Recent progress of iPSC technology in cardiac diseases

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Abstract
It has been nearly 15 years since the discovery of human-induced pluripotent stem cells (iPSCs). During this time, differentiation methods to targeted cells have dramatically improved, and many types of cells in the human body can be currently generated at high efficiency. In the cardiovascular field, the ability to generate human cardiomyocytes in vitro with the same genetic background as patients has provided a great opportunity to investigate human cardiovascular diseases at the cellular level to clarify the molecular mechanisms underlying the diseases and discover potential therapeutics. Additionally, iPSC-derived cardiomyocytes have provided a powerful platform to study drug-induced cardiotoxicity and identify patients at high risk for the cardiotoxicity; thus, accelerating personalized precision medicine. Moreover, iPSC-derived cardiomyocytes can be sources for cardiac cell therapy. Here, we review these achievements and discuss potential improvements for the future application of iPSC technology in cardiovascular diseases.

Keywords Induced pluripotent stem cells · Cardiomyocyte · Disease modeling · Maturation · Differentiation into subtypes

Introduction
Adult human cardiomyocytes neither survive nor proliferate well when isolated from the heart in vivo. Given this, it has been difficult to establish in vitro models of human heart disease using human primary cardiomyocytes. Mouse models are used as an alternative. However, because of differences in electrophysiological properties, such as the different action potential duration and much faster heart rate, and in gene expression patterns, such as MYH6/7, these models do not fully recapitulate human heart disease (Shanks et al. 2009; Nerbonne 2004).

In 2006, mouse-induced pluripotent stem cells (iPSCs) were first generated by inducing four transcriptional factors, OCT3/4, c-MYC, SOX2, and KLF4, into mouse fibroblasts (Takahashi and Yamanaka 2006). Human iPSCs were generated the next year using the same set of transcriptional factors (Takahashi et al. 2007). Currently, human iPSCs can be generated by several methods from several cell sources with high efficiency (Yu et al. 2009; Okita et al. 2011; Kaji et al. 2009; Woltjen et al. 2009; Seki et al. 2010; Huangfu et al. 2008). Human iPSCs show features similar to human embryonic stem cells (ESCs), such as the ability to proliferate unlimitedly and differentiate into the three germ layers, including cardiovascular cells. Therefore, as an alternative for primary cells, many researchers have applied iPSC-derived cardiomyocytes (iPSC cardiomyocytes) to cardiovascular research. One of the most useful applications is disease modeling using patient iPSCs. Because iPSCs maintain the genetic background of the donor cells, in vitro iPSC cardiomyocytes provide the same genetic background as the patient.

Additionally, recent progress in gene editing technologies, such as CRISPR/Cas9, has enabled precise comparisons of disease phenotypes and responses to drugs between the patient iPSC-derived cells and genetically corrected iPSC-derived cells or sometimes between wild-type cells and wild-type cells edited to include the mutation causing the disease (Veres et al. 2014; Musunuru 2013; Smith et al. 2014; Suzuki et al. 2014). As another application of iPSC technology, iPSC cardiomyocytes together with ESC-derived cardiomyocytes (PSC cardiomyocytes) are expected as optimal cell sources for cell-based therapies to treat damaged hearts. It is well documented that directly transplanted PSC cardiomyocytes engraft in the host heart and restore
the heart function when injected into the damaged area by mechanically contributing to the heart contraction or by paracrine effects (Laflamme et al. 2007; Chong et al. 2014; Shiba et al. 2016; Funakoshi et al. 2016; Fan et al. 2020). In addition to direct transplantation, the transplantation of cell sheets derived from PSC cardiomyocytes onto the damaged areas has also been proven to improve cardiac function in damaged hearts (Fig. 1) (Gao et al. 2018; Kawamura et al. 2017; Sekine et al. 2011).

From this perspective, we review the remarkable progress made by applying iPSC technology to cardiovascular research and discuss recent achievements and potential improvements for its clinical application.
Cardiac differentiation of iPSCs

During the last 2 decades, researchers have developed efficient methods to differentiate PSCs into cardiomyocytes in several ways using knowledge based on heart development in vivo (Protze et al. 2019). Yang et al. could differentiate cardiac mesoderm expressing KDR and PDGFRA into cardiomyocytes by manipulating BMP and activin A/Nodal signaling at the early stage of the differentiation, followed by inhibition of the Wnt pathway (Yang et al. 2008). Their following study revealed that individual PSC lines require optimized activin A/Nodal and BMP signaling to efficiently induce cardiac mesoderm (Kattman et al. 2011). As an alternative to BMP and activin A, Lian et al. and Burridge et al. separately developed small compound-based cardiac differentiation protocols. They both showed that a small molecule GSK3-inhibitor, CHIR99021, upregulated Wnt signaling at the early stage of the differentiation, which induced the upregulation of endogenous BMP and nodal signaling. They then added a small compound inhibiting Wnt signaling after the induction of cardiac mesoderm to produce cardiomyocytes efficiently (Lian et al. 2012; Burridge et al. 2014). In addition, researchers have developed several purification methods for PSC cardiomyocytes. For this purpose, several groups have identified cardiac-specific cell surface markers, such as SIRPA, VCAM1, and CORIN, and purified the cells by flow cytometry (Dubois et al. 2011; Uosaki et al. 2011; Zhang et al. 2019b). We developed an efficient purification method using synthetic RNA capable of sensing cardiac-specific microRNAs (Miki et al. 2015). This method is applicable to other cell types too. Tohyama et al. developed a simple method to increase the purity of PSC cardiomyocytes. They focused on differences in glucose and lactate metabolism between cardiomyocytes and nonmyocytes, including undifferentiated iPSCs, and showed that only cardiomyocytes survive in glucose-depleted culture medium containing lactate, an observation they applied to the purification of PSC cardiomyocytes (Tohyama et al. 2013). Collectively, by the combination of efficient cardiac differentiation and purification methods, researchers can generate in vitro iPSC cardiomyocytes efficiently for cardiac disease modeling, drug cardiotoxicity assays, and cardiac cell therapies.

Disease modeling using iPSCs with gene mutations causing heart disease

iPSCs share the same genetic background as the original reprogrammed cells and can differentiate into cardiomyocytes while preserving the genetic information. Therefore, by combination with gene editing technology, iPSC cardiomyocytes are useful tools to analyze the mechanism underlying a disease. In this section, we introduce some of the well-established cardiac disease models using iPSC cardiomyocytes.

Channelopathy

Long QT syndrome (LQTS) is the most common channelopathy that represents a prolongation of QT time in the electrical cardogram and an increased risk of fatal ventricular arrhythmias. One major type of LQTS is congenital LQTS caused by mutations in ion channels, such as KCNQ1, KCNH2, and SCN5A (Wilde et al. 2021; Schwartz et al. 2012). The first LQTS disease model using patient iPSCs was reported by Moretti et al. (2010). They generated iPSCs from patients affected by LQTS type 1 (LQTS1) with a KCNQ1 mutation and demonstrated that the differentiated cardiomyocytes showed a prolonged duration of the action potential compared with control iPSC cardiomyocytes. They also showed that patient iPSC cardiomyocytes had an increased susceptibility to catecholamine-induced tachyarrhythmia and that beta blockade attenuated this abnormality (Moretti et al. 2010). Following this report, several groups established LQTS1 disease models (Ma et al. 2015; Takaki et al. 2019). Wang et al. generated an iPSC-based LQTS1 disease model by overexpressing dominant-negative gene mutations of KCNQ1 using gene editing technology. They revealed that the differentiated cardiomyocytes derived from these mutants showed a prolonged action potential in response to drugs, including nifedipine and pinacidil, highlighting the strength of gene editing for iPSC-based disease modeling (Wang et al. 2014a).

In addition to LQTS1 disease models, there are numerous reports about LQTS type 2 (LQTS2) disease models using iPSC cardiomyocytes from patients with KCNH2 mutations. Itzhaki et al. was the first to report that iPSC cardiomyocytes with KCNH2 mutations show a prolonged action potential with arrhythmogenicity. They also prepared an LQTS cardiac tissue model using iPSCs to evaluate responses to drugs, including potassium-channel blockers, calcium-channel blockers, KATP-channel openers, and late sodium channel blockers (Itzhaki et al. 2011). Another report by Brandao et al. generated a set of isogenic iPSC lines that possess different mutations in different sites in KCNH2 by genetically modifying a control iPSC line using CRISPR/Cas9 gene editing. They demonstrated that the edited lines recapitulated the different LQTS2 mutant phenotypes and different drug responses, showing the strength of the CRISPR/Cas9 gene editing system to analyze the mechanisms underlying individual mutations causing the disease (Brandao et al. 2020). Additionally, Mehta et al. identified that lumacaftor, a drug that acts as a chaperone during
protein folding and used clinically for patients with cystic fibrosis, significantly shortened the QT interval in LQTS2 patient iPSC cardiomyocytes (Mehta et al. 2018). Schwartz et al. tested if the in vitro results can be applied clinically to patients with LQTS2 and demonstrated that the combination of lumacaftor and ivacaftor, which is also commonly used to treat cystic fibrosis (Orkambi, Vertex Pharmaceuticals, Inc.), significantly shortened the QT interval, indicating that the drugs discovered from iPSC-based disease modeling can potentially translate clinically (Schwartz et al. 2019). Similarly, numerous studies have modeled other channelopathies, such as catecholaminergic polymorphic ventricular tachycardia (CPVT) caused by RYR2 or CASQ2 mutations and Brugada syndrome caused by SCN5A mutations (Liang et al. 2016; Jung et al. 2012; Sasaki et al. 2016; Preininger et al. 2016; Li et al. 2020). All these studies demonstrated that patient iPSC cardiomyocytes can recapitulate the disease-specific phenotypes, such as abnormal calcium handling and triggered activity, highlighting the effectiveness of iPSC-based disease modeling for channelopathy research.

**Hypertrophic/dilated cardiomyopathy**

Familial cardiomyopathy is one of the most common inherited heart diseases, and hypertrophic cardiomyopathy (HCM) is one of the most common familial cardiomyopathies. An HCM heart shows ventricular wall thickening with an increased size of cardiomyocytes, a disorganized and disarrayed sarcomere, abnormal metabolism, abnormal calcium sensitivity, and arrhythmogenicity resulting in sudden cardiac death (Ashrafian et al. 2003; Maron 2018). About half of patients with HCM carry mutations in sarcomere genes, such as MYH7 and MYBPC3 (Marian and Braunwald 2017). Lan et al. first reported HCM models using iPSCs from patients with a mutation in MYH7 and demonstrated that the patient iPSC cardiomyocytes recapitulated a series of HCM phenotypes, including cellular enlargement, contractile arrhythmia, and abnormal calcium handling. They also showed that an L-type calcium blocker, verapamil, prevented the development of HCM phenotypes in the cardiomyocytes (Lan et al. 2013). Additionally, several reports have identified possible mechanisms underlying the phenotype in HCM hearts using iPSC cardiomyocytes with mutations in HCM-related genes. Cohn et al. generated the cardiac microtissue assay to analyze iPSC cardiomyocytes from HCM patients. They demonstrated that the p53 pathway was activated along with increased oxidative stress and cytotoxicity due to metabolic stress in HCM cardiomyocytes and that the genetic ablation of p53 attenuated the cellular stress, indicating that the p53 pathway can be a therapeutic target in HCM heart (Cohn et al. 2019). Tanaka et al. identified endothelin-1 as an environmental factor to induce the pathological phenotypes, including the cardiomyocyte hypertrophy and intracellular myofibrillar disarray, in HCM patient iPSC cardiomyocytes. They demonstrated that blocking ET-1 signaling by an ETA (endothelin receptor A)-blocker attenuated the abnormal phenotypes, indicating a potential therapeutic target in the endothelin pathway (Tanaka et al. 2014). Recently, Green et al. used HCM model mice to discover that the novel small compound MYK-461 reduces cardiomyocyte contractility by decreasing the adenosine triphosphatase activity of the cardiac myosin heavy chain. The administration of MYK-461 suppressed the development of ventricular hypertrophy and fibrosis in HCM model mice (Green et al. 2016). Following this study, they demonstrated that iPSC cardiomyocytes with a mutation in MYH7 showed HCM phenotypes, including contraction/relaxation abnormality and metabolic abnormality, and that MYH-461 rescued these abnormalities, showing the usefulness of iPSC cardiomyocytes in preclinical studies (Toepfer et al. 2020). MYK-461 (Mavacamten) is now under a phase 2 clinical trial for patients with symptomatic obstructive HCM.

Dilated cardiomyopathy (DCM) is the other major form of cardiomyopathy. DCM heart manifests with ventricular dilatation, thinning walls, and decreased contractile function, resulting in severe heart failure (Schultheiss et al. 2019; Hershberger et al. 2013). Numerous genes causing DCM have been identified, such as TTN, LMNA, MYH7, TNNT2, MYBPC3, PLN, and DES (Reichart et al. 2019; McNally and Mestroni 2017). Sun et al. first reported the DCM model using iPSC cardiomyocytes from patients with TNNT2 (R173W) mutation. They showed that these iPSC cardiomyocytes have abnormal calcium handling, decreased contractility, and a disorganized sarcomere. They also demonstrated that a beta-adrenergic stimulus worsens the disease phenotypes and that beta-blocker treatment or the overexpression of sarcoplasmic reticulum calcium adenosine triphosphatase (SERCA2) improved them (Sun et al. 2012). In a recent report, Dai et al. used iPSC lines with the same TNNT2 (R173W) mutation and revealed that this mutation destabilized the interaction of troponin with other sarcomere proteins, resulting in the impairment of MYH7-mediated, AMPK-dependent sarcomere-cytoskeleton filament interactions. They therefore applied the AMPK activator A-769662 to DCM patient iPSC cardiomyocytes and observed a partial recovery of the sarcomere disorganization and impaired contractility in A-769662-treated DCM iPSC cardiomyocytes in vitro (Dai et al. 2020). In another report, Hinson et al. (2015) generated DCM models with TTN-mutated iPSC cardiomyocytes, finding the cells had sarcomere insufficiency and impaired responses to mechanical and beta-adrenergic stimuli. Karakikes et al. (2015) generated iPSC cardiomyocytes from a patient harboring the phospholamban (PLN) mutation and showed that the mutation induced abnormal calcium handling, electrical instability, and an abnormal cytoplasmic distribution of PLN protein. In addition to those
studies, there are iPSC-based models for Duchenne muscular dystrophy-related cardiomyopathy (Dick et al. 2013; Lin et al. 2015), Barth syndrome-related cardiomyopathy (Wang et al. 2014b), and DCM related to LMNA, BAG3, and MYH7 mutations (Norton et al. 2011; Yang et al. 2018; Lee et al. 2017, 2019).

Although both HCM and DCM iPSC cardiomyocyte models have provided promising results, including elucidation of the mechanisms underlying these cardiomyopathies, none have recapitulated all the phenotypes, including the disease progression from the early stage to end-stage heart failure in vitro. Additionally, any novel drugs discovered based on these models have not been applied to patients with cardiomyopathies. The reason may be because of the relatively immature phenotype of iPSC cardiomyocytes or the lack of non-myocyte populations that play essential roles for the disease progression. New maturation methods and co-culture platforms containing non-myocytes are required for in vitro cardiomyopathy models to fully recapitulate the phenotypes seen in patients.

### Arrhythmogenic cardiomyopathy/arrhythmogenic right ventricular cardiomyopathy

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited cardiomyopathy traditionally characterized by the fibro-fatty replacement of cardiomyocytes predominantly in the right ventricle, resulting in arrhythmogenicity and sudden cardiac death in young patients. Several desmosome-related genes causing ARVC have been identified, such as DSG2 (desmoglein-2), PKP2 (plakophilin-2), DSC2 (desmocollin-2), and DSP (desmoplakin) (Corrado et al. 2020; Thiene 2015). A series of clinical studies have revealed that the involvement of the left ventricle varies, and there are cases in which the fibro-fatty replacement was observed both in the right ventricle and left ventricle or dominantly in the left ventricle. Since those studies, the disease has been broadly named arrhythmogenic cardiomyopathy (ACM). Currently, patients with ACM show not only mutations in desmosome-related genes, but also in other genes, including DES (desmin), PLN (phospholamban), LMNA (lamin A/C), and CDH2 (cadherin-2) (Chen et al. 2019). In previous studies using model organisms, it has been reported that second heart field progenitors and cardiac mesenchymal stromal cells could be sources of adipocytes in ACM hearts (Sommariva et al. 2016; Lombardi et al. 2009). Given that the epicardium is known to give rise to cardiac mesenchymal stromal cells in the developing heart, several reports have suggested the contribution of the epicardium to the pathogenesis (Yuan et al. 2021). Matthes et al. reported that PKP2 knockdown in epicardium increased lipid droplets, cell migration, and the proliferation of epicardium-derived cells, suggesting the possibility that epicardium cells could contribute to the pathophysiology of ACM (Matthes et al. 2011). More recently, Dorn et al. demonstrated that abnormal cell–cell contacts due to the mutated PKP2 activated an ectopic fat gene program during cardiomyocyte differentiation, which switches the cell fate to adipocytes, and the adipocytes themselves are derived from cardiac progenitors expressing ISL1 and WT1 (Dorn et al. 2018). Although these results indicate the importance of desmosome genes for cell fate decisions in the heart, no previous studies have clearly answered which cell type is the actual source of the lipid accumulation in ACM heart or why fibro-fatty replacement in the left and right ventricles varies among patients with different mutations. (Sommariva et al. 2017).

Using ACM patient iPSCs, researchers have established in vitro ACM models. Kim et al. established iPSC lines with PKP2 mutations and demonstrated abnormal plakoglobin nuclear translocation without abnormal lipid accumulation under a standard differentiation protocol. They then induced both adult metabolism and pathogenic conditions by culturing the cells in an adipogenic cocktail containing insulin, dexamethasone (Dex), IBMX, PPARγ agonist, and indo-methacin, which induced ACM-specific phenotypes, such as lipid accumulation and cell apoptosis, in iPSC cardiomyocytes with PKP2 mutation (Kim et al. 2013). Caspi et al. also reported that combining IBMX, Dex, insulin, and lipid supplementation could worsen the ACM phenotypes in iPSC cardiomyocytes with the same PKP2 mutation (Caspi et al. 2013). Furthermore, Cho et al. showed that in vitro iPSC cardiomyocytes matured when transplanted into neonatal rat hearts in vivo and applied an in vivo maturation method to model ACM using iPSC cardiomyocytes. They observed that the engrafted ACM iPSC cardiomyocytes recapitulated ACM phenotypes, including increased lipid droplets, abnormal intercalated discs, and increased apoptosis, one month following the transplantation, even though the cells did not show any ACM phenotypes in vitro (Cho et al. 2017). All these studies indicate the need to add maturation stimuli, disease-specific pathological stimuli, or in vivo environments to establish more clinically relevant ACM models. These models will further benefit from appropriate differentiation methods for several cell types, including the left and right ventricular cardiomyocytes, and the development of the co-culture platforms to observe cellular interactions precisely to fully understand the mechanisms in ACM heart using iPSC-based disease models.

### Modeling drug-induced cardiotoxicity

Drug-induced cardiotoxicity is a major hurdle for drug discovery. About 30% of phase 2 clinical trials have failed due to safety issues, including cardiotoxicity, and cardiotoxicity
is one of the leading causes for drug withdrawals even after new drugs appear in the market (Onakpoya et al. 2016; Arrowsmith and Miller 2013). One of the frequent side effects of these drugs is the blockade of the I_{Kr} current, resulting in QT interval prolongation and fatal ventricular arrhythmia, such as Torsade de Pointes (TdP). Traditionally, researchers have used immortal cell lines expressing hERG (KCNH2 encoding the I_{Kr} channel) to test if the compounds of interest block the I_{Kr} channel and prolong the QT interval. However, these in vitro cell lines are quite distinct from human cardiomyocytes. Although mouse models can be used to test drug-induced cardiotoxicity, they do not fully recapitulate human cases due to the different electrophysiological properties and faster beating rate. As a result, traditional methods for drug testing show low specificity and cannot precisely predict the risk of arrhythmogenicity (Gintant et al. 2016). In addition to the arrhythmogenicity, reducing left ventricular (LV) contractile function is a major side effect of several drugs, such as cancer drugs. For example, anthracyclines as well as tyrosine kinase inhibitors and trastuzumab are known to induce contractile abnormality and heart failure in a series of clinical studies (Chen et al. 2008; Lenneman and Sawyer 2016; Han et al. 2017).

iPSC cardiomyocytes provide an alternative tool to test the cardiotoxicity of drugs in vitro. Navarette et al. tested if human iPSC cardiomyocytes respond to torsadogenic hERG blockers, finding behavior consistent with the reported drug effects in the literature. They showed that torsadogenic hERG blockers, such as sotalol and quinidine, induced early afterdepolarization and ectopic beats in iPSC cardiomyocytes with a significantly higher rate than untreated controls (Navarrete et al. 2013). Kawatou et al. successfully generated an in vitro TdP model using 3D cardiac tissues derived from human iPSCs. Upon treatment with I_{Kr} channel blockers, the tissues showed tachyarrhythmia with characteristics of TdP, including a typical polymorphic extracellular field potential and spiral wave re-entry (Kawatou et al. 2017). Kopljar et al. established the motion field imaging-based contraction measurement assay to evaluate the effect of chronic exposure to cardiotoxic drugs, including doxorubicin. They demonstrated that chronic exposure suppressed the contractile motions of iPSC cardiomyocytes and increased the release of cardiac biomarkers, including cTNI and FABP3 (Kopljar et al. 2017). Sharma et al. classified tyrosine kinase inhibitors (TKIs) in terms of cardiotoxicity by measuring alterations in cardiomyocyte viability, contractility, electrophysiology, calcium handling, and signaling. They revealed that TKIs with strong cardiotoxicity effects inhibited vascular endothelial growth factor receptor 2 (VEGFR2) and platelet-derived growth factor receptor (PDGFR), while TKIs with low cardiotoxicity effects did not, and that insulin and IGF1 suppressed the TKI-mediated cardiotoxic effects in iPSC cardiomyocytes (Sharma et al. 2017). All these results indicate that human iPSC cardiomyocytes are useful platforms to test the cardiotoxic effects of drugs and study the mechanisms underlying the drug-induced cardiotoxicity.

At the national level, the Japan iPSC Cardiac Safety Assessment (JiCSA) has reported the efficient torsadogenic risk assessment using the multi-electrode array (MEA) and iPSC cardiomyocytes (Ando et al. 2017; Yamazaki et al. 2018). At the international level, several regulatory agencies, including the FDA, European EMA, Health Canada, and Japan NIHs, have started the Comprehensive In Vitro Proarrhythmia Assay (CIPA) initiative to develop new assays that enable a more precise prediction of clinical proarrhythmia risk (Colatsky et al. 2016). The consortium recently reported the result of an international multisite study that characterized the electrophysiological effects of 28 drugs with known clinical torsadogenic risk on 2 commercially available iPSC cardiomyocyte lines across 10 experimental sites, highlighting the importance of standardizing commonly available and easy assays for all researchers and companies (Blinova et al. 2018).

In addition to in vitro cardiotoxicity models, researchers have recently used iPSC cardiomyocytes to identify populations at high risk for drug-induced cardiotoxicity to develop personalized precision medicine. Burridge et al. demonstrated that patient iPSC cardiomyocytes could predict the susceptibility to doxorubicin-induced cardiotoxicity (DIC). They showed that iPSC cardiomyocytes from patients with breast cancer who experienced DIC were more sensitive to doxorubicin toxicity than iPSC cardiomyocytes from patients who did not have DIC (Burridge et al. 2016). Stillitano et al. also demonstrated that iPSC cardiomyocytes could reproduce the susceptibility to develop an arrhythmogenic drug response. They prepared iPSC cardiomyocytes from patients presenting QT prolongation in response to sotalol and those who did not. They confirmed that the response to sotalol in in vitro iPSC cardiomyocytes was strongly correlated to the inter-individual differences observed in vivo (Stillitano et al. 2017). Those results indicate that researchers can predict the patient’s response to drugs by analyzing the patient’s iPSC cardiomyocytes in vitro. In another report, Matsa et al. provided evidence that iPSC cardiomyocytes can be used in vitro to predict and validate patient-specific drug safety and efficacy. They generated iPSCs from several individuals whose primary heart tissues are also available for analysis and compared their transcriptomes, showing that the NRF2 pathway, which is related to the cellular defense against oxidative stress, varies among patients. They then demonstrated that gene expression patterns related to NRF2-mediated oxidative stress pathways were preserved between iPSC cardiomyocytes and their original primary heart samples. They classified the iPSC cardiomyocytes into low risk, medium risk, and high risk for cardiotoxicity based on the expression of NRF2 pathways and showed that
several cardiotoxic drugs, such as tacrolimus and rosiglitazone, induced the toxicity only to iPSC cardiomyocytes in the high-risk group and not to iPSC cardiomyocytes in the low risk (Matsa et al. 2016). Taken together, recent advances in cardiotoxicity study using iPSC cardiomyocytes suggest a paradigm shift from traditional animal models or tests using immortal cell lines to more useful and precise drug toxic tests, which may contribute to more efficient drug discovery in the future.

**Cardiac cell therapy**

In addition to disease modeling, PSC cardiomyocytes are promising sources for cardiac cell therapy (Laflamme and Murry 2011). For the past decade, researchers have demonstrated the efficacy of the transplantation of PSC cardiomyocytes to damaged hearts. Detailed analyses of the grafted cardiomyocytes have revealed that they couple with the host heart, proliferate and gradually mature during the long-term engraftment, resulting in improved heart function (Funakoshi et al. 2016; Shiba et al. 2012; Chong and Murry 2014). However, there are still several hurdles to overcome for their clinical application in the cell-based therapies. One is the limited engraftment of the injected cardiomyocytes. Only a low percentage of injected cells successfully engraft in the host heart, probably because of cell death or rapid washout from the heart in the acute phase following the injection. Several groups have reported ways to improve survival and engraftment. Laflamme et al. (2007) demonstrated that a pro-survival cocktail, including insulin-like growth factor 1 and cyclosporine, with heat shock treatment prior to the transplantation improved the survival and engraftment of the transplanted PSC cardiomyocytes. Hattori et al. (2010) reported that small PSC cardiomyocyte aggregates, but not single-cell injections, could improve the engraftment because the cells were more likely to be trapped inside the heart due to the relatively larger size of the aggregates. Bargehr et al. (2019) demonstrated that co-transplantation with PSC-derived epicardium could induce the proliferation of the engrafted cardiomyocytes due to several secretion factors derived from the epicardium, resulting in a larger graft size with improved therapeutic effects. Though promising, further investigations to enhance the survival and proliferation of the injected cardiomyocytes for a larger graft size are required. Another issue to overcome is graft-related ventricular arrhythmia. Recently, several groups have successfully established large animal models for cardiac cell therapy using monkeys or pigs with subacute myocardial infarction. They have shown long-term engraftment of the injected cardiomyocytes and therapeutic efficacy along with electrical coupling of the engrafted cardiomyocytes to the host heart. However, fatal ventricular arrhythmia began a few days after the injection and continued for a few weeks (Chong et al. 2014; Shiba et al. 2016). A catheter-based analyses identified that those arrhythmias arose from the micro-re-entry or abnormal impulse generation of the grafted cardiomyocytes (Liu et al. 2018; Romagnuolo et al. 2019). Because these arrhythmias worsen the outcomes of a cell therapy, they must be controlled, presumably by efficient anti-arrhythmia drugs or improving the quality of the transplanted cardiomyocytes with respect to the electrical stability.

**Recent progresses on quality of iPSC-derived cells for disease study and treatment**

Although we have shown great achievements by applying iPSC technology to cardiovascular research, further improvements are needed for clinical application. In this section, we discuss recent progress and potential improvements in the quality of the differentiated cells.

**Generation of the targeted cardiac subtypes**

As mentioned above, researchers have developed efficient differentiation methods for iPSC cardiomyocytes in vitro following knowledge in developmental biology. Using any of those cardiac differentiation methods, we can generate iPSC cardiomyocytes at high purity. However, the resulting cardiomyocytes represent a mixture of several cardiac subtypes, including ventricular cells, atrial cells, and pacemaker cells.

To generate each cardiac subtype independently, several groups have focused on retinoic acid (RA) signaling for atrial differentiation in the developing heart (Zhang et al. 2011; Devalla et al. 2015). More recently, Lee et al. demonstrated that atrial and ventricular cardiomyocytes are derived from distinct mesoderm populations by manipulating the concentrations of BMP4 and Activin A in the early mesoderm stage, resulting in a clear distinction of the atrial and ventricular fates (Lee et al. 2017). Additionally, several groups have successfully established a differentiation protocol for sinus node pacemaker cardiomyocytes (Birket et al. 2015a; Protze et al. 2017). Thus, researchers can now precisely control cell fate in vitro and generate specific cardiac subtypes, enabling cardiac subtype-specific disease modeling. For example, Laksman et al. established atrial fibrillation (AF) models using PSC-derived atrial cells and demonstrated that flecainide and dofetilide, drugs clinically used for AF patients, modulate the re-entrant arrhythmogenic activation in differentiated atrial cardiomyocytes (Laksman et al. 2017). A more recent study of theirs reproduced the clinical cardiotoxicity of ibrutinib, a drug for leukemia, by demonstrating that it has a toxic effect on atrial cardiomyocytes, but not on ventricular cardiomyocytes (Shafata talab et al. 2019). Additionally, Goldfracht et al. generated...
atrial- and ventricular-specific engineered heart tissues and demonstrated that lidocaine, a sodium channel blocker used to treat ventricular arrhythmia, decreased the conduction velocity only in the ventricular tissues and not in the atrial tissues (Goldfracht et al. 2020). Zhao et al. generated heteropolar cardiac tissues containing atrial and ventricular ends and showed their spatially different responses to several drugs (Zhao et al. 2019). Although no previous reports have established models of sinus node-specific diseases, such as sick sinus syndrome, the above results suggest that precise differentiation into each cardiac subtype provides more precise platforms to analyze disease phenotypes and drug responses. Furthermore, in the developing heart, it is well known that there are two distinct subpopulations in the ventricle: trabecular and compact cardiomyocytes. Recent reports demonstrated the successful generation of trabecular and compact ventricular PSC cardiomyocytes by manipulating neuregulin and Wnt signaling, respectively (Mikryukov et al. 2021; Funakoshi et al. 2021). The specified ventricular cardiomyocytes would be helpful to analyze trabeculation and compaction-related diseases, such as LV non-compaction. However, generating right ventricular (RV) and LV cardiomyocytes independently in vitro remains a challenge. LV cardiomyocytes are developed from the first heart field (FHF), while RV cardiomyocytes are from the second heart field (SHF). Andersen et al. demonstrated that differentiated ventricular cells are mixed populations of FHF-derived and SHF-derived cells using a double reporter cell line, HCN4 (FHF marker)-GFP and TBX1 (SHF marker)-RFP. They showed HCN4+ cells expressed the FHF markers TBX5 and HAND1, while TBX1+ cells expressed the SHF markers FGF8, FGF10, and SIX2, and further identified CXCR4 as a potential cell surface marker to isolate SHF progenitors (Andersen et al. 2018). Zhang et al. (2019b) also generated a double reporter cell line, TBX5-clover2 and NKX2.5-tagRFP, and showed that the TBX5+/NKX2.5+ population has FHF features, while the TBX5−/NKX2.5+ population has SHF features. Although these studies give promising results, none have demonstrated the direct differentiation of RV and LV cardiomyocytes that express the global gene expression patterns or functions observed in in vivo hearts. Further investigations are essential to fully understand the mechanisms underlying the generation of LV and RV cardiomyocytes in vitro, which will enable the generation of disease models showing the different phenotypes between the left and right ventricles, such as ARVC (Fig. 2).

The cardiac cell type may have profound implications for cardiac cell therapy, since there is still no understanding what happens if cell types are misplaced in the heart. For example, we have no information on the effects of...
transplanting atrial cells or pacemaker cells into the ventricle or RV cells into the left ventricle. Nor do we know if the contamination of the pacemaker cells can cause graft-related arrhythmias upon transplantation into the ventricle. Further investigations are required to develop safer and more efficient cardiac cell therapy.

**Non-myocyte populations in the heart and ideal platforms to recapitulate their interactions with cardiomyocytes**

In the human heart, there are not only cardiomyocytes but also several non-myocyte populations, including the epicardium, endocardium, fibroblasts, endothelial cells, smooth muscle cells, and inflammatory cells, all of which interact tightly with cardiomyocytes both in normal heart development and in disease conditions. Therefore, researchers are also focusing their efforts on generating non-myocyte populations in vitro. The epicardium is an epithelial cell population that consists of the outer epithelial layer spreading around the heart and is known to be a cell source for cardiac fibroblasts and smooth muscle cells through the epithelial-to-mesenchymal transition (EMT) (Cao and Poss 2018). Several groups have successfully generated PSC-derived epicardium by manipulating BMP, Wnt, and RA signaling. They also demonstrated that the differentiated epicardium has the potential to differentiate into fibroblasts and smooth muscle cells by adding known factors to induce EMT, such as fibroblast growth factors (FGFs), transforming growth factor-beta (TGFβ), and platelet-derived growth factor (PDGF) (Witty et al. 2014; Iyer et al. 2015). Using the differentiated epicardium and its derivatives, Zhang et al. (2019a) demonstrated that the differentiated cardiac fibroblasts are sensitive to both pro- and anti-fibrosis drugs and that those fibroblasts enabled the analysis of the crosstalk between cardiomyocytes and fibroblasts through BMP/ANP-NRP1 pathways in cardiac fibrosis. The endocardium is a specialized endothelial cell population that consists of an inner layer of the heart and is well known as a cell source for coronary endothelial cells, valvular endothelial cells, and valvular interstitial cells (Zhang et al. 2018). Recently, several groups have successfully established differentiation protocols of the endocardium from PSCs by manipulating BMP and FGF signaling (Mikryukov et al. 2021; Neri et al. 2019). Neri et al. demonstrated that the differentiated endocardium undergoes EMT, expresses markers of valve interstitial cells when cultured on mouse atrio-ventricular canal cushions, and recapitulates the features of mitral valve prolapse using iPSCs derived from a patient harboring a mutation in DCHS1, a possible cause for mitral valve prolapse (Neri et al. 2019). These studies indicate that differentiated non-myocytes are useful tools for modeling diseases in which non-myocytes play important roles.

In addition to non-myocyte-specific disease modeling, researchers have established co-culture systems to recapitulate the environment of the in vivo heart more precisely, including tight interactions between cardiomyocytes and non-myocytes. Recently, Giacomelli et al. (2020) generated 3D microtissues with healthy-control iPSC cardiomyocytes and iPSC fibroblasts from patients with PKP2 mutation causing ACM and demonstrated that microtissues containing the mutant fibroblasts showed the same arrhythmic behavior observed in patients with ACM. The study also noted that there was a higher proportion of activated myofibroblasts in the mutated fibroblasts, supporting the notion that epicardial cells are a source of the fibro-fatty substitution seen in ACM heart. In a more recent report, Hofbauer et al. generated PSC-derived self-organized 3D cardiac chamber-like structures, named cardioids, containing cardiomyocytes, endothelial cells, and fibroblasts. Using cardioids with the knockout of HAND1, an essential transcription factor for cardiac chamber morphogenesis, they showed an evident defect in cardiac cavity self-organization, indicating that self-organization and genetic cardiac defects can be modeled in this cardioid platform. They additionally demonstrated that the differentiated epicardium spontaneously differentiated into fibroblasts and smooth muscle cells when co-cultured with cardioids and established a cardiac injury model showing the accumulation of extracellular matrix derived from non-myocytes following cryoinjury (Hofbauer et al. 2021). Yang et al. showed the importance of inflammatory cells for disease modeling. They established an immunocardiac co-culture platform containing PSC cardiomyocytes and PSC macrophages to recapitulate the abnormal inflammatory infiltration of macrophages in the heart with COVID-19 infection. They demonstrated that macrophages induced an increase of reactive oxygen species and apoptosis in cardiomyocytes by secreting inflammatory cytokines following exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Yang et al. 2021).

The above studies give evidence that non-myocytes play important roles in recapitulating disease conditions and that the co-culture of multiple cell types can be an ideal platform to analyze the mechanism of heart disease. Further progress, such as the development of co-culture platforms containing all necessary cell types, are required to fully recapitulate the complex interactions for each cell type in the human heart in vivo during both normal development and disease conditions (Fig. 2).

**Maturation of differentiated cardiomyocytes**

Although we are currently able to generate PSC cardiomyocytes with high efficiency, these cells are typically in an immature state; they display a low level of sarcomere organization, insufficient mitochondrial oxidation capacity,
and underdeveloped calcium handling, thus resembling fetal cardiomyocytes of the early developmental stage (Karbassi et al. 2020). These immature properties hinder drug screenings or disease modeling of adult-onset heart disease, since the cellular characteristics of fetal-like immature cardiomyocytes are quite distinct from those of adult-like mature cardiomyocytes. As we mentioned above, researchers have used their understanding of early heart development in vivo to establish efficient in vitro PSC differentiation protocols for many cell types in the heart. However, regarding cardiac maturation, there is a lack of knowledge about the in vivo heart in the perinatal, postnatal, and adult stages.

A major change in the maturation status occurs at birth, when proliferative fetal cardiomyocytes switch their phenotypes to a non-proliferative type. One of the most remarkable changes at birth is seen in the blood circulation from the maternal circulation to the systemic circulation, which increases the workload of the newborn heart. Following this observation, electrical stimulation and mechanical stress can promote the maturation of PSC cardiomyocytes (Ronaldson-Bouchard et al. 2018; Feric and Radisic 2016; Nunes et al. 2013). However, cardiomyocytes in vivo usually switch their phenotype rapidly in the postnatal period. For example, in mice, during the first week after birth, cardiomyocytes lose their proliferative phenotype, undergo mitotic quiescence, and shift to the hypertrophic phase (Masters and Riley 2014; Porrello et al. 2011). These observations suggest key factors in the early postnatal period in addition to changes in the workload to induce the rapid phenotypic switch. In rodent studies, several factors have been identified to induce this phenotypic switch. Puente et al. demonstrated that an oxygen-rich postnatal environment was an upstream event that induced reactive oxygen species (ROS) and oxidative damage, resulting in cell cycle arrest during the first postnatal week in mice (Puente et al. 2014). Cardoso et al. focused on a postnatal shift from anaerobic glycolysis to mitochondrial fatty acid utilization in the heart and demonstrated that the latter regulated cell cycle arrest and the maturation status in cardiomyocytes (Cardoso et al. 2020). Similarly, Nakano et al. revealed that the high blood glucose level suppressed cardiac maturation in the perinatal stage in vivo, further supporting the idea that the energy substrate plays an important role in cardiomyocyte maturation (Nakano et al. 2017). Additionally, Hirose et al. provided evidence that the inactivation of thyroid hormone signaling reduces cardiomyocyte maturation and delays cell cycle arrest in postnatal mice (Hirose et al. 2019). The concentration of several hormones, including thyroid hormone and steroid hormone, are dramatically increased in the perinatal period, resulting in an “hormonal surge”, compared to the early fetal periods, suggesting the important roles of these hormones for the phenotypic switch in the postnatal stage (Li et al. 2014; Rog-Zielinska et al. 2014). Given these potential factors in cardiomyocyte maturation, researchers have successfully developed methods to promote the maturation level of PSC cardiomyocytes. Several groups reported that culturing cells with lipid-rich media as well as hormonal stimuli, including T3 and Dex, improved the sarcomere organization, mitochondrial function, and calcium handling in PSC cardiomyocytes (Yang et al. 2014, 2019; Gentillon et al. 2019; Mills et al. 2017; Rog-Zielinska et al. 2015; Parikh et al. 2017). We recently developed an efficient maturation protocol using the combination of T3, Dex, PPARγ agonist, and palmitate in low glucose-containing media. The PSC cardiomyocytes treated by this protocol showed structural maturation, withdrawal from the cell cycle, and the ability to utilize exogenous fatty acids efficiently as an energy source, which is one of the representative features of the adult-like metabolism in the heart (Funakoshi et al. 2021).

In addition to these factors, Sakamoto et al. demonstrated that ERRγ signaling plays an essential role in postnatal cardiac maturation in mice (Sakamoto et al. 2020). Coincident with this finding, we recently reported that an ERRγ agonist enhanced maturation with T-tube formation in iPSC cardiomyocytes in vitro (Miki et al. 2021). Garbern et al. demonstrated that the inhibition of mTOR signaling, which acts as a nutrient sensor and a cell cycle regulator, by a small compound, Torin 1, enhanced the maturation of iPSC cardiomyocytes via p53-induced quiescence (Garbern et al. 2020). All of these results indicate that mimicking the in vivo perinatal period can mature PSC cardiomyocytes in vitro. Using these approaches, several groups have shown the importance of maturation in disease modeling using patient iPSCs. Birket et al. (2015b) used a maturation method that combines T3, Dex, and IGF1 to reveal that mature iPSC cardiomyocytes derived from HCM patients with the MYBPC3 mutation recapitulated the contraction defect in the patients. Feyen et al. (2020) showed that iPSC cardiomyocytes matured in lipid-rich media recapitulate the contraction deficit in the DCM model or electrophysiological abnormality in the long QT syndrome model, but immature PSC cardiomyocytes do not. Also using lipid-rich media, Knight et al. (2021) demonstrated that mature iPSC cardiomyocytes develop pathological hypertrophy with relaxation defects in response to either a pro-hypertrophic agent or HCM-related gene mutation. In a more recent report, Bliley et al. developed 3D engineered heart tissues that show optimal preload and afterload to promote the maturation level of iPSC cardiomyocytes. They applied these engineered heart tissues to model ACM using iPSCs from patients with a mutation in DSP. They observed representative phenotypes of ACM, including abnormal desmosome distribution and impaired systolic and diastolic function, in the engineered tissues, but not if the cells were cultured in typical 2D or 3D culture systems (Bliley et al. 2021). However, even with these adjustments, the maturation level of PSC cardiomyocytes is still comparable to that in...
early postnatal heart and not in adult heart. Future research is needed to elucidate the mechanisms underlying the maturation process from the postnatal stage to the adult stage in order to model late adult stage-onset diseases.

Several groups have reported the important roles of non-myocyte populations in the maturation of cardiomyocytes. Hortells et al. (2020) demonstrated that during the postnatal period in mice, periostin-expressing cardiac fibroblasts contribute to the phenotypic switch from immature cardiomyocytes to mature ones. Wang et al. provided evidence that cardiac fibroblasts also undergo the phenotypic switch from neonatal to adult stage and that this switch regulates the maturation of cardiomyocytes. They also showed that adult-like fibroblasts can enhance the maturation level of neonatal cardiomyocytes (Wang et al. 2020). Coincident with those findings, several groups have established co-culture methods with non-myocytes to enhance the maturation in PSC cardiomyocytes. For example, Giacomelli et al. have generated 3D cardiac microtissues containing iPSC cardiomyocytes, endothelial cells, and epicardium-derived fibroblasts. The 3D microtissues showed improved sarcomere structures, enhanced contractility, and mitochondrial respiration and were electrophysiologically more mature due to the increased level of intracellular cyclic AMP (Giacomelli et al. 2020, 2017). Bargehr et al. (2019) showed PSC-epicardium can enhance the contractility, myofibril structure, and calcium handling of 3D engineered heart tissues when mixed with PSC cardiomyocytes in heart tissues. Presumably, the combination of these co-culture platforms using appropriate non-myocyte populations with compounds or hormones that enhance maturation would further promote the maturation of PSC cardiomyocytes (Fig. 3).

As for cardiac cell therapy, in most previous reports using large animal models, researchers transplanted immature cardiomyocytes and observed graft-related arrhythmia for a few weeks following the transplantation (Shiba et al. 2016; Romagnuolo et al. 2019). Although no previous studies have identified the causes of the graft-related arrhythmia, one possible mechanism is the relatively immature phenotype of the transplanted cardiomyocytes, since they have fetal-like immature electrophysiological properties in vitro. We recently demonstrated that the maturation status in in vitro cardiomyocytes prior to the cell transplantation affected the graft quality, including the expression of CX43, a gap junction protein that increases the electrical stability of the grafted cells, suggesting that the transplantation of mature cardiomyocytes could reduce the occurrence of the graft-related arrhythmias (Funakoshi et al. 2021). Studies transplanting mature cardiomyocytes into large animal may answer this question.

**Combination of iPSCs with other emerging technologies**

Recent advances in artificial intelligence and machine learning are remarkable. For example, AlphaGo defeated a world champion of Go, which is considered one of the most complex board games, suggesting that artificial intelligence has already surpassed human performance in some aspects (Silver et al. 2016). In the biology field, machine learning
has enabled researchers to classify cellular images, build genomic connections, and discover new drugs (Webb 2018). Likewise, in studies related to cardiac disease, researchers have begun to apply these emerging technologies to analyze comprehensive data derived from iPSC cardiomyocytes. Maddah et al. (2020) established an image-based deep learning method to detect and quantify variations in the cellular structures from the immunostaining of PSC cardiomyocytes for drug toxicity. Juhola et al. (2021) applied machine learning to evaluate drug effects in iPSC cardiomyocytes derived from patients carrying mutations causing CPVT and showed that it could predict the drug effect with high accuracy. In addition, a recent report by Theodoris et al. showed the efficiency of combining machine learning and iPSC technology in drug discovery. They established a method to combine gene network analysis and machine learning, resulting in the discovery of new drug candidates that correct dysregulated gene networks in iPSC endothelial cells carrying the NOTCH1 mutation that causes aortic valve (AV) stenosis. The compound identified as the most efficacious rescued not only the iPSC endothelial cells but also patient-derived AV cells and treated AV disease in a mouse model (Theodoris et al. 2021). Current machine learning systems allow us to predict 3D protein structures and protein–protein interactions with remarkably high accuracy and are available to all researchers, which can accelerate the discovery of the new proteins (Jumper et al. 2021; Baek et al. 2021). Collectively, strategies that combine iPSC technology and machine learning could be an effective method to discover candidate therapies in the near future.

Conclusions

Since iPSCs were first discovered 15 years ago, researchers have applied related technology to cardiovascular research and achieved remarkable progress in many aspects, including disease modeling, platforms for drug discovery and toxicity, and cell-based therapy. Recent progress in methods for cell differentiation, maturation, and the generation of 3D culture platforms with appropriate non-myocyte populations are further accelerating this field. Although no studies have comprehensively recapitulated the environment of the adult human heart using differentiated cells, we assume in vitro models will succeed by combining these methods. Additionally, new technologies for data analysis, such as machine learning, will allow us to apply in vitro models for the discovery of novel therapies effectively. While many challenges remain, a new door has opened for the clinical application of iPSC technologies for cardiovascular diseases.

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Declarations

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