

REVIEW

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Toward the latest advancements in cardiac regeneration using induced pluripotent stem cells (iPSCs) technology: approaches and challenges

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Abstract

A novel approach to treating heart failures was developed with the introduction of iPSC technology. Knowledge in regenerative medicine, developmental biology, and the identification of illnesses at the cellular level has exploded since the discovery of iPSCs. One of the most frequent causes of mortality associated with cardiovascular disease is the loss of cardiomyocytes (CMs), followed by heart failure. A possible treatment for heart failure involves restoring cardiac function and replacing damaged tissue with healthy, regenerated CMs. Significant strides in stem cell biology during the last ten years have transformed the in vitro study of human illness and enhanced our knowledge of the molecular pathways underlying human disease, regenerative medicine, and drug development. We seek to examine iPSC advancements in disease modeling, drug discovery, iPSC-Based cell treatments, and purification methods in this article.

Keywords Cardiac regeneration, iPSC, Stem cell-based therapy, Regenerative medicine, Signaling pathways

Background

Stem cell biology and regenerative medicine are new branches of sciences. For this reason, there has been a great interest in stem cells in the past decades due to their therapeutic application in regenerative medicine [1–3] Fathi, Farahzadi, et al.,. Although cardiac progenitor cells have been described to exist within the myocardium, the myocardium has no intrinsic regenerative capacity because of a lack of postnatal mitosis. For this reason, many studies have been investigated on all types of stem cells and even extracellular vesicles derived from them [4].

Takahashi and Yamanaka started the project that would eventually become iPSC in 2006. By introducing the four transcriptional factors OCT3/4, c-MYC, SOX2, and KLF4 into mouse fibroblasts, they created mouse iPSCs [5]. iPSCs are somatic cells that have been genetically

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modified to resemble embryonic stem cells (ESCs) by expressing certain transcription factors [6]. There are various ways to induce pluripotency in cells, including nuclear transfer, exposure to factors expressed in pluripotent cells, and overexpression of certain transcription factors [7]. Human induced pluripotent stem cells (hiPSCs) and their derivatives provide a suitable supply of human cells and have been used in a number of scientific domains, including the modeling of illness and the identification of new drugs [8]. Worldwide, cardiovascular disease caused 17,921,000 fatalities in 2017. Some cardiac conditions, including long QT syndrome (LQTS), are linked to innate factors, such as mutations in the gene encoding a sodium ion channel, which lead to arrhythmias. Another condition called myocardial infarction (MI) causes the oxygen level in the cardiac muscles to fall, and instead of the CMs regenerating, the human heart forms an extensive scar that reduces blood flow and contractility [9]. Stem cells are one of the recommendations for treating the post-infarction heart since adult mammalian hearts do not have a large ability for regeneration in various injuries. Only cardiac progenitor cells seem to have the capacity to develop into highly functioning CMs and it is exceedingly difficult to acquire enough cardiac progenitor cells from a patient's biopsy [10, 11]. Cellular metabolism plays a significant role in the maturation of iPSC-derived cardiac tissue models and the formation of CMs, and alterations in cellular metabolism lead to cardiac pathogenesis. iPSC-derived CMs (iPSC-CMs) vary from mature human CMs in several ways, including contractile function, electrophysiology, metabolism, and structure. iPSC-CMs resemble fetal CMs in several respects, such as the absence of pathways relevant to the adult phenotype and particular gene expression. In iPSC-CMs, aerobic glycolysis is the primary source of ATP generation, with limited assistance from oxidative phosphorylation. The use of iPSCs in clinic may be limited by these various metabolic characteristics. Due to these problems, researchers have created a number of methods for maturing iPSC-CMs, such as 3D culture, electrical pacing, extended culture, or adding hormones and fatty acids to the media used to grow cells. These methods improve the functional and structural maturity of iPSC-CMs [12]. According to studies, utilizing the aforementioned maturation techniques may boost the expression of genes in iPSC-CMs that control cardiac metabolism, including CD36, ACAT1, PDK4, ATP5, LPL, PPARA, and DGAT1. Additionally, fatty acid supplementation and extended culture were used to enhance the expression levels of the genes ESRRA and PPARGC1A, which are related to mitochondrial biogenesis, and ACADVL, PPARD, and SCD, which are related to fatty acid oxidation. PGK1, ALDOA, LDHA, HK1 and HK2 are among the glycolysis-related genes whose expression levels may

be decreased by extended culture and fatty acid supplementation [13, 14].

Differentiation of iPSCs into cardiomyocytes

Differentiating CMs from hiPSCs is a crucial step in producing artificial cardiac muscle cells and tissues. Engineering efforts to purify the hiPSC-CMs and various techniques to stimulate hiPSC-CM development are investigated. In reality, ESCs and iPSCs develop in a similar manner and may become any kind of specialized human cell [15, 16]. Although there may be phenotypic differences between CMs produced from ESCs and iPSCs due to variations in gene expression. Based on *in vivo* heart development, researchers have created a variety of techniques to differentiate PSCs into CMs during the past two decades [17, 18]. Human ESCs (hESCs) may generate CMs on their own or with the aid of growth factor-directed methods. It is possible to enhance the production of CMs by treating hESCs with a variety of growth factors, including Vascular endothelial growth factor (VEGF), bone morphogenetic protein 4 (BMP-4), activin A, basic fibroblast growth factor (bFGF) and the peptide Wnt inhibitor dickkopf homolog 1 (DKK-1). Due to their resemblance to hESCs, the majority of these growth factors aid in the differentiation of iPSCs [19]. However, due to the fact that cardiac differentiation effectiveness often depends on cell line, these methods' effectiveness in human ES cell lines varies. Additionally, using several growth factors is expensive. Due to their accessibility, potency, and capacity to penetrate cells, small molecules may be used to control the signaling pathways involved in the self-renewal and lineage differentiation of stem cells.

Signaling pathways involved in differentiation

BMP-4 and Wnt/ β -catenin signaling pathways were shown by Ren et al. to be able to trigger cardiac differentiation in hiPSCs. They found that pre-treatment with BMP-4 of hESCs and iPSCs, followed by post-treatment with a small molecule Wnt inhibitor, significantly boosts the formation of CMs with normal electrophysiological function and pharmacological responsiveness [20]. Wnt proteins have a role in embryonic development and control a variety of cellular functions in the adult organism, including cell division and proliferation, gene transcription, polarity, and migration. Wnt proteins also have a role in the development and differentiation of the heart. These proteins have the ability to activate both canonical (β -catenin dependent) and non-canonical (β -catenin independent) Wnt signaling, which are intracellular signaling pathways. Mesoderm development depends on canonical Wnt/ β -catenin signaling, which is weak during cardiac specification. Additionally, the proliferation of CMs depends on Wnt/ β -catenin signaling [21–23].

Mazzotta et al. performed a research by using small-molecule inhibitors to analyze functions of canonical/non-canonical Wnt signaling in hESCs and they suggested that canonical signaling regulate mesoderm induction and non-canonical signaling modulate cardiovascular development [24]. Wnt family consists of 19 various members in mammals. Activation of Wnt/ β -catenin signaling initiates with interaction between Wnt ligands and Frizzled receptor which is seven-pass transmembrane receptor and its co-receptor, LRP5/6 (low-density lipoprotein receptor-related protein). Destruction complex consists of 2 kinases, glycogen synthase kinase 3 (GSK3 β) and casein kinase 1 (CK1), AXIN protein and the tumor suppressor gene product adenomatous polyposis coli (APC) [25, 26]. Activation of Wnt/ β -catenin signaling is depicted in Fig. 1 [25]. There are some other signaling pathways that adjust cardiac maturation. Activation of serine/threonine-protein kinase Akt, results in growth, differentiation and metabolism. In addition, PI3K/Akt signaling pathway is essential and is activated during CM

differentiation. Inhibition of PI3K may lead to downregulation of Nkx-2.5 and GATA-4 which are cardiac marker genes. It also has been indicated that inhibition of the PI3K/Akt signaling pathway in the early cardiomyogenesis cause the suppression of Wnt/ β -catenin signaling (Fathi, Valipour, et al., [27]). Yang et al. could differentiate CM cells from hESC embryoid bodies. They added different factors (activin A, BMP4, bFGF, VEGF, and DKK1) to serum-free media of hESC and generate KDR^{low}/C-KIT^{neg} populations that have cardiac, endothelial, and vascular smooth muscle potential. By plating in monolayer culture, these KDR^{low}/C-KIT^{neg} cells differentiate and generate populations consisting of greater than 50% contracting CMs. These populations derived from the KDR^{low}/C-KIT^{neg} give rise to colonies that contain three lineages (CMs, endothelial cells and vascular smooth muscle cells) when plated in methyl cellulose cultures [28].

As was previously indicated, early embryonic differentiation requiring Wnt for mesodermal specification;

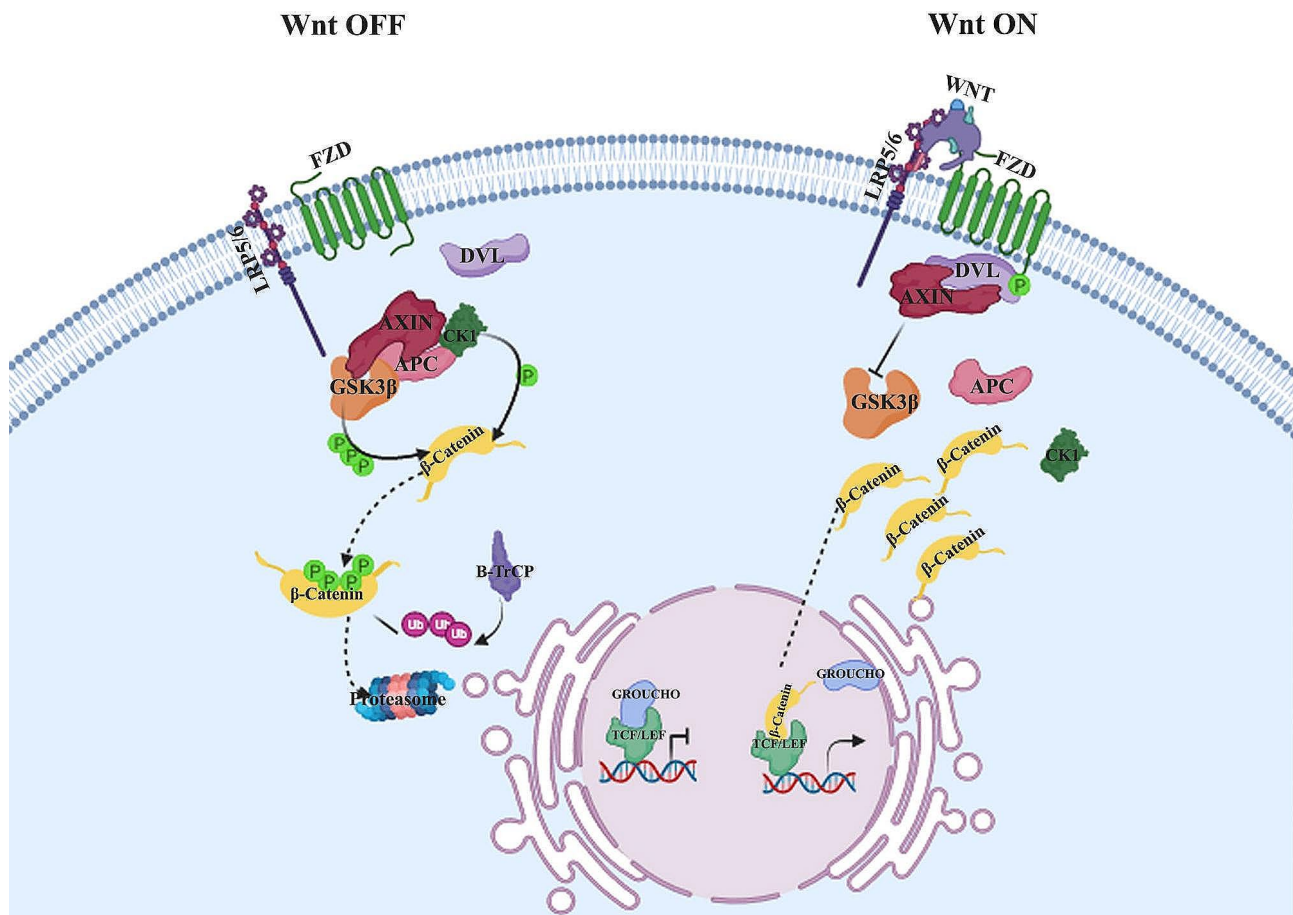


Fig. 1 The graphical representation of Wnt/ β -Catenin-dependent signaling. **(A)** When Wnt ligand is absent (off state), β catenin is phosphorylated by kinases (GSK3 and CK1) of the Destruction complex. Then, phosphorylated β -catenin ubiquitinate to be targeted for degradation in the proteasome. **(B)** Binding of Wnt ligand to Frizzled receptor and LRP5/6 (on state), allows the Destruction complex to decompose which induce the stabilization of β -catenin. β -catenin accumulates in cytoplasm and then is transferred to the nucleus and starts gene transcription. This figure adapted from Fig. 1 of reference number 25 [25]

however, later Wnt signaling prevents cardiac specification, hence suppression of Wnt signaling may be required for the development of CMs [29]. This model is shown in Fig. 2 [30]. Using extrinsic stimuli like BMP4 and CHIR, WNT inhibition with XAV939 or IWR1, and enrichment of CMs by providing lactate as an energy source within 15 days, Kadari et al. were able to produce CMs from a variety of hiPSC lines. During the first two days of the induction phase, basal media containing insulin were administered. They demonstrate how the cells seem to be under a lot of stress when utilizing basal media without insulin right away. In order to reduce cell death and its detrimental effects on cardiac specification, they thus opted to maintain insulin for the first two days and stop using it during the specification phase [31]. According to research by Aguado et al., cells with high expression of the shelterin complex protein TRF1 and relatively long telomeres develop into CMs more quickly and effectively than cells with low expression. The differentiation of CMs produced from iPSCs with long telomeres was also enhanced by the use of ascorbic acid, but the differentiation of CMs produced from iPSCs with short telomeres was unaffected [32]. Ascorbic acid, growth hormones including BMP4 and FGF2, and small compounds that target the canonical Wnt signaling were shown to work well together to improve the differentiation efficiency and maturity of iPSC-CMs in a separate study by Yassa et al. [32].

In addition to mentioned signaling pathways, TGF β family signaling is another one that involves in cardiac regeneration. TGF β family have crucial roles in differentiation, apoptosis and proliferation. In addition, TGF β has a role in various diseases such as cardiac hypertrophy and cardiac abnormality. This family include BMPs, TGF β isoforms, inhibins/activins and growth and differentiation factors. As Dronkers et al. stated in their review article, TGF β family can be split into two clusters of TGF β and BMP. BMPs bind to receptor and cause to phosphorylation of SMAD1/5/8, while TGF β isoforms function with activins toward phosphorylation of SMAD2/3. SMAD4 binds to this phosphorylated SMADS, creating a complex and then enters the nucleus which turn on

TGF β target genes. TGF β family complex has 3 types of receptors include ALK 1–7 (activin-like kinase or type I receptors), ActR2A, ActR2B, TGFBR2 and BMPRII (type II) and TGFBR3 (type III receptors). TGF β can also send signals through other pathways so called “SMAD-independent pathways” such as Rho, PI3K/Akt and MAPK [33, 34]. TGF β family signaling is shown in Fig. 3 [33].

Drug discovery and disease modeling

In medication research, disease modeling, and even patient-specific cellular disease models, hiPSCs have become a popular platform. By enabling the generation of an endless supply of patient-specific human cells, iPSCs may revolutionize cardiology. Any human body cell type may be differentiated from somatic cells via iPSC reprogramming [35, 36]. From 2009 to 2018, it cost around USD 985 million to develop a new drug and release it into the market. Even though rigorous testing was conducted throughout the research period, 90% of medications fail to pass clinical trials. This is mostly due to the employment of unrelated cell culture techniques or inaccurate animal models that do not accurately mimic the human system. Because they offer human cell resources that are suited for research objectives, hiPSCs and their derivatives have been exploited in a broad range of scientific domains [8, 37, 38]. The iPSC can be developed into CMs while maintaining its genetic information and shares a genetic background with the initial reprogrammed cells [17]. The modeling of patient-specific iPSCs, a powerful tool for investigating illnesses and finding new drugs, as shown in Fig. 4 recently [39, 40]. The official and necessary procedure for releasing a new medicine into the market is overseen by the European Medicines Agency (EMA) in Europe and the Food and medicine Administration (FDA) in the USA, as Ovics et al. explained in a review study. Chemical and biological research, pre-clinical development, clinical trials, and one post-marketing phase are the three pre-marketing stages that make up drug development [41]. A wide range of cardiovascular models, such as LQTS, dilated cardiomyopathy, hypertrophic cardiomyopathy and arrhythmogenic right

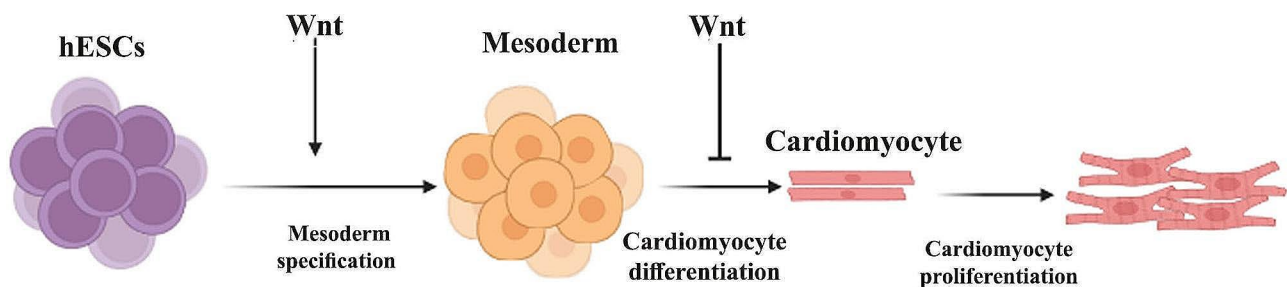


Fig. 2 The roles of Wnt/ β -catenin signaling in development of cardiomyocytes. Activation of this pathway is essential for mesodermal specification and suppression of this pathway is required during cardiomyocytes differentiation. This figure adapted from Fig. 2 of reference number 30 [30]

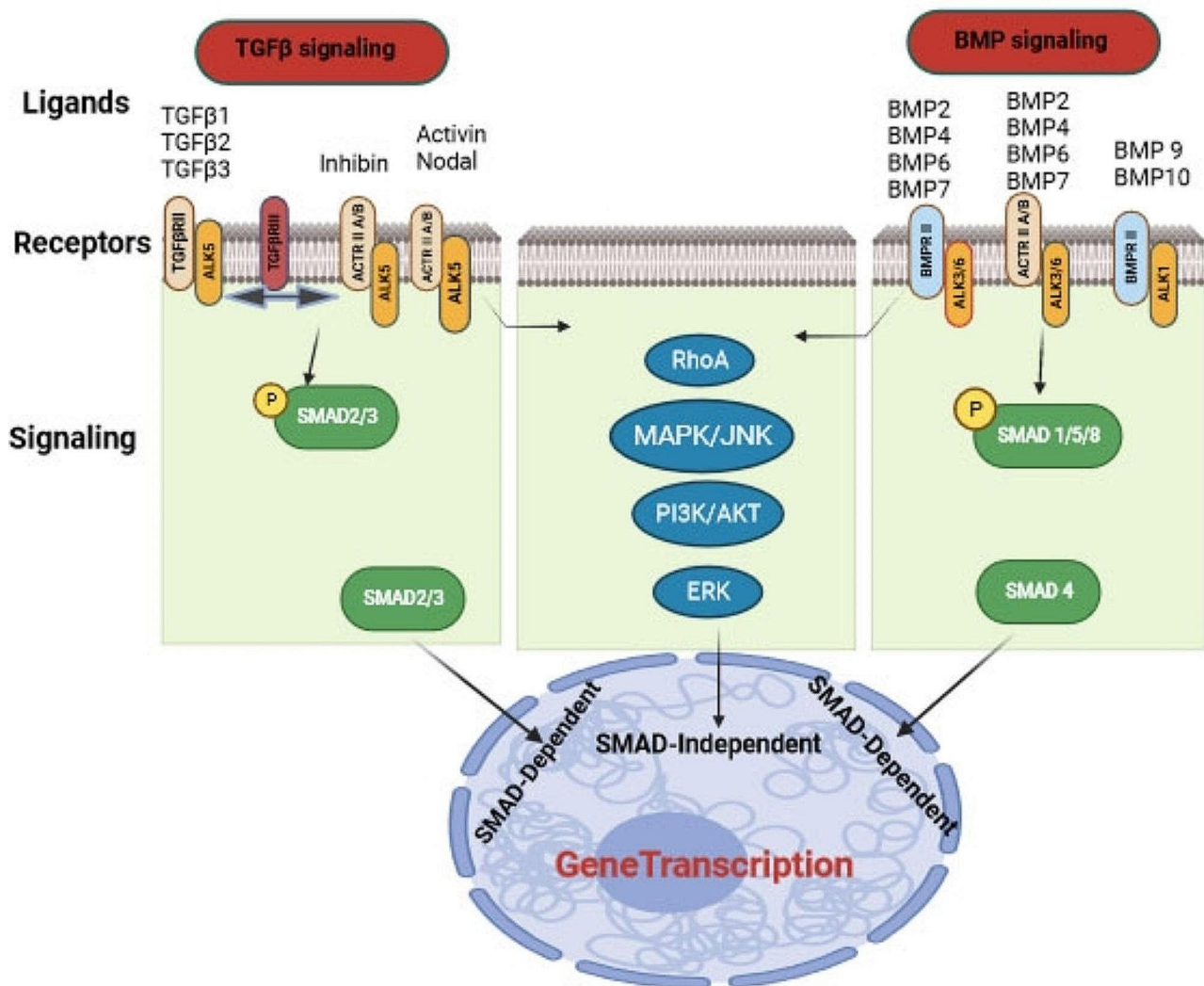


Fig. 3 This figure depicts two clusters of TGFβ family signaling, BMP signaling and TGFβ signaling. BMPs (on the right site) bind to BMP type receptor or activin IIA or IIB which send signals via ALK1 or ALK 2, 3 ,6. As a result, SMAD 1, 5, 8 are phosphorylated and by joining SMAD4, this new made complex translocates to the nucleus. On left site, binding of TGFβ 1, 2, 3 to TGFβ receptor II and binding of Nodal, activating and inhibin to activating receptor IIA, respectively activate signaling through ALK5 and ALK4. This signaling leads to phosphorylation of SMAD 2, 3 and then SMAD4 binds to phosphorylated SMAD 2, 3. This complex enters the nucleus and cause gene transcription. This figure adapted from Fig. 1 of reference number 33 [33]

ventricular cardiomyopathy, were derived from patients’ iPSCs [8].

Long QT syndromes

A dysfunctional ion channel is a genetic condition known as LQTS. LQTS is often defined by a lengthening of the QT interval on the ECG, which may result in cardiac arrest and is typically brought on by mental or emotional strain [42–44]. It has been discovered that diseases are brought on by mutations in the genes encoding for cardiac ion channels, ion channel subunits, or proteins that impact how an ion channel functions. 17 genes have been linked to congenital LQTS, according to research. Less than 5% of clinically confirmed instances of congenital LQTS have been linked to mutations in genes other

than KCNQ1, KCHN2, and SCN5A, which account for more than 75% of cases [45]. Because of the underlying chromosomal abnormality, the LQTS subtype has been divided into many subgroups. LQTS type 1, type 2, and type 3 have distinct connections between genotypes and phenotypes that have been documented.

The potassium channel gene KCNQ1 on chromosome 11 is the basis for LQT1, while the KCNH2 gene on chromosome 7 is the basis for LQT2. Gain-of-function mutations in SCN5A on chromosome 3 are the basis for LQTS type 3. The action potential is prolonged as a result of an increase in the myocardial sodium current (INa), which occurs during the plateau period [46–48].

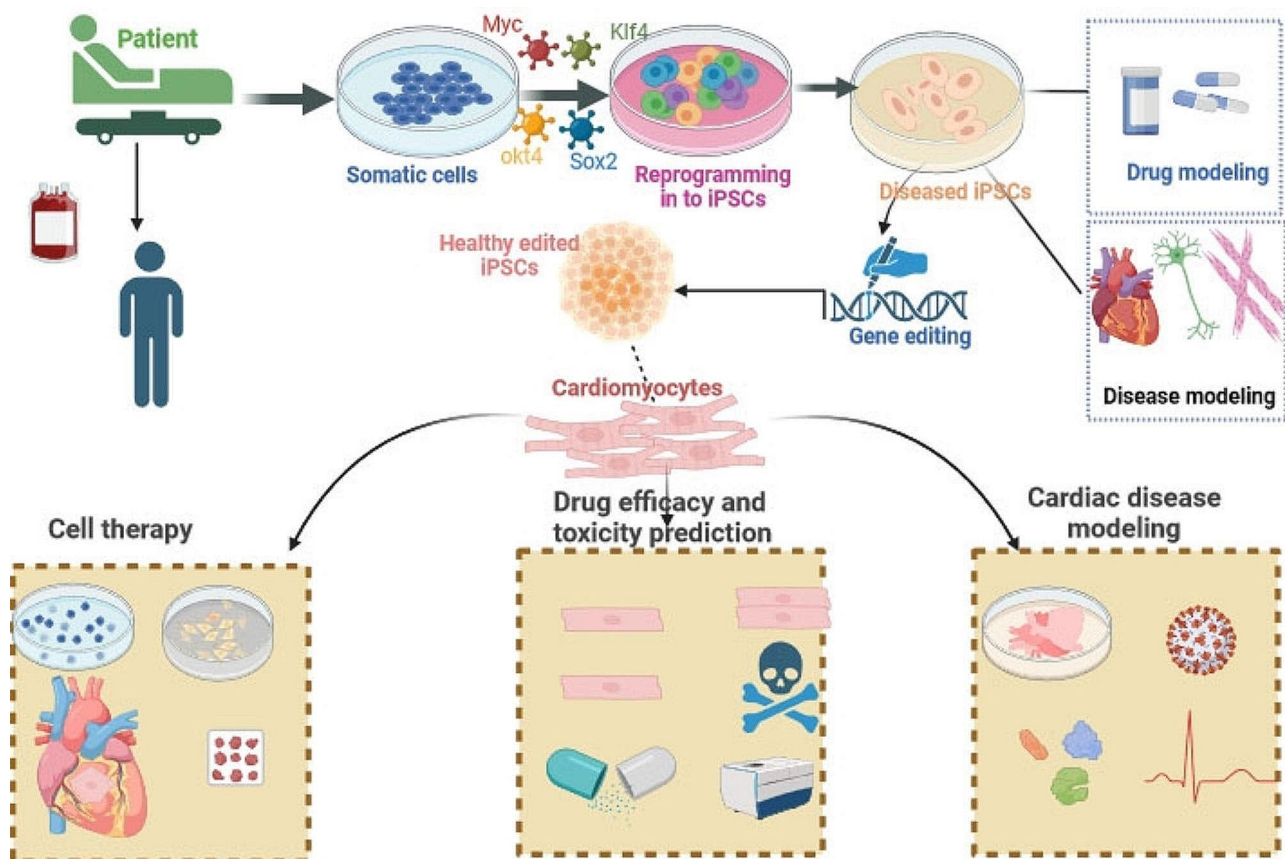


Fig. 4 Various applications of iPSC-derived cardiomyocytes

Role of iPSC research

The first LQTS iPSC-CM model was released by Moretti et al. and a number have since been published. From the dermal fibroblasts of two patients with LQT1 who had the R190Q mutation in the *KCNQ1* gene, they created iPSC-CMs. Individual cells were identified as having a “ventricular,” “atrial,” or “nodal” phenotype based on the expression of cell-type-specific markers and recordings of action potentials in single cells. The “ventricular” and “atrial” action potential length was considerably extended in the mutant cells. Additionally, the IKs current was reduced by 70 to 80% in cells with the R190Q-*KCNQ1* mutation, and the activation and deactivation characteristics of the channel were altered. Additionally, they demonstrated that beta-blockade decreased the phenotypic of mutant cells having an enhanced vulnerability to catecholamine-induced tachyarrhythmia [49]. Wang et al. used genetic modification techniques to overexpress the dominant negative gene mutation of *KCNQ1* in order to create an iPSC-based model for the LQTS1 disease. They have shown that as compared to unedited control cells, editing iPSCs generated from cardiomyocytes display LQTS features and a lengthened action potential time. They demonstrated that the addition of nifedipine or pinacidil decreased the iPSC cardiomyocytes’ action

potential length, strengthening the safety and effectiveness of isogenic iPSC lines in drug development [50]. In a different work, Garg et al. generated LQT2 iPSC-CM from T983I-*KCNH2* variant peripheral blood mononuclear cells. In mutant cells, patch-clamp tests showed that action potential duration lengthening decreased the rapidly activating delayed rectifier K⁺ current (IKr). In order to do this, they utilized a strong IKr activator called ICA-105,574, which improved IKr density and standardized action potential length in mutant cells [51]. This raised the possibility of medication for people with LQT2-specific disorders. Mesquita et al. developed iPSC- LQTS2 from two patients with c.1600 C>T in *KCNH2*, p.R534C in hERG mutation and inserted the same mutation in a control iPSC line, and then differentiated into CMs. Electrophysiology tests revealed that the mutant iPSC line’s action potential duration was much longer than that of the control cell line. Action potential lengthening was seen in the control cell line after treatment with E4031, an IKr inhibitor, whereas IKr was reduced in LQT2-iPSC [52]. In order to create a collection of isogenic iPSC lines with different *KCNH2* mutations that produce LQT2, Brandao et al. (2020) genetically altered an iPSC line using CRISPR/Cas9 gene editing. They indicated that genetically modified hiPSC-CMs can have electrophysiological

differences related to the KCNH2 mutation. Thus, it can be helpful in improving the diagnosis, prognosis and the classification of patients with congenital LQTS [53]. Wang et al. designed iPSC line of LQT patient harboring two mutations: a heterozygous KCNQ1 c.656G>A and a heterozygous TRPM4 c.479 C>T. They found that the QT interval in LQT patients was reduced by lidocaine, a sodium channel blocker, and verapamil, a calcium channel blocker. Verapamil dramatically decreased the number of ICD discharges. The relative decrease in QT interval after verapamil administration was similarly shown to be greater for LQT cardiomyocytes when compared to lidocaine [54].

Arrhythmogenic right ventricular cardiomyopathy

Inherent heart muscle disease called arrhythmogenic right ventricular cardiomyopathy (ARVC) increases the risk of ventricular arrhythmias and sudden cardiac death, especially in young people and athletes, as fatty fibrous tissue replaces the right ventricular myocardium as well as that of the left ventricle. It affects at least 1 in 1000 persons under the age of 35, and it may account for up to 10% of fatalities from undetected cardiac disease in this age range [55–57, 110]. ARVC is familial and frequently autosomal dominant in roughly 50% of cases. Changes in five desmosomal protein-encoding genes, including DSG2, DSC2, PKP2, and DSP, which may result in cell death and fragmentation, have been linked to ARVC. Desmosomal protein deficiency may alter electrocardiographic parameters, cardiomyocyte gap junction structure, and sodium channel function. These mutations, which cause the premature termination of related proteins, are categorized as insertions, deletions, or nonsense mutations [58–61]. Desmosomal proteins have a role in the Wnt/ β -catenin signaling pathway and are crucial for cardiac myogenesis. Desmosome protein alterations block Wnt/ β -catenin, which helps to generate mesoderm precursor adipose [62–64]. TGF-3 and TMEM43 are two non-desmosomal genes that have been found in ARVC. The first is for transmembrane protein 43, and the second is for transforming growth factor 3 [65, 66]. Desmosome gene mutations are present in more than 50% of the afflicted people, most often in the PKP2 gene, which codes for plakophilin-2. Reduced β -catenin activity and aberrant plakoglobin nuclear translocation are caused by PKP2 mutation in iPSC-CMs [62, 67].

Role of iPSC research

Ma et al. developed the first ARVC in vitro model with PKP2 gene mutation using patient iPSC-CMs [68]. From the fibroblasts of two ARVC patients with PKP2 mutations, Kim et al. created iPSC lines. By producing an adult-like metabolic energetics from an embryonic condition and activating the peroxisome proliferator-activated

receptor-gamma, or PPAR γ , they were able to produce an ARVC in-vitro model. In mutant PKP2 iPSC-CM, this model showed enhanced lipogenesis, apoptosis, and deficiencies in calcium handling [69]. By knocking down PKP2, Matthes et al. evaluated the role of the desmosomal protein PKP2 in epicardial and epicardium-derived cells, which led to increased lipid indicators, cell migration, and proliferation. Their study suggested that epicardium cells may be important for understanding the pathogenesis of ARVC [70]. In especially for ARVC, Capsi et al. (2013) emphasize the special potential of hiPSC technology to represent congenital cardiac diseases. They develop two ARVC patients with PKP2 mutations into hiPSC-derived CMs. In the ARVC model, there was a decrease in PKP2 and connexin-43 protein expression [71]. The second most common ARVC variation is called desmoglein-2 (DSG2) [72]. An ARVC iPSC-CM model was created by Hawthorne et al. from a patient who had the DSG2 c.2358delA mutation. Less DSG2 mRNA, altered DSG2 protein distribution, atypical calcium regulation, and shorter action potentials were all seen in mutant cells. Additionally, elevated levels of pro-inflammatory cytokines have been seen, which may be connected to both conventional and non-canonical NF- κ B activation [73].

Dilated cardiomyopathy

The term “dilated cardiomyopathy” (or “DCM”) refers to a set of non-ischemic diseases of the heart muscle that are characterized by dilated left ventricles and dysfunctional contraction [74–76]. There are two types of the illness: familial/hereditary and non-familial. Familial factors account for 30–50% of DCM. Over 40 distinct genes, including TTN, TNNT2, PLN, MYH7, LMNA, and DES, have been identified as having genetic alterations [41, 77]. The TTN mutation, which is a frequent cause of dilated cardiomyopathy and occurs in around 25% of familial instances, is one such factor. The biggest known protein in humans, titin is a sarcomere protein encoded by the TTN gene [78, 79].

iPSC models

Hinson et al. demonstrated that sarcomere insufficiency, inconsistent cell signaling activation, and limited cell development were present in iPSC generations from DCM patients with TTN mutations. These cells responded to β -adrenergic stress with reduced effects [80]. Huang et al. used CRISPR-Cas9 technology to mimic hiPSC-CMs of DCM with A-band TTN mutations. This model showed aberrant sodium channel activity, sarcomere abnormalities, and contraction failure. With A-band TTN variations that improve contractility and lessen DCM symptoms, they also demonstrated the therapeutic potential of sarcomere modulators in DCM

[81]. Patients with TTN mutations' skin fibroblasts were used by Schick et al. to create iPSC-CMs. Mutated iPSC-CM showed defects in sarcomeric structure, diminished sensitivity to isoproterenol that mimicked β -adrenergic stimulation, and increased angiotensin-II levels [82]. Jeon et al. were able to create the hiPSC line YCMI007-A from a patient who had p. TNNT2 gene mutation causing Arg-205Trp. These cells might be useful for the DCM model since they could develop into three germ layers and had a normal karyotype [83]. Perea-Gil et al. [101] used the CRISPR technique of gene editing to produce iPSC-CMs from a patient with the TNNT2- p.R183W mutation for an in vitro DCM model. They showed that the concomitant administration of the two kinase inhibitors Gö 6976 and SB 203,580 may boost the gene expression of the serine and glycine-encoding genes and restore contractile failure in cells with different mutations, including TNNT2, LMNA, and TTN. They also showed that DCM symptoms may be reduced by activating transcription factor 4 and downstream elements like a crucial enzyme in the serine biosynthesis pathway [84]. According to research by Dai et al., troponin connections with tropomyosin are dysfunctional in iPSC-CMs made from DCM patients with the TNNT2-R173W mutation using the CRISPR/Cas9 technique. This limits the binding of PKA to other proteins. This results in a deficiency in the MYH7-mediated and AMPK-dependent connections between sarcomeres and cytoskeleton filaments. They demonstrated that TNNT2-R173W mutant cells' poor contractility could be repaired and sarcomere protein interactions improved by activating AMPK by A-769,662 [85]. Lamin A and lamin C are two major LMNA gene products that are expressed in the majority of differentiated somatic cells [86, 87]. Siu et al. produced two lines of DCM-derived iPSC-CMs from two patients. The LMNA exon 4 of one patient contained an R225X mutation, while the exon 4 of the second patient had a frameshift mutation. They demonstrated that pharmacological blockade of the ERK2 pathway with MEK1/2 inhibitors, U0126 and selumetinib significantly reduced the apoptotic effects of electrical stimulation for mutant LMNA iPSCs [88]. Lee et al. modeled DCM with LMNA mutation using iPSC-CMs. Platelet-derived growth factor (PDGF) signaling pathway was activated in mutant cells and aberrant calcium transients were seen in mutant iPSC lines. Sunitinib and crenolanib are used to inhibit platelet-derived growth factor receptor- β (PDGFRB) expression in K117fs iPSC-CMs, which reduces the unique calcium transient and decreases CAMK2D levels. LMNA in DCM is brought on by the pathogenic mutation K117fs [89]. Three DCM patients' peripheral blood mononuclear cells were transformed to produce three hiPSC lines by Lee et al. The c.1129 C>T LMNA mutation was present in all cases. All lines of iPSC have been

shown to have normal morphology and high levels of pluripotent markers, making them suitable as an in vitro model for LMNA mutations in DCM [90].

Hypertrophic cardiomyopathy

The most prevalent congenital cardiomyopathy, hypertrophic cardiomyopathy (HCM), is characterized by left ventricular hypertrophy without a clear hemodynamic cause, such as severe hypertension or aortic stenosis. Adults are thought to have a prevalence of HCM that falls between 0.02 and 0.23% [91–93]. Most often, sarcomere mutations are the genetic basis of HCM. Heart failure, arrhythmias, and sudden cardiac death are all linked to HCM [94]. Myosin binding protein C (MYBPC3) and -myosin heavy chain (MYH7) mutations are the most common, followed by TNNI3 and TNNT2 mutations. However, 20 sarcomeric and myofilament-related proteins contain 450 mutations that have been linked to HCM [95–97]. Myosin heavy chain beta (MHC- β), which is produced in skeletal and heart muscles and is a crucial protein in the thick filament of the human heart and contributes to contraction, is encoded by MYH7 [41, 98, 99].

iPSC models

Han et al. produced iPSCs from an HCM patient with an Arginine442Glycine missense mutation in the MYH7 gene in more recent years. In HCM iPSC-CMs compared to control iPSC-CMs, electrophysiological abnormalities, disordered sarcomeres, and a substantial increase in genes important for cell proliferation were seen. They discovered that using verapamil plus the histone deacetylase inhibitor trichostatin A (TSA) abolished irregular heartbeats and reduced calcium irregularity in HCM CMs [100]. Ten family members with HCM, half of whom had the Arg663His missense mutation in the MYH7 gene, were used to create iPSC-CMs by Lan et al. The cellular expansion, calcium cycling dysregulation, contractile arrhythmia, and intracellular calcium increase were all characteristics of mutant cells. Verapamil, mexiletine, lidocaine, and ranolazine have all been shown via drug testing to restore unfavorable alterations in mutant cells [101]. Using CRISPR/Cas9 technology, Cohn et al. created four isogenic iPSC-CM models of the MYH7 and MYBPC3 mutations. Due to delayed relaxation kinetics, mutated cell line generated hypercontractility. They demonstrated that myofilament calcium delivery, variation location, and extracellular calcium level are not related to hypercontractility. Studies using RNA sequencing revealed p53 activation, oxidative stress, and cytotoxicity, all of which may be reversed by genetically altering p53. As a direct myosin inhibitor, verapamil and blebbistatin were also put to the test. Verapamil decreased twitch tension in R403Q+/- cardiomyocytes by 31.7% and blebbistatin decreased twitch force by 35.3% [102]. Escribá et al.

created iPSC-CMs using CRISPR/Cas9 genome editing from two siblings who had HCM including the MYBPC3 mutation. Their research demonstrated that the MYBPC3 variation leads to mitochondrial bioenergetic dysfunction, which forces mitochondria to operate at greater efficiency in order to meet the escalating energy requirements of mutant cells. A seriously damaged person also showed abnormal excitation-contraction coupling [103]. Table 1 displays the stated models for disease modeling [49, 104–106].

Purification methods

Regenerative cell treatments need large amounts of pure PSCs. Animal models have shown several positive benefits of cardiac replacement treatment employing CMs derived from PSCs, including ESCs and iPSCs, to injured hearts. Thus, for the clinical application of PSC-CMs, 1×10^7 – 1×10^9 purified PSC-CMs are needed. Additionally, cell contamination should be avoided since it may result in unexpected events [107, 108]. There are several ways to clean up iPSCs. Here, we quickly go through two of the most popular techniques.

Antibody-based cell purification

The most effective approach for cell purification to date is antibody-based cell enrichment. Antibodies against cell type-specific surface proteins may tag the target cells and separate them using FACS or magnetic-activated cell sorting (MACS). The fundamental benefit of these approaches is their specificity and sensitivity. For PSC-CMs, researchers have created a few purification techniques. For instance, identifying CORIN, Signal-Regulatory Protein Alpha (SIRPA), and Vascular Cell Adhesion Molecule 1 (VCAM1) as cardiac-specific cell surface proteins. However, no research revealed surface markers that are exclusive to CMs. Because the MACS approach need specialized antibodies to the cellular surface proteins, it is challenging to utilize with cell types like CMs that lack certain cell surface proteins [109–111]. Rossler et al. compared the purification of hiPSC-CM cultures using lactate or MACS and their research revealed no considerable difference between these methods. They stated that purified cells via lactate or MACS had comparable features in structure, proteome, function and can be utilized in 3D cell cultures [112]. KDR (Flk-1) was

previously employed in investigations to identify cardiac progenitor cells; however, since this receptor is expressed on a variety of mesoderm populations, it was unable to enrich solely hPSC-CMs [113, 114]. In addition, although markers like SIRPA and VCAM1 are expressed in other cell types, cell sorting utilizing antibodies should first allow enrichment of cardiomyocytes. Therefore, contamination from non-CMs or non-specific cells might reduce the effectiveness of the transplant [115]. Liew et al. performed a research and introduced a novel surface marker, JAK2, to purify and sort live cells of ventricular CMs. They isolated CMs which were SIRPA positive with expressed MLC2A and MLC2V proteins and their research demonstrated that isolating ventricular SIRPA+/JAK2+ cells can lead to higher purity [116].

miRNA-based methods

There are further purification techniques that rely on spotting miRNA (microRNA) activity in live target cells [115]. Non-coding RNA molecules called miRNAs are involved in the control of gene expression. RNA polymerase II and III carry out the transcription of miRNAs, producing precursors that go through a sequence of cleavage events to create mature miRNA. In other words, the majority of miRNAs are transcribed from DNA sequences as primary miRNAs and processed into precursor miRNAs and mature miRNAs [117, 118]. MiRNAs are typically 21–23 nucleotides in length and can bind to the 3' untranslated regions (3'UTR) of messenger RNAs (mRNAs) to downregulate their target genes [119]. To quickly and effectively purify large numbers of PSC-derived cells, Tsujisaka et al. devised a technique that combines miRNA-responsive mRNA switch (miR-switch) with MACS (miR-switch-MACS). The miR-switch is a two-part synthetic mRNA. One is a protein-coding region, and the other is a complementary sequence to the target miRNA called a miRNA target site. Because of the contact between the target miRNA and the miR-switch, translation from the miR-switch is suppressed if the cell expresses the target miRNA. In contrast, the miR-switch is translated if the target miRNA is not expressed by the cell. To specifically suppress CD4 expression in iPSC-CMs, they created a miR-208a-responsive CD4-coding mRNA (miR-208a-CD4 switch) that encodes the CD4 cell surface protein. Because they could successfully filter iPSC-CMs among transfected cells (>95% purity) and maintain more than 50% of iPSC-CMs based on the computation of the number of CMs before and after MACS, they employed 300 ng miR-208a-CD4 switch to harvest significant amounts of iPSC-CMs [108]. A powerful technique for cell purification based on endogenous miRNA activity was disclosed by Miki et al. (2015) They discovered numerous miRNA that were co-expressed on differentiation days 8 and 20

Table 1 This table briefly indicates mentioned disease models in regenerative medicine

Diseases	Mutated genes	Features
LQT	KCNQ1, KCNH2, SCN5A	Prolonged AP duration
ARVC	PKP2, DSG2, DSC2, DSP	Right ventricular dilation
DCM	TTN, TTN2, LMNA	Systolic dysfunction and left ventricular dilation
HCM	MYH7, MYBPC3	Left ventricular hypertrophy

in hPSC-CMs, thus they created artificial mRNAs known as miRNA switches that can detect these miRNAs. They created artificial mRNAs called miRNA switches that code for fluorescent proteins that are marked with sequences that are targets of miRNAs produced by the cells of interest. CMs may be isolated and sorted from diverse cell types using synthetic miRNA switches. Because the fluorescent protein expression differential between CMs and non-CMs was greatest utilizing the miR-208a switch, they decided to employ miR-208a as a marker for CM-specific miRNA [120].

Stem cell-based therapy

Any treatment for diseases employing various types of viable human stem cells, such as adult stem cells, iPSCs, and ESCs, is referred to as stem cell-based therapy. Two methods for iPSC-based cell treatment are autologous and allogeneic cell transplantation. In allogeneic transplantation, iPSCs donate from someone other than the patient, employing human leukocyte antigen (HLA) that match donor in order to prevent immunological rejection by the host. In autologous transplantation, the patient's own iPSCs develop into target cells. The immune system in humans is significantly regulated by HLA [8, 121]. In 2014, the first transplantation of autologous iPSC-derived cells was performed on a Japanese woman in her seventies suffering from age-related macular degeneration. A degeneration of the retinal epithelium, a layer of cells that supports photoreceptors necessary for vision, is the cause of age-related macular degeneration. Following surgery, the patient did not encounter any severe complications. During the first year, there was no evidence of immunological rejection of the transplanted iPSC-derived retinal pigment epithelium cells, and the patient's visual acuity did not increase or decrease without further treatment [122, 123]. There are several obstacles that make the autologous approach difficult to use. For instance, safety testing for selecting the finest iPSC line from 30 distinct lines is estimated to cost US\$500,000, and the total cost per patient is approximately \$1,000,000. In addition, autologous transplantation is a lengthy procedure. There are more obstacles for autologous stem cell therapies than for allogeneic transplantation. During allogeneic therapy, the cell product may be extensively characterized and examined over a lengthy period of preclinical evaluation before being administered to a variety of patients. But in the autologous approach, the available time for this evaluation is much shorter [124]. In a rat model of MI, Guan et al. evaluated how well hiPSC-CM transplantation improved myocardial function. They discovered that animals had reduced mortality than the control group four weeks after receiving an iPSC-CM transplant. When iPSC-CMs were put into the control group instead of cell-free fluid, this model of

left ventricular dysfunction was attenuated. Their study showed that hiPSC-CM grafts survived in infarcted rat hearts for 4 weeks and restored myocardial function [125]. Li et al. investigated the effects of secreted exosomes from hiPSC-derived endothelial cells on survival of CMs after MI and they suggested that these exosomes could reduce the injury of glucose and oxygen deprivation, improve the Ca²⁺ homeostasis and restore heart function [126]. Ye et al. injected three types of hiPSC-derived cells (CMs, endothelial cells and smooth muscle cells) into injured hearts of a porcine model with MI and indicated engraftment of all three lineages cells at the site of injury after almost 4th week of injection. Their study showed enhancements in contractile performance, myocardial wall stress, metabolism and no development of arrhythmias [127]. The immaturity and heterogeneity of heart cells are two challenges for iPSC-based treatment. Regarding these problems, better methods to deal with the variability of the iPSC-CMs have been established, along with separation strategies based on particular cardiac markers, such as VCAM1. The population of cardiac cells in the CM consists of nodal-like, atrial, pacemaker, ventricular, and functional cells [76]. In a study, Funakoshi et al. [39] examined the engraftment efficiency of CMs at various stages of development in numerous days of mouse mesodermal cells and found that day 20 had the highest engraftment ratio. They administered day 20 cm to immune-deficient animals with infarcted hearts, and they saw a significant improvement in function and echocardiogram of the heart, demonstrating the therapeutic potential of these cells [128]. One of the issues with iPSC transplants is arrhythmias that has been observed in non-human primates [129]. Marchiano et al. distinguished ion channel genes that are expressed after hPSC-CM transplantation which lead to automaticity and arrhythmias. They utilized CRISPR/Cas9 technology to overexpress gene KCNJ2, knock out CACNA1H, HCN4, SLC8A1 genes and created adult like CMs that have decreased automaticity and contracted in response to exterior stimulations. These cells were transplanted in vivo and engrafted into host CMs which indicate no arrhythmias. They manifested that automaticity and risk of arrhythmias can be reduced by inducing the expression of adult like ion genes in PSC-CMs [130].

Challenges and future perspectives of iPSCs in vitro models

The creation of iPSC lines was a significant development in the area of regenerative medicine. These cells may develop into a variety of cell types, including vascular endothelial cells, smooth muscle cells, and CMs, making them an innovative and trustworthy model system for studying the molecular and genetic causes of human disorders [76]. iPSC-CMs are a viable alternative to primary

cells and animal models since they express a number of cardiac-specific genes, have a human genome, and possess key characteristics of human CMs. Although iPSC-CMs provide opportunities for drug development and testing of therapeutic approaches, these models have several limitations. Imperfect replication of adult disease phenotype and intricate intercellular communication of disease, is the primary limitation of iPSC-CMs which is because of their embryonic structure and lack of intercellular interactions between various cell types. Among the embryonic structures of iPSC-CMs are short sarcomeres, variable calcium ion concentration, and less mitochondria. Moreover, the complexity of certain diseases involving multiple genes, heterogeneous diseases, and individuals carrying distinct gene mutations are obstacles for iPSC technology. Consequently, it may be impossible to produce a single compound for a spectrum of diseases using a single patient-derived cell line. In addition, it is challenging to simulate the physiological state of the heart and the intercellular interactions of different cell types using a 2D iPSC-CMs culture. As a result, efforts are being made to develop 3D culture systems [8, 131]. Standard 2D cell culture is unable to replicate the complex physical and environmental interactions that occur in vivo. Therefore, they are unable to develop some cardiac disease phenotypes. ECM scaffolds, cellular components, and fluidic microenvironments may all be obtained from 3D culture models. Hydrogel scaffold-based models with biowires, engineered cardiac/heart tissues, HoC, and designed human myocardium are examples of 3D cardiac models. Scaffold-free 3D models include cardioids, cardiac spheroids, and cell sheets as another category. Researchers have taken a variety of steps to undertake morphological and functional maturation of iPSC-CM, to enhance CMs models, and to learn the underlying processes in addition to developing disease-treating therapies. Although 3D models may replicate disease-specific models that haven't been replicated in 2D models, it is essential to reach a full CM maturation in the development of novel medications, therefore perhaps patient-specific iPSC models couldn't totally replace animal models or conventional cell line assays. [132]. Maturation is a difficult process. A variety of elements, including as mechanical cues, electric signals, biochemical adhesion, and cell to cell contacts, influence the growth of CMs in vivo. It is impossible to produce a significant number of CMs for regenerative medicine quickly and at high capacity. As a result, it is anticipated that additional techniques will be introduced to supplement the ones now in use, resulting in improved iPSC-CM maturation induction and cell transplantation [133]. Overall based on the review article written by Gill et al. hurdles and challenges for the clinical translation of stem cell therapy can be summarized in heterogeneity of stem

cells, uncontrolled differentiation, migration, survival of transplanted stem cells and immunogenicity of these cells after transplantation [134] and for more detailed information it is suggested to read the mentioned article.

Conclusion

These days, iPSC technology has developed into an interesting tool for scientists to model diseases, offering new prospects in the area of modeling cardiac disease that make it feasible to research complicated cardiac arrhythmia syndromes and to reproduce patient phenotypic in vitro models. The development of iPSC technology seems to be useful for making notable advancements in understanding the fundamental principles of cardiomyogenesis, and it may aid in future biomedical research by facilitating the testing of novel medications and potentially significant therapeutic applications. One of the most significant advancements for iPSC technology is specifically giving a new source for individualized cardiac myocytes in the future and effective methods for cell differentiation towards chosen somatic cell types.

Abbreviations

iPSC	Induced pluripotent stem cell
CMs	Cardiomyocytes
PSCs	Pluripotent stem cells
hPSC	Human Pluripotent stem cells
PSC	CMs-PSC-derived CMs
iPSC	CMs-iPSC-derived CMs
hiPSCs	Human Induced pluripotent stem cells from
ESCs	Embryonic stem cells
bFGF	Basic fibroblast growth factor
MI	Myocardial infarction
VEGF	Vascular endothelial growth factor
DKK	1-Peptide Wnt inhibitor dickkopf homolog 1
BMP	4-Bone morphogenetic protein 4
Wnt	Wingless-related integration site
XAV939	Wnt Signaling Inhibitor
TRF1	Telomere Repeat binding Factor 1
LRP5/6	Low-density lipoprotein receptor-related protein
TGF	β -Transforming growth factor beta
FGF2	Fibroblast growth factor 2
GSK3 β	Glycogen synthase kinase 3
CK1	Casein kinase 1
APC	Adenomatous polyposis coli
PI3K	Phosphoinositide 3-kinase
Akt	Protein kinase B
BMPs	Bone morphogenetic proteins
SMAD	Small mother against decapentaplegic
ALK	Activin-like kinase
ActR2A	Activin receptor type-2 A
ActR2B	Activin receptor type-2B
TGFBR1	Transforming growth factor beta receptor 2
BMPR2	Bone morphogenetic protein receptor type 2
TGFBR3	Transforming growth factor beta receptor III
MAPK	Mitogen-activated protein kinase
PKP2	Plakophilin 2
TTN	Titin
TNNT2	Troponin T2, cardiac type
PLN	Phospholamban
LMNA	Lamin A/C
DES	Desmin
PKA	Protein kinase A
AMPK	AMP-activated protein kinase
CAMK2D	Calcium/calmodulin dependent protein kinase II delta

MEK	Mitogen-activated protein kinase kinase
ERK	Extracellular signal-regulated kinase
TNNI3	Troponin I3, cardiac type
FACS	Fluorescence-activated cell sorting
MACS	Magnetic-activated cell sorting
SIRPA	Signal-Regulatory Protein Alpha
VCAM1	Vascular Cell Adhesion Molecule 1
miRNA	MicroRNA
mRNAs	Messenger RNAs
3'UTR	3' untranslated regions
FDA	Food and medicine Administration
EMA	European Medicines Agency
LQTS	Long QT syndrome
INa	Sodium current
IKr	Potassium current
IKs	Delayed rectifier K ⁺ current
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein 9
ARVC	Arrhythmogenic right ventricular cardiomyopathy
PPAR γ	Peroxisome proliferator-activated receptor-gamma
DSG2	Desmoglein-2
DCM	Dilated cardiomyopathy
PDGF	Platelet-derived growth factor
PDGFRB	Platelet-derived growth factor receptor- β
HCM	Hypertrophic cardiomyopathy
MYBPC3	Myosin binding protein C
MYH7	Myosin heavy chain 7
MHC	β -Myosin heavy chain beta
TSA	Trichostatin A
HLA	Human leukocyte antigen
JAK2	Janus kinase 2
KDR	Kinase insert domain receptor (KDR is also known Flk1)
Flk1	Fetal Liver Kinase 1
KIT	KIT proto-oncogene receptor tyrosine kinase
MLC2A	Myosin light chain 2 A
MLC2V	Myosin light chain 2 V

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Author contributions

S.P.F as well as R. F. had main contribution in design, and manuscript writing; E. F. cooperated in final editing and critical review of the manuscript; B. V. collaborated in drawing the figures.

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