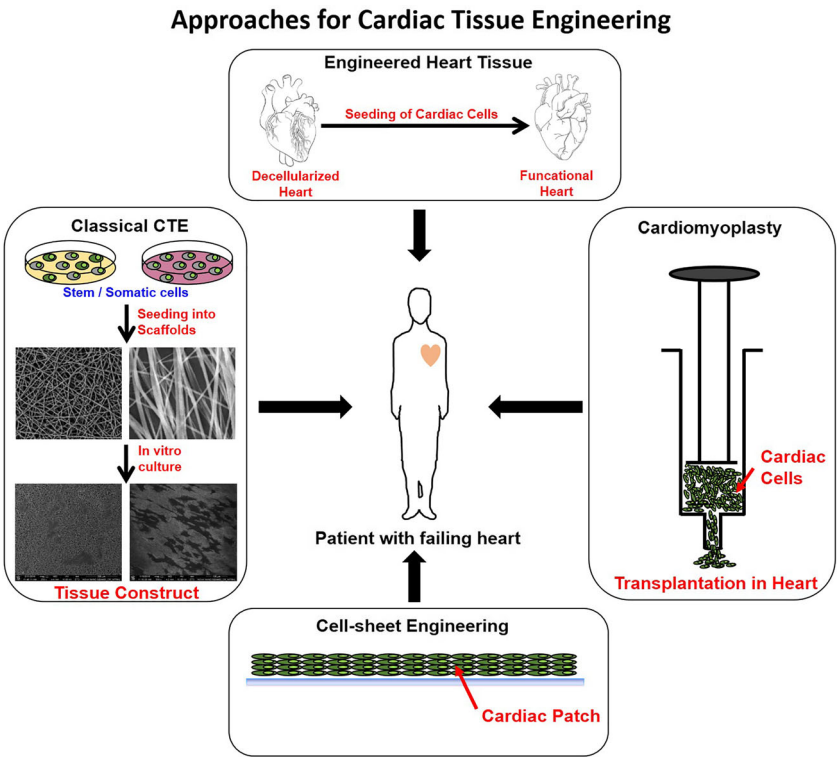

Fig. 2 Various approaches of cardiac tissue engineering using iPSCs-derived cardiomyocytes

pig [244] models of myocardial infarction. However, iPSCs-CMs grown on fibrin sheets were also found to migrate into other tissues such as lung and spleen, as were observed in cardiomyoplasty and therefore, needs improvement.

For surgical application, a cardiac patch should be sufficiently thick (~1 cm) to replace the myocardium and should have sufficient mechanical strength so that it can be held in hand or surgical instrument while applying on the heart. At the same time, the patch should be contractile, vascularized and electro-conductive to integrate into the host myocardium (reviewed in [245]). The "classical" tissue engineering approaches have previously been successful in the development of human tissues such as trachea, blood vessels, skin replacements, bladder replacements and cartilage replacements (reviewed in [246]). Several tissue engineered products are already approved by the FDA and are available commercially. Thus, such approaches have also been tried for the development of 3D cardiac patches. In "classical" cardiac tissue engineering approach, the stem cell-derived CMs are seeded on a variety of biomaterial-based scaffolds and cultivated in bioreactors to develop a 3D cardiac construct. Both fibrous and porous scaffolds as well as hydrogel have been fabricated by using a variety of techniques such as electrospinning, solvent casting, salt leaching, soft lithography and micro-patterning [247]. A number of natural biomaterials such as Collagen I, fibrin, silk fibroin, chitosan, alginate and synthetic biomaterials such as poly-caprolactone (PCL), polyglycolic acid (PGA), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), Poly-L-Lactic acid (PLA), polylactic co-glycolic acid (PLGA) and polyglycerol sebacate (PGS) etc. have been used for the development of scaffolds for cardiac tissue engineering (Table 3) (reviewed in [280]). More recently, marine sponge derived biomaterials have also been investigated for fabricating 3D scaffold for iPSCs-CMs [249]. Interestingly, commonly used printing papers, chromatography papers and nitrocellulose membranes have also been tested as scaffold for development of beating cardiac tissue from iPSCs-CMs [281]. Hybrid of natural and synthetic biomaterials has also been used to improve the mechanical strength, electrical conductivity, hydrophilic nature and anisotropy of the scaffolds [275]. For example, coating of PLGA fibrous scaffolds with polypyrrole (PPy) polymer could provide sufficient topological, mechanical and electrical for differentiation of iPSCs into CMs in both stimulated and unstimulated protocols [272]. In other studies, Matrigel™ (Fig. 3) [264] or gelatin-coating of nanofibrous 3D scaffold of PCL improved their hydrophilic nature for cell attachment and resulted in better iPSCs proliferation and CMs differentiation than those cultured as monolayer on tissue culture dishes [198]. Parallel-alignment of PCL nanofibers in electrospun scaffolds could control the cell orientation and mimic the architecture of the heart tissue (Fig. 4) [264]. Seeding of iPSCs-CMs on aligned nanofibrous scaffold of PLGA and culture on tissue culture plates also showed that CMs can align themselves, establish gap junctions and show improved electrical coupling [273].

Polymerix mixtures and 3D culture were also found to improve the differentiation and functionality of tissue engineered cardiac constructs. The iPSCs-CMs cultured on

Table 3 Various biomaterials used for fabrication of scaffolds for cardiac tissue engineering

Biomaterials	Co-polymer/ Cross-linker	Purpose/ Type of Scaffold	Seeded cell type	References
Natural Biomaterials				
Alginate	PEG monoacrylate-fibrinogen	3D Bio-printed Scaffold	iPSCs-CMs	[248]
Chitin	–	Porous 3D Scaffold	iPSCs-CMs	[249]
Chitosan	Polypyrrole	Porous Scaffold	CMs	[250]
Collagen	Elastin, PCL	Cardiac Patch	CMs	[251]
Decellularized ECM	Gelatin methacrylate	Bio-printed Scaffold	CPCs	[252, 253]
ECM	–	CTE Scaffold	iPSCs-CMs	[254]
Fibrin	Collagen	Encapsulation in Hydrogel	iPSCs-CMs	[255]
Fibrin gel	–	Cardiac Patch	iPSCs-CMs	[243]
Fibrin glue	–	Cardiac Patch	MSCs	[256]
Gelatin	–	Encapsulation in Hydrogel	iPSCs-CMs	[236]
Gelatin (Photoactive)	–	3D Bio-printed Scaffold	iPSCs	[257]
Gelatin methacryloyl	Graphene oxide	Encapsulation in Hydrogel	CMs	[258]
Omenta (Bioink)	–	3D Bio-printed Scaffold	iPSCs-CMs	[259]
PEG	–	Encapsulation in Hydrogel	iPSCs	[234]
PEG-PCL	Collagen binding peptide	Encapsulation in Hydrogel	iPSCs	[235]
Soybean oil	Epoxidized acrylate	Bio-printed Scaffold	MSCs	[260]
Synthetic Biomaterials				
PAA	–	Encapsulation in Hydrogel	CMs	[261]
PCL	Gelatin	Nanofibrous Scaffold	MSCs	[262]
PCL	Polyethylene oxide	Nanofibrous Scaffold	iPSCs	[263]
PCL	–	Nanofibrous Scaffold	iPSC-CMs	[264]
PCL	–	Nanofiber	Cardiac cells	[265]
PGS	Fibronectin, Fibrin, Collagen	Honeycomb Scaffold	CMs, EC, Fibroblasts	[266, 267]
PHA	PCL	Porous Scaffold	CPCs	[268]
PLA	PEG, PANI	Nanofibrous Scaffold	Cardiac fibroblast	[269]
PLGA	Chitosan	Porous	CMs	[270]
PLGA	Gelatin-elastin	Fibrous Scaffolds	CMs	[271]
PLGA	PPy	Nanofibrous Scaffold	iPSCs	[272]
PLGA	–	Nanofibrous Scaffold	iPSC-CMs	[273]
PLLA	ECM proteins	Nanofibrous Scaffold	Cardiac fibroblast	[274]
Polyaniline	Polyetersulfone	Nanofibrous Scaffold	iPSCs	[275]
poly(N-isopropylacrylamide)	–	Cell sheet engineering	CMs	[237]
PU	Laminin or gelatin	Micro-patterned Scaffold	CPCs	[276]
PVA	Alginate	Encapsulation in Hydrogel	CMs	[277]
PVA	PVP	Cardiac Patch	CMs	[278]
PPy	Chitosan	Encapsulation in Hydrogel	CMs	[279]
Tetraaniline-PEGD	Thiolated hyaluronic acid	Encapsulation in Hydrogel	MSCs	[262]

PEG polyethylene glycol, *PLGA* poly(L-glycolic acid), *PCL* poly(ε-caprolactone), *PU* Polyurethanes, *PLLA* Poly (L-lactic acid), *PHA* Polyhydroxyalkanoate, *PAA* propylacrylic acid, *PVA* Polyvinyl alcohol, *PVP* Polyvinyl pyrrolidone, *ECM* Extracellular matrix, *PGS* poly(glycerol sebacate), *PANI* Polyaniline, *PPy* Polypyrrole, *PEG* Polyethylene glycol hydrogel, *PEGD* polyethylene glycol diacrylate, *iPSCs* Induced Pluripotent Stem Cells, *MSCs* Mesenchymal Stem Cells, *CPCs* Cardiac Progenitor Cells, *CMs* Cardiomyocytes, *EC* Endothelial cells

PCL, PEG and carboxylated PCL mixture provided a better culture system for matrix-integrin interactions for improved contractility and maturation [282]. Similar benefits of combinatorial polymer on cell survival and functionality of iPSCs were also observed with natural biomaterials such as collagen, gelatin, laminin and heparin sulfate [283]. In other study, scaffolds fabricated from collagen and fibrin blend was also shown to improve the improved compaction and syncytia formation in iPSCs-CMs [255]. Shadrin et al. [242] developed a cardiac patch by differentiating iPSCs into CMs in a 3D culture, which showed excellent myocardial structure and electromechanical coupling comparable to the adult myocardium.

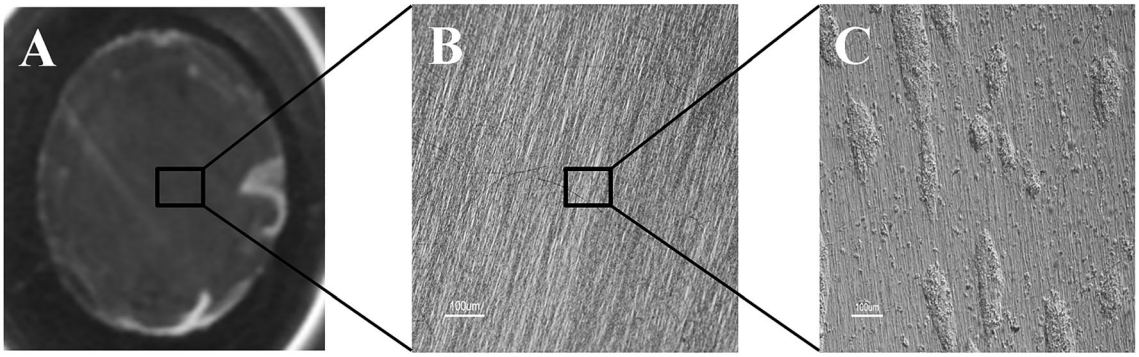

Fig. 3 In vitro culture of human iPSCs on electrospun PCL nanofibres. A and B: Optical microscopy (**a**) and Scanning Electron Microscopy (**b**) of electrospun PCL nanofibers. **c** Colonies of human iPSCs cultured on PCL nanofibers

In vitro culture of iPSCs-CMs on 3D scaffolds (derived from natural ECM of decellularized heart tissue) was also found to have improved CMs functionality and maturation in terms calcium signaling, beat kinetics and response to pharmacological stimuli [254]. In our study, we electrospun PCL nanofibers [264] and cultured iPSCs on Matrigel™- coated aligned

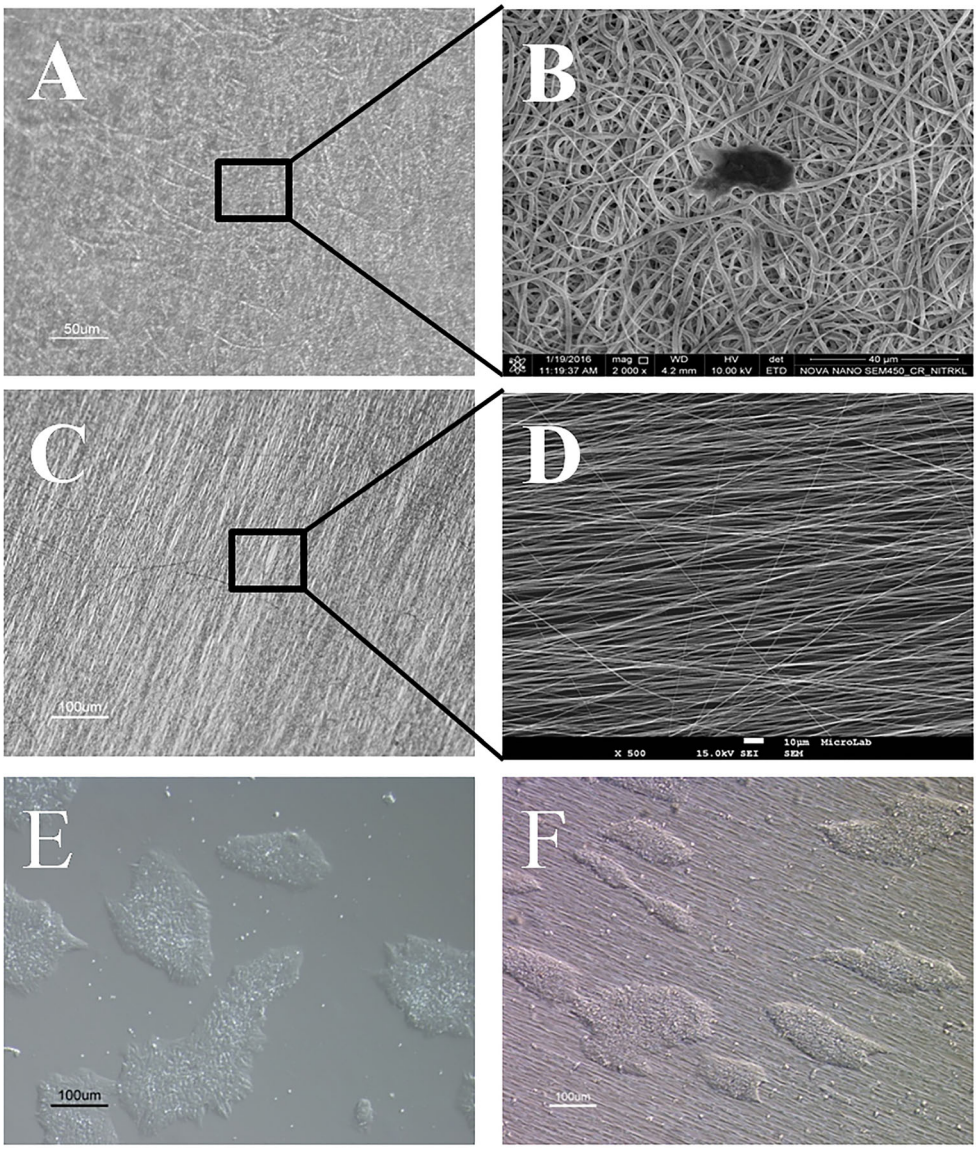

Fig. 4 Human iPSCs cultured on aligned PCL nanofibers coated with Matrigel™. **a** and **b** Random PCL nanofibers; **c** and **d** Aligned PCL nanofibers (**c** and **d**). Growth of human iPSCs on Matrigel™-coated tissue culture plates (**e**) and electrospun aligned PCL nanofibers (**f**)

nanofiber that promoted cell attachment, proliferation and alignment of iPSCs on scaffold (Figs. 3 and 4). A number of strategies such as triculture with fibroblast and endothelial cells, use of pro-angiogenic factors, incorporation of natural ECM and growth factors, incorporation of electro-conductive nanoparticles, exposure of electric or magnetic fields, micro-patterning etc. are being investigated worldwide to improve the post-transplantation functionality of the tissue engineered cardiac patches [247, 255, 275, 284].

Cardiac tissue-like structures can be 3D printed from iPSCs-derived CMs and other cardiac cells by mixing them with bioinks and rapid prototyping. Several bioinks such alginate, collagen I, fibrinogen, Matrigel™, or even decellularized ECM have been tested and found suitable for generating multi-layered 3D cardiac tissues. Maiullari et al. [248] developed a multi-cellular heart tissue from iPSCs-derived CMs by co-printing it with endothelial cells encapsulated in hydrogel strands of alginate and PEG-fibrinogen. In yet another approach, the iPSCs-derived CMs were bioprinted into cardiac patches by using ECM from decellularized omental tissue as bioink [259]. Gao et al., [257] used multiphoton-excited 3D printing technology to fabricate a scaffold from photoactive gelatin polymer, on which human iPSCs could differentiate into CMs, endothelial cells and smooth muscle cells to form a cardiac patch. Transplantation of these iPSCs-derived cardiac patches into mice model showed significant improvement in cardiac function [257]. The 3D printing technologies provide an advantage over other methods in that it can recapitulate the native heart anatomy and vessel architecture through mathematical modeling for prototyping. Studies have shown that iPSCs-derived CMs could also be developed as cell spheroids, along with endothelial cells and dermal fibroblasts, and 3D printed to form a tubular cardiac constructs to function as a cardiac pump [285]. In yet another approach, iPSCs-derived CMs were co-cultured with fibroblast and ECM-derived collagen on a pre-printed holder to generate engineered heart muscle (EHM), which was scalable in a GMP system [286].

The iPSCs-CMs have also been used for generation of engineered heart tissue (EHT) by decellularization of cadeveric heart using detergents and re-seeding with iPSCs-CMs, fibroblasts and endothelial cells. Decellularized heart tissue maintains the natural architecture of native heart and ECM and therefore, upon re-seeding promotes the proliferation of human iPSCs and provides cues for tissue-specific differentiation [287]. Such strategies have successful in the development of beating heart in mouse model and created great hope in regenerative medicine. It was seen that re-population of decellularized heart with iPSCs-derived cardiovascular progenitor cells lead to proliferation and differentiation of seeded cells into CMs, smooth muscle cells and endothelial cells and beating heart could be obtained. Unfortunately, the EHT was insufficient for pumping blood due to slow electric conduction caused by lack of gap-junctions and limited number of CMs. Nevertheless, iPSCs-CMs derived EHT has created lot of hope for future development.

Current Limitations and Challenges in the Derivation and Applications of iPSCs

Limitations and Challenges in Cellular Reprogramming, Derivation and Culture of iPSCs

Cellular reprogramming possesses several challenges in the field of regenerative medicine such as incomplete reprogramming, clonal diversity, genomic integration of the reprogramming factors, lower efficiency and longer time duration required reprogramming the cells. Despite tremendous research, the precise mechanism behind reprogramming and functionality of reprogrammed iPSCs has remained elusive. In many cases, incomplete reprogramming of iPSCs was reported to occur wherein iPSCs showed similarities with ESCs in their morphology and phenotype but exhibited aberration in chromatin at transcription level [56, 288, 289]. Thus, in many cases, transplantation of iPSCs lead to development of teratoma and immune-rejection in mice models [290]. In some cases, even the transplantation of fully-reprogrammed iPSCs did not overrule the development of teratoma, as was seen with ESCs. Being patient-specific cells, it was expected that the iPSCs will have matching MHC haplotype with the donor and therefore, it will increase the immunological compatibility and eliminate the need for post-transplantation immune-suppression therapy. However, in a recent study, MHC matching failed to prevent the long-term rejection of iPSCs-derived neurons (in brain) in non-human primates [291]. Apparently, during reprogramming event, the antigenic properties of the somatic cells are also reprogrammed [292]. Nevertheless, a number of other studies have shown that fully differentiated autologous iPSCs-derivatives are well tolerated by the host without using immune suppression regimen [292, 293]. Some studies have also shown that generation of large number of MHC homozygous iPSCs could serve as a source of allogenic cells for transplantation [294]. However, further research is required in this direction.

Another challenge in iPSC research has been the diversity in the clonal characteristics of iPSCs due to epigenetic memory or genetic instability [295]. In many cases, iPSCs were shown to have DNA methylation and histone acetylation signatures similar to their parental cells [296, 297] or have an aberrant DNA methylation [56]. The residual somatic cell memory in iPSCs results in their slow proliferation and differentiation ability. Such variation also affects the functionality of the iPSCs and increases the risk of immunogenicity and tumorigenicity of the cells. Consequently, clonal diversity

restricts the direct application of iPSCs in cell therapy, disease modelling and personalized medicine. In a human clinical trial, Mandai et al. [105] observed that iPSCs-derived retinal epithelial cells from one of the patients passed the in vitro tumorigenesis test but was not usable for transplantation in human due to presence of three deletions observed in the DNA sequences. Variation in the success of iPSCs-derived cells may also occur due to variability in the differentiation of individual cells.

A yet another challenge is the integration of DNA in the genome of the somatic cells, which may lead to unpredictable effects in the reprogrammed cells. Thus, attempts have been made to develop strategies for excision of integrated genes or use non-integrating vectors. However, such strategies have resulted in lower efficiency of reprogramming (0.001–0.01%). To overcome the problem of low reprogramming efficiency, new approaches such as use of epigenetic modifiers and/or small chemical compounds are being investigated.

The derivation of iPSCs and their characterization and differentiation are expensive and time-consuming process requiring ~$10,000 - $25,000 per cell line and at least ~6–9 months of time in an established laboratory. Generating a clinical-grade iPSCs may cost up to $800,000 [107]. Further, the setting up iPSCs facility involves exorbitantly high cost for infrastructure and intensive capital investment in clinical trials. Most developing and undeveloped countries have ill-developed research infrastructure for stem cell therapies. Therefore, extensive research is required to expedite the process of iPSCs derivation and reduce the cost of their maintenance, differentiation and transplantation. Further, the technology is still in its developing phase and a consistent regulation by various regulatory agencies is still not available. The current iPSCs culture procedures in human do not meet the stringent standards of GMP for their scalable culture. The protocols for iPSC isolation and CM differentiation differ from lab-to-lab and can contribute to variability and reproducibility of overall outcomes of the iPSC-based cell therapy [298, 299]. Differences in the iPSC isolation and differentiation methods, including the cell culture media and expertise of the technician, dramatically impact the colony morphology of iPSCs, cellular homogeneity, differentiation efficiency and their functionality in terms of gene and protein expression [300, 301]. These variations in each step of the iPSC isolation, culture and differentiation may accumulate and can result in altogether different outcome than expected. Thus, rigorous quality control measures, standardized protocols and ‘gold standard’ control iPSC lines are needed to ensure that the newly isolated iPSCs meet the standard. GMP compliant raw materials and protocol, single cell assay technologies and big data analytics of transcriptome and proteome data may help identifying and minimizing the sources of variability.

Limitations and Challenges with In Vivo Survival and Retention of iPSC-CMs

The iPSC-CM based cell therapy by cardiomyoplasty suffers from low cell retention, variable bio-distribution and poor survival of implanted cells [302, 303]. The intramyocardially injected iPSCs can undergo massive displacement from the site of injection and accumulate in the lung [304], corroborated by their venous drainage and rapid exit through injection channels during heart contraction. The efficiency of iPSC-derived CM cardiomyoplasty may further reduce due to acute inflammation and injury caused by injection procedure and blockage of the microcirculatory network by the injected cells. The local ischemic may result in the low survival of the transplanted cells and thus, compromises their functional efficiency. Inefficient functional integration into host tissue has also been reported to occur due to disorganized communication between transplanted CMs and host ECM or due to poor electromechanical synsytium formation between iPSC-derived CM and native CMs in the heart. In one study, it was shown that overexpression of N-cadherin, which is critical for CM adhesion, into iPSC-derived CMs not only improved their functional integration but also augmented their reparative ability in the failing hearts of mouse MI model [305].

Several studies on cardiomyoplasty of iPSC-derived CM reported functional improvements in CVDs but the functional benefits were either transient or did not result in the formation of structure myocardium [306]. Some studies even suggested that the beneficial effects of iPSC-derived CMs may actually be due to their cytokine-mediated paracrine effects such as anti-inflammation, anti-apoptosis, anti-fibrosis and pro-angiogenesis action mediated by cytokines such as TNFα, PIGF1, GCSF, VEGF, SDF1α, VCAM1 and PAI1 [136]. The retention, survival, bio-distribution and engraftment of iPSC-derived CMs may be improved by use of biomatrices or scaffold-based 3D cardiac constructs [302]. Cardiac tissue constructs, seeded with high concentration of iPSC-derived CMs with improved conductive properties through incorporation of electrocondutive materials (e.g. carbon nanofibers, PPy etc.) into scaffolds are thus, being investigated [272, 304]. Currently, the biggest limitation in cardiac tissue engineering lies in delivering oxygen to all cells within the construct, which limits their engraftment and survival after transplantation. In the native myocardium, capillaries of ~7 μm diameter are spaced at distances of ~20 μm, with each myofiber located between two capillaries, to meet the high oxygen demands of metabolically active CMs [307]. Angiogenesis occurs by proliferation of endothelial cells under the influence of VEGF, PDGF and FGF2 and the vascular network is stabilized by smooth muscle cells and pericytes directed by PDGF-β and its receptor (PDGFR-β), angiopoietins and their receptors (Tie-1 and -2) [308]. Thus, several approaches such as tri-

culture of CMs with endothelial cells and fibroblasts, supplementation of culture medium with synthetic oxygen carriers (e.g. perfluorocarbon) and incorporation of proangiogenic factors and peptides (e.g. VEGF, HGF, antiopoietin-1, MCP1) etc. are being investigated to promote neovascularisation or de novo vasculogenesis during cardiomyogenesis but remains a daunting task to be accomplished [309].

Conclusion

The iPSCs technology is a rapidly evolving field, offering a wide range of applications in cardiac regenerative medicine through cell-based therapy, scaffold-free cardiac patches and cardiac tissue engineering. It also offers opportunities to isolated iPSCs-CMs from patients affected with genetic diseases for the purpose of correcting the underlying genetic mutations or understanding their pathophysiology. Newer small molecules are being identified for improving the nuclear reprogramming of somatic cells and their differentiation into CMs. New culture methods and transplantations devices are also being developed for their efficient clinical applications. It has also become possible to directly reprogram the somatic cells into CMs without an intermediately pluripotent state, which further opens the possibility of direct in vivo reprogramming for treatment of end-stage cardiac diseases. However, iPSCs technology is also uncovering a number of newer challenges such as clonal diversity, immno-rejection and tumorigenesis which were actually in genesis of iPSCs research itself. Finally, deriving clinical grade iPSCs-CMs following GMPs, scalability, high cost and regulatory compliances for clinical use of iPSCs are to be addressed before the bed-side availability of the technology.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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