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Modelling inherited cardiac disease using human induced pluripotent stem cell-derived cardiomyocytes: progress, pitfalls and potential

van Mil, Alain[1,2], Balk, Geerthe Margriet[1,2], Neef, Klaus[1,2], Buikema, Jan Willem[2,3], Asselbergs, Folkert W[2,4,5,6], Wu, Sean M[3,7,8], Doevendans, Pieter A[2], Sluijter, Joost P.G[1,2*]

1. Department of Cardiology, Division Heart and Lungs, Experimental Cardiology Laboratory, Regenerative Medicine Center, University Medical Center Utrecht, Utrecht, the Netherlands
2. Department of Cardiology, Division Heart and Lungs, University Medical Center Utrecht, Utrecht, the Netherlands
3. Stanford Cardiovascular Institute, Stanford University School of Medicine, Stanford, CA, USA
4. Institute of Cardiovascular Science, Faculty of Population Health Sciences, University College London, London, United Kingdom
5. Durrer Center for Cardiovascular Research, Netherlands Heart Institute, Utrecht, the Netherlands;
6. Farr Institute of Health Informatics Research and Institute of Health Informatics, University College London, London, United Kingdom
7. Department of Medicine, Division of Cardiovascular Medicine, and Stanford University School of Medicine, Stanford, CA, USA
8. Institute of Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA, USA

*Corresponding author:

Professor of cellular and translational cardiology, Division Heart and Lung, Dept. of Cardiology, Experimental Cardiology Laboratory, UMC Utrecht Regenerative Medicine Center, University Medical Center Utrecht | Internal mail no G03.550 | P.O. Box 85500 | 3508 GA Utrecht, the Netherlands | T: +31 88 75 575 67 | F: +31 30 25 226 93 | J.Sluijter@umcutrecht.nl

Abstract

In the past few years, the use of specific cell types derived from induced pluripotent stem cells (iPSCs) has developed into a powerful approach to investigate the cellular pathophysiology of numerous diseases. Despite advances in therapy, heart disease continues to be one of the leading causes of death in the developed world. A major difficulty in unravelling the underlying cellular processes of heart disease is the extremely limited availability of viable human cardiac cells reflecting the pathological phenotype of the disease at various stages. Thus, the development of methods for directed differentiation of iPSCs to cardiomyocytes (iPSC-CM) has provided an intriguing option for the generation of patient-specific cardiac cells. In this review, a comprehensive overview of the currently published iPSC-CM models for hereditary heart disease is compiled and analysed. Besides the major findings of individual studies, detailed methodological information on iPSC generation, iPSC-CM differentiation, characterization and maturation is included. Both, current advances in the field and challenges yet to overcome emphasize the potential of using patient-derived cell models to mimic genetic cardiac diseases.

Introduction

Advances in the field of molecular biology and genomics have induced a shift in medicine towards a more precise, personalized and causal approach. In the field of cardiology this approach is thought to be especially beneficial for patients with inherited cardiac disease[1], currently often lacking sufficient standard of care. Unravelling the underlying mechanisms of cardiac disease and developing new treatment options have been hampered by limitations of available models. While established transgenic small animal models of inherited cardiac disease do provide some insights into the pathogenic mechanisms of these diseases[2,3], substantial

differences between the physiology of cardiomyocytes from small animal models and humans limit extrapolation of results, leading to a failure to translate findings from small animals to humans[4]. Likewise, transgenic non-cardiac human cell lines, e.g. hERG-overexpressing HEK-293 cells, were shown to model cardiac diseases insufficiently since they do not recapitulate the complex cardiac phenotype, e.g. sarcomere organization, calcium handling, metabolism and (electro)physiology[5].

The development of induced pluripotent stem cells (iPSC) by Yamanaka et al.[6] has helped to overcome these challenges by enabling the generation of patient-specific iPSC-derived cardiomyocytes (iPSC-CM), carrying disease-specific mutations. With these cells, researchers are able to recapitulate the disease phenotype in vitro. With refined protocols for iPSC generation and differentiation into somatic cell types, including cardiomyocytes, a sustainable source of non-transgenic human cardiac cells has become available for *in vitro* disease modelling. Cardiomyocytes generated from iPSCs have several advantages over human embryonic stem cells (ESCs) or organ derived stem cell models, as iPSCs can be generated from a variety of easily accessible cell sources, including cells from the skin, urine and blood[7–9]. Furthermore, the generated cardiomyocytes are donor (patient) specific, enabling genotype-phenotype associations and offering a personalized drug-screening platform for individualized patient therapy.

Since the first study in 2010[10], much progress has been made using patient specific-iPSC models to characterize cardiac diseases and study their molecular pathogenesis. Over 90 studies using iPSC-CM models are now available, including long QT syndromes (LQTS), catecholaminergic polymorphic ventricular tachycardia (CPVT), arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVC), familial dilated cardiomyopathy (DCM), familial

hypertrophic cardiomyopathy (HCM), and many more. In this review, we provide an in-depth overview of the current iPSC-CM models of inherited cardiac diseases. Methods for differentiation and characterization of iPSC-CMs, including functional parameters like cellular electrophysiology, calcium handling and contraction kinetics are evaluated. Finally, challenges, limitations and future perspectives of iPSC-CM models of inherited cardiac disease will be discussed.

Generation of iPSC-CM models

1. iPSC generation

Patient-specific iPSC-CM models rely on the generation of iPSC lines from a tissue sample. To minimize the invasiveness of this procedure, a trend from using dermal fibroblasts from skin biopsies towards blood or urine cells can be observed[8,9] (Figure 1A). Similarly, introduction of reprogramming factors[6] into patient's cells is moving from integrating vectors, e.g. retro- and lentiviruses, towards non integrating vectors, e.g. Sendai virus[11] or virus-free methods like episomal transfection[12], or mRNA delivery[13], avoiding insertional mutagenesis and potential transgene reactivation (Figure 1B). Details on methods used for generation and characterization of 192 iPSC lines (covering 152 different mutations; published in 91 papers) are provided in Supplementary Table 1.

2. iPSC-CM differentiation

The directed differentiation of cardiomyocytes from pluripotent stem cells is currently performed by either co-culture with visceral endoderm-like (END-2) cells[14], embryoid body formation in suspension[15], or monolayer cell culture with supplementation of specific differentiation factors[16].

Although details, efficiencies and yields vary considerably between individual studies, the chemically defined monolayer differentiation protocol is increasingly used, likely due to its high efficiency and ease of use (Figure 1C and Supplementary Table 1)[17,18].

3. iPSC-CM characterization

No consensus has been reached on the definition of a bona fide *in vitro* human cardiomyocyte sufficiently recapitulating its *in vivo* counterpart. iPSC-CMs have frequently been described displaying an immature, foetal-like phenotype, e.g. lacking mature sarcomeric organization[19], low ratios of multinucleation[20], underdeveloped t-tubule networks[21] and altered Ca^{2+} handling[22]. Cardiac maturation involves changes in gene expression levels, structural reorganization (e.g. myofibrils) and, importantly, functional changes (Ca^{2+} handling, contractility and action potential characteristics) rather than the mere expression of certain markers[23]. Thus, analyses of electrophysiological properties, contraction and contractile force, cell-cell coupling, metabolism, mitochondrial content and morphology, cell size and morphology, and sarcomere density and organization need to be considered. These differences between iPSC-CMs and adult cardiomyocytes have to be taken into account when establishing disease-in-a-dish models and interpreting results.

Studies within the scope of this review characterized iPSC-CMs to some degree, including immunofluorescence imaging, electron microscopy, fluorescence-activated cell sorting and qRT-PCR. Most studies (53 of 91) report at which day of the differentiation protocols spontaneously contracting cells were first observed (day 6-22 days, mean: day 11). Characterization of the iPSC-CMs was carried out on average on day 30 (between day 1 and day 150) after start of the differentiation protocol. A substantial fraction of studies (26 of 91)

included functional measurements, e.g. multi-electrode arrays (MEA), to assess electrophysiological maturation of the iPSC-CMs. Details on characterization and assessment of maturation of patient specific iPSC-CMs are listed in Supplementary Table 1.

Differences in protocols for generation of patient specific iPSC lines, differentiation to cardiomyocytes, and characterization of iPSC-CMs make studies on disease modelling difficult to compare and reproduce. However, the following studies highlight the evidence that iPSC-CMs exhibit clinically relevant phenotypes and that the use of these disease models in pathophysiological and molecular research can result in the discovery of new therapies for inherited cardiac diseases.

iPSC-CM models of cardiac disease

To provide an in-depth overview of the current iPSC-CM models of inherited cardiac diseases, Supplementary Table 1 provides detailed information on all studied patient iPSC-CM lines, with a quantitative outline in Figure 1D. Supplementary Table 2 lists the cardiac diseases, prevalence and known associated genes, both with and without iPSC-CMs studies. The major results are discussed in the text and Figure 2 shows the subcellular localization of all mutated genes.

a. Long QT Syndrome (LQTS)

The long QT syndrome (LQTS) is an autosomal dominant cardiac disease, affecting up to 1 in 1,000 live births. It is associated with over 500 different mutations in at least 15 genes[24] encoding ion channel (interacting) proteins. Patients may only show a prolonged repolarization phase (the QT phase) on ECG measurements, but this can predispose to potentially life-threatening ventricular arrhythmias, so-called *Torsades de pointe*, and sudden cardiac arrest. In

the last few years, patient-specific iPSC-CM research has mainly focused on LQTS1, LQTS2, and LQTS3, but Andersen-Tawil syndrome (LQTS7), Timothy syndrome (LQTS8), and calmodulinopathies (LQTS14/15) have also been investigated.

Mutations in the genes *KCNQ1* and *KCNH2* (Figure 2) are most common and cause LQTS1 and LQTS2, respectively. The *KCNQ1* gene encodes for the α-subunit of the voltage-gated K^+ channel mediating the slow delayed rectifier K^+ current (I_{KS} current). The KCNH2 or hERG channel is a K^+ channel needed for the rapid delayed rectifier current I_{Kr}. Both the I_{Ks} and the I_{Kr} are outward currents. A total of seven different mutations in the *KCNQ1* gene have been studied[25–34]. Multiple LQTS1 iPSC-CM models showed a dominant negative effect of a *KCNQ1* mutation leading to a diminished I_{Ks} current due to a sarcolemmal deficiency of KCNQ1 channels[25,26,29]. In addition, electrophysiological abnormalities, including a prolonged corrected field potential duration were recorded in LQTS1 iPSC-CMs[26]. Cross-talk between the *KCNQ1* mutation and Ca^{2+} handling abnormalities was reported as well, including the observation that Ca^{2+} antagonists could rescue the electrophysiological phenotype[28]. Other studies reported protective effects of β-adrenergic antagonists[25] or ML277, a selective I_{Ks} activator[29].

KCNQ1 is also affected in Jervell and Lange-Nielsen syndrome (JLNS), which is characterized by life-threatening arrhythmias like ventricular tachycardia and bilateral deafness. JLNS is caused by a homozygous or compound heterozygous mutation in *KCNQ1* or *KCNE1*[35], disrupting the function of the voltage-gated K^+ channel needed for the I_{Ks} current. One study using engineered and patient-derived JLNS iPSC-CMs showed that both mutations led to the electrophysiological JLNS phenotype, with increased action potential duration (APD) and sensitivity to proarrhythmic drugs[36].

Similarly to LQTS1 iPSC-CM models, LQTS2 iPSC-CM models showed APD prolongation[37,38]. In total, eight different *KCNH2* mutations were modeled[28,31,45,37–44]. In general, the LQTS2 clinical phenotype was mimicked *in vitro* by a diminished I_{Kr} current and arrhythmia, caused by a decrease in hERG function due to the *KCNH2* mutation. A study on the *KCNH2* N996I mutation reported only a mild increase in APD without early-after depolarizations (EADs)[42], agreeing well with the mild KCNH2 N996I clinical phenotype[46]. Concerning the molecular mechanisms, trafficking defects were found to occur in the LQTS2 models[40,44] possibly due to a defect in the glycosylation of KCNH2, which could be alleviated by blocking Calpain proteins[44], consequently rescuing the electrophysiological phenotype. As shown for LQTS1, LQTS2 also showed Ca^{2+} handling disturbances which could be rescued with Ca^{2+} antagonists[39]. One study showed that RNAi-mediated knockdown of the mutant allele could rescue the electrophysiological phenotype, suggesting an at least partially dominant negative effect[44].

LQTS3 is caused by gain-of-function mutations in the gene *SCN5A*. In total, nine LQTS3 associated mutations in *SCN5A* have been studied using iPSC-CMs[39,47–54]. This gene encodes the main cardiac Na^+ voltage-gated channel NaV1.5, essential for the Na^+ current that determines the fast upstroke of the cardiac action potential. LQTS3 iPSC-CM studies have shown prolonged APD, possibly due to a slower inactivation of the sodium channel[49] or a faster channel recovery from inactivation[47]. While one study reported an arrhythmogenic effect of mexiletine due to hERG interaction[47], this was not observed in other studies, possibly due to a dosage effect[50]. Detection of EADs varied with the mutation studied, with one study not recording any EADs or delayed after-depolarizations (DADs)[48], which may be the result of significant cardiomyocyte subtype variability[48,49]. Next to LQTS3, mutations in the SCN5A gene can cause Brugada

syndrome (BrS), conduction disease and atrial standstill[55]. However, mixed phenotypes are often seen, known as 'overlap syndrome'. In an iPSC-CM model of overlap syndrome, electrophysiological abnormalities were found, including a significantly diminished I_{Na} current density due to the loss-of-function mutation[51]. BrS was studied in three papers using iPSC-CMs[56–58]. In one of those, three iPSC-CM models were made from patients with BrS where no BrS-associated mutations were found[56]. While a BrS-associated *SCN5A*-1795insD mutation line did show effects due to a reduced sodium channel function, no abnormalities were seen in the aforementioned three iPSC-CM models, indicating that SCN5A dysfunction may not be a prerequisite for BrS. The fact that BrS often only presents in adulthood and is thought to be associated with ventricular fibrosis[59] raises the question whether an iPSC-CM model can successfully model such a multifactorial disease at this stage.

Andersen-Tawil Syndrome (ATS) or LQTS7 is a rare autosomal dominant genetic disorder linked to mutations in *KCNJ2* (ATS1). Patients with mutations in *KCNJ2* account for ~70% of cases, while the genetic cause of the remaining 30%, labelled as type 2 (ATS2), remains unknown[60]. *KCNJ2* encodes the voltage-gated, inward-rectifying potassium channel (Kir2.1, Figure 2) that contributes to the inward-rectifier potassium current (IK1). So far three ATS1 mutations have been studied using an iPSC-CM model to elucidate the pathogenesis and find potential drug candidates[61]. ATS iPSC-CMs recapitulated the abnormal electrophysiological phenotype of ATS, showing strong arrhythmic events and irregular Ca2+ release, which could be suppressed by the antiarrhythmic agent, flecainide, through modulation of the NCX current.

Timothy syndrome (TS, LQTS8) is characterized by cardiac arrhythmias in combination with, amongst others, dysmorphic facial features and syndactyly. Up to 2005, only 18 cases were reported[62]. TS is caused by a mutation in the gene *CACNA1C*, encoding for the sarcolemmal

voltage-gated Ca^{2+} channel (Figure 2), CaV1.2, the main cardiac L-type calcium channel, which is indispensable for the generation of the cardiac action potential and excitation-contraction coupling[63]. To date, one TS iPSC-CM model has been studied, which showed DADs and a slow intrinsic beating rate in ventricular iPSC-CMs, agreeing well with bradycardia found in TS patients[64].

LQTS-associated calmodulinopathies (LQTS14 and 15) arise due to mutations in one of the three Calmodulin genes, affecting the Ca^{2+} binding properties of this protein[65]. Clinically, the patients present with life-threatening arrhythmias that are often resistant to conventional therapies. LQT14-iPSC-CMs showed prolonged repolarization with altered rate-dependency, which resulted in augmented inward current during the plateau phase and failure to adapt to high pacing rates[66]. Importantly, this study used addition of simulated IK1 by dynamic-clamp, to overcome the extremely low IK1/Kir2.1 expression in iPSC-CMs and mimic a mature action potential profile. Two studies using iPSC-CM models of LQT15 were able to show a dominant negative effect of single heterozygous *CALM2* mutations on the suppression of L-type Ca2+ channel (LTCC) inactivation[67,68]. Consistent with clinical phenotypes[69], LQT15-iPSC-CMs exhibited significantly lower beating rates and prolonged APD, compared with control cells, including allele-specific knockout cells.

b. Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

Affecting around 1 in 10,000 people, CPVT is characterized by stress-induced ventricular arrhythmia, potentially causing sudden cardiac death in young individuals[70]. While one subtype of CPVT (CPVT1) is caused by mutations in the ryanodine receptor type 2 gene (*RYR2*), a second subtype is caused by a mutated calsquestrin-2 gene (*CASQ2*). Both RYR2 and CASQ2

are needed for correct Ca^{2+} handling in the cardiomyocyte: while the RYR2 channel allows for Ca^{2+} flow out of the sarcoplasmic reticulum in the case of depolarization, CASQ2 is an abundant Ca^{2+}-binding protein in the sarcoplasmic reticulum. Next to RYR2 and CASQ2, mutations in triadin (*TRDN*), *CALM1/2*, *KCNJ2*, and *TECRL* are also known (Figure 2 and Supplementary Table 1, 2).

Altogether, 16 different mutations causing CPVT have been investigated[71,72,81–86,73–80]. Induction of the arrhythmic phenotype by stressors including adrenergic agonists and pacing was observed in all CPVT iPSC-CM models. It was postulated that the deviant effects of adrenergic stimulation in CPVT are caused by altered CaMKII signaling[76]. Pacing also induced a negative inotropic effect as well as DADs in both CPVT1[71] and CPVT2 models[75]. Importantly, a more immature phenotype of CPVT iPSC-CMs was observed compared to control iPSC-CMs[75,79,80], raising the question whether this comparison is adequate. However, the validity of the CPVT iPSC-CM model has been shown[78], as responses to dantrolene agrees well with the responses in patients, depending on their respective mutations.

c. Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia (ARVC)

ARVC is an inherited primary cardiomyopathy associated with increased risk of ventricular arrhythmia and sudden cardiac death. One in 5,000 people are thought to be affected worldwide[87]. Forty to fifty percent of ARVC patients harbour mutations in desmosomal proteins, including plakoglobin, plakophilin, desmoplakin, desmoglein 2 and desmocollin 2[88]. The desmosome is needed for cell-to-cell attachment, connecting the cytoskeleton of neighbouring cells (Figure 2). So far, six different mutations have been studied using iPSC-CM models[89–94]. In these studies, on baseline (before treatment with adipogenic medium), desmosome abnormalities

were observed, and in two studies darker[89] or clustered[90] lipid droplets were seen. Signalling pathways including the canonical Wnt pathway and the PPARγ pathway were found to be upregulated, which became more pronounced after treatment with so-called 3 Factor or 5 Factor adipogenic medium[90,91]. The latter caused metabolic changes that could not be reversed by introducing a wild-type plakoglobin transcript[91]. The same study showed that, by enriching for Isl+ cardiac progenitor cells and thus the amount of right ventricular (RV) cardiomyocytes, Isl1+ cells confer the dominant RV pathology seen in ARVC patients, such as lipogenesis. Additionally, the reduction in desmosomal protein levels in ARVC iPSC-CM models is in close agreement with histopathological data from ARVC patients[95]. Nonetheless, it remains questionable whether iPSC-CMs treated with the 3 Factor and/or 5 Factor method, protocols using different small-molecule compounds and hormones to switch metabolism and accelerate pathogenesis, truly reflect the physiological environment *in vivo*, although PPARγ upregulation might reflect a more mature iPSC-CMs model[19]. In addition, the clinical ARVC phenotype may be provoked by exercise, possibly causing more mechanical stress on the cardiomyocytes[96]. It is challenging to model all the external influences *in vitro* in an iPSC-CM model, but this will likely help to elicit a more accurate disease phenotype.

d. Dilated Cardiomyopathy (DCM)

Dilated cardiomyopathy (DCM) is the most common cardiomyopathy, with a prevalence of 1 in 250. The aetiology is divers and can be metabolic, valvar, neuromuscular or genetic. The clinical phenotype of DCM includes left ventricular dilation and impaired systolic function, ultimately leading to heart failure. The inherited form of DCM is associated with mutations in more than 20 different genes, coding for proteins in the cytoskeleton, nuclear lamina, and sarcomere[97]. Lamin

A/C (LMNA) DCM is among the most malignant subtypes, its clinical phenotype is characterized by early-onset atrial fibrillation and conduction delay as well as sudden cardiac death[98]. Mutations in the desmin (*DES*) gene, in turn, can cause HCM, DCM[99] or ARVC[100]. Of all DCM cases, 30% arise due to mutations in the titin (*TTN*) gene[101].

Recent studies using iPSC-CM disease modelling have aimed to elucidate the molecular pathogenesis of mutations in lamin A/C (*LMNA*)[102], desmin (*DES*)[103], cardiac muscle troponin T (*TNNT2*)[27,104,105], phospholamban (*PLN*)[106], RNA-binding motif protein 20 (*RBM20*)[107,108], *TTN*[109,110] and *BAG3*[111] (Figure 2 and Supplementary Table 1). A total of 13 mutations have been studied, in some cases both homozygous and heterozygous forms. A significant amount of these studies describe disrupted sarcomeres, decreased contractile force and Ca^{2+} handling impairment, which is in agreement with what is seen in heart failure patients[112]. In addition, as seen *in vivo*, β-adrenergic blockers and Ca^{2+} antagonists were proven to attenuate the phenotype[105,107], including sarcomeric disarray and increased apoptosis. Importantly, by using targeted gene correction, two iPSC-CM studies on PLN R14Del could fully revert the in vitro disease phenotype[106,113].

e. Hypertrophic Cardiomyopathy (HCM)

Hypertrophic cardiomyopathy is estimated to be one of the most prevalent hereditary heart diseases in the world[114], affecting up to 1 in 500 individuals worldwide. It is characterized by left ventricle hypertrophy in the absence of a causative hemodynamic burden. Genetically, it is associated with mutations in sarcomeric protein-coding genes, most frequently myosin heavy chain-β (*MYH7*) and cardiac myosin binding protein C (*MYBPC3*)[115,116,117], but also *TPM1*[118,119] (Figure 2). To date, more than 1400 different mutations are known to cause HCM[118].

Ten HCM-causing mutations in *MYH7*, *MYBPC3*, *TPM1*, *ALPK3*, and *PRKAG2* (HCM-like) were studied using iPSC-CM models[27,115,117–124]. These *in vitro* models recapitulate some of the disease phenotypes by reporting Ca^{2+} handling irregularities, cardiomyocyte hypertrophy, sarcomere disarray, arrhythmias and hypercontractility[118,120,121,123]. Several pathways were shown to be affected by the mutations, including Endothelin-1 signalling[119], the canonical Wnt-pathway[121], and most prominently, calcineurin/NFATc4 signaling[119–121]. Both NFATc4 nuclear localization and disrupted Ca^{2+} handling were found to be causal factors in the development of the pathognomonic cellular hypertrophic phenotype. Some conflicting results were also found: while one study observed decreased RyR2 and SERCA2a expression levels[121], another reported the opposite[118]. Additionally, the type of arrhythmogenic events differs significantly; for some mutations, DADs were prominent arrhythmic events[120], while in others, they were accompanied by EADs[118] or no DADs were seen[121]. The exact cause of these heterogeneities is not clear but could be attributed to the maturity of the cells, as the time point of measurement differs significantly between the studies.

f. Left Ventricular Non-compaction Cardiomyopathy (LVNC)

LVNC is characterized by a prominent LV trabecular meshwork, deep intertrabecular recesses in the ventricular wall and a thin compacted layer[125]. Major clinical manifestations include systolic and diastolic dysfunction, systemic embolism, and arrhythmias. LVNC has similar traits as, and shares many of its associated genes with DCM and HCM[126]. However, mutations in *MIB1* have been shown to cause LVNC without additional phenotypes[127]. As an isolated myocardial trait, prevalence is very low, but as a morphologic trait shared by different cardiomyopathies, it is more common. So far, one iPSC-CM LVNC disease model has been developed[128]. In this study

it was shown that iPSC-CMs generated from LVNC patients with a mutation in the cardiac transcription factor TBX20 revealed reduced baseline proliferative capacity by ~50%, which recapitulates the cell cycle defects thought to play a role in LVNC pathogenesis. Additionally, the LVNC iPSC-CM model was associated with perturbed transforming growth factor beta (TGFβ) signalling through PRDM16, a gene in which specific mutations are known to cause LVNC[129].

g. Hypoplastic Left Heart Syndrome (HLHS)

Three iPSC-CM models of hypoplastic left heart syndrome[130–132] have been established. HLHS is a rare congenital heart defect that is characterized by a severely underdeveloped left heart. HLHS iPSC-CMs showed an immature cellular phenotype and a more foetal-like gene expression profile, consistent with observations *in vivo*[133]. In one study mutations in the *MYH6* gene were correlated to defective heart development, as they documented defective cardiomyogenesis in heterozygous *MYH6* mutated iPSC lines[131], whereas a more recent study found several *NOTCH* mutations and showed re-activation of NOTCH signalling to partly rescue the *in vitro* disease phenotype[132].

h. GATA4 Congenital Heart Disease

Congenital heart defects (CHD) are the most prevalent of all human developmental malformations with an incidence of 6–8 per 1,000 live births[134]. Atrial and ventricular septum defects are the most common type of CHD, present in 50% of all cases of CHD. Mutations in *GATA4*, a cardiac transcription factor, have been shown to cause cardiac septal defects and cardiomyopathy[135]. Mechanistically, the *GATA4* G296S mutation results in a reduced affinity for

its binding element, decreased transcriptional activity, and disrupted interaction with Tbx5. Using iPSC-CMs, this mutation was shown to impair contractility, calcium handling, and metabolic activity, through disruption of transcriptional cooperativity[136]. Namely, the mutation disrupted 1) TBX5 recruitment, associated with dysregulation of genes related to cardiac septation, 2) GATA4 and TBX5-mediated repression at non-cardiac genes and 3) enhanced open chromatin states at endothelial/endocardial promoters.

i. Monogenic diseases with cardiac traits

RASopathies: Cardiofaciocutaneous syndrome (CFCS) and Noonan syndrome with multiple lentigines (NSML)

The genes that are mutated in CFCS and NSML (formerly known as LEOPARD syndrome) encode proteins that function in the RAS/MAP kinase pathway. CFCS is characterized by the simultaneous occurrence of multiple congenital abnormalities and mental retardation. Along with e.g. macrocephaly and hyperkeratosis, most patients present with congenital heart defects like pulmonic stenosis or HCM[137]. To date, around 60 cases of CFCS have been published[138], and two *BRAF* mutations were characterized using iPSC-CMs, where an upregulation of a foetal gene program, including ANP, was shown as well as arrhythmogenicity[139,140]. The model also showed the involvement of fibroblasts in developing the pathognomonic cardiomyocyte phenotype[139]. NSML is another very rare inherited disorder, with approximately 85% of patients carrying a mutation in the *PTPN11* gene and commonly developing HCM, in addition to many other disease features. The HCM phenotype was recapitulated in an NSML iPSC-CM model where cardiomyocyte enlargement and preferential nuclear localization of NFATc4 was shown[10].

Duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD) is a severe X-linked neuromuscular disorder that affects approximately 1 in 3,500 new-born males. DMD is caused by mutations in dystrophin, which encodes a rod-shaped cytoplasmic protein that anchors the sarcomere to the extracellular matrix. DMD usually presents in early childhood with progressive muscle weakness. Because of therapeutic improvements for DMD, patients have a longer lifespan, but resulting in cardiomyopathy to become a prevalent cause of mortality[141]. The cardiac phenotype observed in DMD patients includes arrhythmias, structural alterations and hemodynamic abnormalities, and patients still die young.

Nine different mutations in the dystrophin gene, affecting different isoforms of the protein, have been studied using iPSC-CM models[142–146]. Part of the initial work done on DMD iPSC-CMs models focused on gene expression and its rescue[142,143]. CM differentiation was observed to be less efficient in the DMD cell lines[143], and iPSC-CMs showed increased cellular damage, apoptosis and altered Ca^{2+} handling[144,145]. The use of 3D-engineered cardiac tissues has shed some light on the impaired response to external cues in DMD iPSC-CMs[146]. Guan et al.[144] found elevated cardiac injury markers *in vitro* with DMD iPSC-CMs, which is in agreement with clinical data[147].

Other Myopathies

Myotonic Dystrophy type 1 (DM1) is a genetic multisystem disorder with a minimum prevalence of 8–10 in 100,000[148]. DM1 can occur from birth to old age, and symptoms can vary significantly between patients, from minor muscle pain to severe muscle weakness, respiratory issues, myotonia and cardiac conduction defects. DM1 results from an unstable trinucleotide

(CTG) repeat expansion within the 3' end of the dystrophia myotonica protein kinase (*DMPK*) gene[149]. As there is no effective therapy, a recent study aimed to develop genome therapy in DM1 iPSCs to reverse the phenotype for developing autologous stem cell therapy for DM1[150]. While DM1 iPSC-CMs showed presence of nuclear RNA foci and aberrant splicing of MBNL1/2, insulin receptor, and cardiac troponin T, removal of mutant transcripts by genome-treatment in DM1 iPSC-CMs led to reversal of the disease phenotype.

Another gene related to multisystem disorders featuring cardiomyopathy and skeletal myopathy is *HSPB5*, encoding a small heat shock protein[151]. The 343delT mutation has been shown to cause early-onset skeletal myopathy presenting with limb weakness and hypotonia in early childhood. In an iPSC-CM model of early-onset skeletal myopathy, aggregation of insoluble mutated protein and induction of a cellular stress response was observed[152]. *In vitro* refolding of 343delT in the presence of wild-type rescued its solubility, concordant with the recessive inheritance of the disease.

Friedreich's ataxia (FRDA)

Friedreich's ataxia is an autosomal recessive disorder causing neurodegeneration and cardiomyopathy. It is estimated to occur in up to 1 in 20,000 Caucasians and is caused by an excessively expanded GAA repeat within the first intron of the frataxin gene, leading to epigenetic silencing of the gene. Frataxin is a small mitochondrial protein involved in iron sulphur cluster biosynthesis. FRDA usually presents with progressive ataxia between 10 and 15 years of age, causing dysarthria, muscle weakness and spasticity. Around two thirds of all FRDA patients also suffer from cardiomyopathy[153].

In FRDA iPSC-CMs, fibrillary disarray, mitochondrial damage, and calcium handling deficiency were observed under standard culture conditions[154,155]. However, challenging the iPSC-CMs with increasing concentrations of iron led to hypertrophy, mitochondrial damage and Ca^{2+} handling disruptions[156,157]. Deferiprone, a drug counteracting iron overload in β-thalassemia, was reported to relieve stress-stimulation in FRDA iPSC-CMs[157], consistent with clinical studies reporting a reduction in heart hypertrophy in FRDA patients[158]. However, the GAA repeat length reportedly changed during iPSCs generation[154], which has to be taken into account when directly comparing the *in vitro* findings to the clinical phenotype.

Barth syndrome (BTHS)

Barth syndrome is caused by a mutation in the *TAZ1* gene coding for tafazzin, a CoA-independent phospholipid acyltransferase. This enzyme is necessary for the maturation of cardiolipin, a component of the inner mitochondrial membrane. The mutation results in a multidimensional phenotype: dilated cardiomyopathy, skeletal myopathy, neutropenia, growth retardation and increased urinary excretion of 3-methylglutaconic acid in early childhood[159]. So far, one BTHS iPSC-CM model exists[160]. Using modified RNA to rescue TAZ1 expression and CRISPR/Cas9-based scarrless genome editing to correct the *TAZ1* mutation, this study showed that the *TAZ1* mutation was causal for the biochemical phenotype including cardiolipin levels, mitochondrial and ATP deficits, as well as of the functional cellular phenotype, including sarcomeric disarray and diminished contractility. Normalizing mitochondrial ROS production could attenuate this phenotype, and the therapeutic agent bromoenol lactone increased cellular cardiolipin levels, thereby improving sarcomere organization as well as twitch strength.

Storage diseases: Pompe, Fabry, and Danon disease

In the US, around 1 in 40,000 new-borns have Pompe disease[161], also called Glycogen Storage disease Type II. It is caused by an autosomal recessive mutation in the acid alpha-glucosidase (*GAA*) gene, encoding for a lysosomal glycogen-degrading enzyme. Depending on the residual function in the mutated *GAA* gene, there are early and late onset forms. The classic clinical presentation of infantile-onset Pompe disease includes generalized muscle weakness as well as cardiomegaly and hypertrophic cardiomyopathy[162]. Enzyme replacement therapy is the golden standard, using recombinant human GAA (rhGAA). The first successful iPSC generation from an early onset Pompe disease patient was not possible without the introduction of an inducible *GAA* transgene[163], whereas two other studies were able to generate early and late onset Pompe iPSCs without the addition of GAA[164,165]. The early onset Pompe disease iPSC-CMs were shown to have the typical cellular Pompe phenotype, including large glycogen-containing vacuoles, multiple large lysosomes and autophagosomes as well as deteriorating mitochondria, in close agreement with previous histological findings[166]. RhGAA was able to partially rescue this phenotype in Pompe iPSC-CMs. Research on a late-onset Pompe cell line recapitulated part of the aforementioned findings and reported a successful attenuation of the cellular phenotype using a lentiviral GAA rescue[164]. A more hypertrophic phenotype, as expected with Pompe disease cardiomyocytes, could not be observed, which could partly be due to the immaturity of the examined iPSC-CMs. Notably, Raval et al.[165] were able to detect protein hypoglycosylation in Pompe iPSC-CMs, which is associated with altered Ca^{2+} handling[167] that, in turn, may cause cardiac hypertrophy[120].

Fabry disease, another storage disease, is caused by an X-linked alpha-galactosidase A (GLA) deficiency, leading to progressive build-up of globotriaosylceramide (GL-3) in

lysosomes. Clinically, the deficiency results in renal failure, left ventricular hypertrophy and possibly strokes[168]. Similar to Pompe disease, enzyme replacement therapy is the current treatment option. In the first Fabry iPSC-CM model[169], low GLA activity and the accumulation of GL-3 was shown to cause abnormal sarcomeric structures, consistent with observations in endomyocardial biopsies[170]. Glucosylceramide synthase inhibition and recombinant GLA were shown to both prevent and reverse the cellular phenotype. Subsequent studies of Fabry iPSC-CMs showed cellular hypertrophy, impaired contractility, decreased metabolism[171], and increased IL-18 levels in both iPSC-CMs and patient sera, in parallel with LV hypertrophy progression[172]. Importantly, IL-18 neutralization reduced progression of hypertrophy *in vitro*.

Danon disease (DD) is an X-linked lysosomal and glycogen storage disorder characterized by impaired autophagy caused by mutations in the *LAMP2* gene, encoding the lysosomal-associated membrane protein type 2. Patients present with severe cardiac and skeletal muscle abnormalities resulting in HCM, heart failure and sudden cardiac death[173]. In an iPSC-CM model of DD, the impairment of autophagic flux, mitochondrial damage, and increased apoptosis were observed[174]. Additionally, DD iPSC-CMs were significantly larger and exhibited increased calcium decay times compared to healthy iPSC-CMs, thereby recapitulating the hypertrophy and decrease of contractile function seen in DD patients. While DD causes early-onset fatal cardiomyopathy in male patients, female patients show a later onset and less severe clinical phenotype, which has been attributed to random inactivation of the *LAMP2* gene on the X chromosome[175]. Indeed, a recent study using DD iPSC-CMs provided evidence that this random inactivation is responsible for the phenotype in female patients[176].

Other diseases with cardiac traits

iPSC-CMs have also been used to model familial transthyretin amyloidosis (ATTR), an autosomal-dominant protein-folding disorder leading to multi-organ failure and death[177]. Hepatocytes of ATTR patients with mutations in the transthyretin (*TTR*) gene produce mutant TTR that forms aggregates and fibrils in target organs, mainly the heart and peripheral nervous system. Patient specific iPSCs were differentiated into hepatic, neuronal, and cardiac lineages thereby modelling the three major tissue types involved in this disease. The demonstration that hepatic secreted mutant TTR decreased iPSC-CM cell survival, supports the use of patient-specific iPSC as models for studying disorders affecting multiple organs.

An iPSC model of abetalipoproteinemia (ABL) has also been used to illustrate a disease involving more than one organs[178]. ABL, or Bassen-Kornzweig syndrome, is a rare autosomal recessive disorder of lipoprotein metabolism, resulting from mutations in the gene encoding the microsomal triglyceride transfer protein (*MTTP*)[179]. In addition to expression in the liver and intestine, MTTP is also expressed in cardiomyocytes, a finding that may explain why several ABL patients exhibit cardiac arrhythmias and heart failure[180]. ABL iPSC-CMs failed to secrete apoB and show elevated lipid storage defects, associated with ABL disease, and are hypersensitive to metabolic stress[178].

iPSC-CM models may also be useful to potentially improve risk management of coronary artery disease (CAD), as shown by a study using iPSCs with an aldehyde dehydrogenase 2 (*ALDH2*) mutation (ALDH2*2), which is linked to an increased risk of CAD and more severe outcomes, and occurs in 8% of the human population[181,182]. It was shown that the ALDH2*2 mutation resulted in elevated ROS and toxic aldehydes, inducing cell cycle arrest and apoptosis, especially during ischemia.

j. Patient-independent applications of iPSC-CMs

Next to monogenetic diseases affecting the heart, several studies have established iPSC-CM models for acquired cardiac diseases as well. Diabetic cardiomyopathy was modelled by switching fatty acid-adapted wild-type iPSC-CMs to a glucose-rich medium containing endothelin 1 and cortisol, resulting in induction of a diabetic stress phenotype, showing hypertrophy, lipid accumulation and peroxidation[183]. An iPSC-CM model of Takotsubo cardiomyopathy has also been established, which showed that enhanced β-adrenergic signalling and a higher sensitivity to catecholamine-induced toxicity are associated with the disease phenotype[184]. In addition, iPSC-CM disease modelling has been successfully extended towards infectious diseases affecting the heart, namely coxsackievirus-induced viral myocarditis[185,186], and endotoxin-induced inflammation[187], showing that iPSC-CMs possess the reaction system involved in both viral and endotoxin-induced inflammation. In the viral myocarditis models, multiple drugs, including Interferon β1, but not Interferon α, were shown to attenuate the infection by abrogating virus proliferation *in vitro*.

iPSC-CM models have been used successfully to recapitulate cardiotoxic drug effects, as reviewed in greater detail elsewhere[188]. A deeper insight into the individual susceptibility of breast cancer patients to doxorubicin-induced cardiotoxicity (DIC) was provided using patient specific iPSC-CMs[189]. However, the use of this model for the discovery of novel DIC cardioprotectants might be limited, as one of two tested cardioprotectants increased toxicity, possibly due to the use of immature iPSC-CMs. In another study, the effect of the drug combination Sofosbuvir and Amiodarone, known to cause severe bradycardia *in vivo*, was studied using iPSC-CMs. Indeed, this combination, among others, was shown to cause impaired Ca^{2+} handling *in vitro*[190]. iPSC-CMs have also been used to screen for cardiovascular toxicities

associated with anticancer tyrosine kinase inhibitors[191]. Importantly, the results correlated with clinical phenotypes and this study led to the finding that toxicity can be alleviated via cardioprotective insulin/IGF signalling. Additionally, efforts to establish alcoholic cardiomyopathy and peripartum cardiomyopathy iPSC-CM models are ongoing.

Discussion

Using human iPSC-CMs for disease modelling has certainly made progress in recent years with more than 25 iPSC lines carrying new disease specific mutations published every year since 2013 (Supplemental Table 1). Currently, as shown in our review, over 150 cardiac disease specific iPSC lines have been described, some available from repositories and cell banks. The relevance of using these iPSC-CMs as disease models is well established as they reflect pathophysiological characteristics of a broad range of inherited cardiac diseases.

iPSC-CM models of LQTS, CPVT and ARVC mimicked the electrophysiological abnormalities and drug responses seen in these inherited arrhythmic diseases which emphasizes the potential use of these models to develop patient-specific clinical regimens and as drug screening platforms. A striking example of the predictive value is the susceptibility to arrhythmogenic effects of cisapride seen in LQTS (and HCM) iPSC-CMs, which was previously observed in patients and the reason to withdraw this drug from the market[27]. In another study, a panel of genetically diverse iPSC-CMs reproduced the patients susceptibility to develop a cardiotoxic response to sotalol, a QT-prolonging drug[192]. Moreover, an iPSC-CM model of LQTS3 could be treated with a combined pacing and pharmacological approach similar to the treatment regimen used in these patients[47].

iPSC-CM models of DCM and HCM also agreed well with *in vivo* characteristics, showing impaired Ca^{2+} handling, decreased contractile force and sarcomeric disarray in DCM, and Ca^{2+} handling irregularities, cardiomyocyte hypertrophy and arrhythmias in HCM. Additionally, iPSC-CM models of LQTS, DCM, HCM, LVNC, CHD, Pompe disease, NSML, and Takotsubo cardiomyopathy enabled researchers to gain new insights on the molecular pathogenesis leading to the disease phenotype observed in these models and patients, providing potential new leads for individualized therapies[10,44,104,119–121,136,165,184,193]. As one example, a DCM *TNNT2* R137W iPSC-CM model showed epigenetic activation of phosphodiesterase (PDE) expression, a newly discovered pathological mechanism in this DCM subtype, presenting the potential of using PDE inhibitors in these patients[104].

Genome editing has particularly helped to progress iPSC-CM disease modelling. In several disease models, genome editing was used to reverse mutated, disease causing genes to the healthy wild type[28,106,110,113,150], validating the pathogenicity of the mutation by showing a reversal of the disease phenotype, and providing an ideal diseased cell to healthy cell comparison to gain novel insight on the involved signalling pathways. Moreover, these studies lay the groundwork for developing targeted gene therapy for patients with hereditary cardiac disease.

In addition to the successes of inherited cardiac disease models, iPSC-CM models of anti-cancer related drug toxicity produced novel insight on how to combat drug toxicity, provided a cardiac safety index for tyrosine kinase inhibitors[191], and showed patient-specific vulnerability to doxorubicin to potentially guide decision making of using doxorubicin[189].

To summarize the progress, the presented studies have shown that iPSC-CM disease models reflect important clinical phenotypes and can be used to elucidate the molecular

mechanisms and cellular phenotypes of a large variety of cardiac diseases. Moreover, iPSC-CM models can be used to predict patient responses to novel or existing drugs.

However, despite the progress, pitfalls are still present and several limitations and challenges need to be considered when using iPSC-CMs for cardiac disease modelling.. Firstly, patient characteristics and the choice of cell type for reprogramming will influence individual iPSC line characteristics and hence the quality as a disease model system. It has been postulated that while the pathogenic mutation is the 'first hit', age, gender, ethnicity, comorbidities, and environmental factors, or so-called 'second hits' may play an important role in developing a clinical phenotype[194]. For example, ethnicity can modulate the clinical presentation of HCM[195], as well as show differences in basic muscle metabolism as measured by CK levels[196]. Most papers do not include detailed patient characteristics, and the 'second hit' hypothesis has yet to be tested in iPSC-CM models.

The cell type used for generation of the iPSC line can also influence characteristics of iPSC-CM modelling of cardiac disease, since the original cell type-specific epigenetic pattern can persist in reprogrammed iPSC lines, and differentiation efficiency is affected[197,198]. In the studies covered by our review, iPSCs were generated either from skin biopsies, urine or blood samples, but none of the studies assessed potential differences between iPSCs, and derivative iPSC-CMs, generated from different cell types of the same donor. Furthermore, the X-inactivation status in female iPSCs, which can either be retained or erased during the generation of iPSCs, depending on the protocol used[199], has not been assessed.

Secondly, the choice of controls is another critical aspect when assessing and comparing results from studies using iPSC-CM models. Some papers have used asymptomatic carriers as

controls, which offer some insight in the importance of second hits. Most often the cellular phenotype of iPSC-CM models has been compared to healthy control iPSC-CMs, sometimes including (gender-matched) family members, to ensure a similar genomic background. Isogenic control iPSC lines, generated by genome editing, so far are the ideal controls as they have the same genomic background of the original disease model iPSC line and provide a relevant and true 'healthy control[31,42].

Thirdly, there is considerable variability in protocols for differentiation and characterization of iPSC-CMs (Supplementary Table 1). This may lead to observed variances in differentiation efficacy, defined as the ratio of cardiomyocyte marker positive cells at a specific time point of the differentiation protocol, and kinetics, defined as the time point in the differentiation protocol for the emergence of a specific cardiomyocyte marker, e.g. spontaneous beating. iPSC-CM models also differ frequently by the cardiomyocyte subtype generated, with varying ratios of ventricular, atrial, and nodal cells, affecting comparability of essential read-outs, like electrophysiology and contractile force. Depending on the disease studied, culture conditions for iPSC-CMs can affect outcomes notably. The arrhythmic phenotype of CPVT is present only when stimulated with adrenergic agonists, comparable to the clinical phenotype, whereas the ARVC iPSC-CM phenotype is not as overt at baseline as after stimulation with adipogenic medium.

Finally, we have compared assays used to assess cardiomyocyte maturation in the different studies. Only few studies compare iPSC-CM gene expression levels to adult cardiac tissue and assess sarcomeric and mitochondrial organization or other morphological features (Supplementary Table 1). Although electrophysiological properties of spontaneously beating iPSC-CMs have been measured to some extent, spontaneous beating is not standard in adult

cardiomyocytes. Preferably, iPSC-CMs should be paced to analyse potential arrhythmogenicity of their respective mutations. Furthermore, with several specific cardiac diseases manifesting exclusively during adulthood, the relatively immature iPSC-CMs might only partly recapitulate the disease phenotypes. Therefore, assessment of maturation of iPSC-CMs used for cardiac disease modelling is advisable and we have listed key characteristics and related recommended measurements in Table 1.

As outlined so far, significant progress has been made with iPSC-CM disease models, while several pitfalls still need attention. Several suggestions to improve and exploit the full potential are currently discussed[200] and several research groups have started to implement them.

In order to make results reproducible and inter-comparable between research groups, standardization of protocols for the generation of iPSC lines and iPSC-CMs is essential. Standardization is needed on various methodological levels, from selecting patients (as cell donors), cell types and reprogramming protocols, in order to minimize confounding effects on the iPSC lines and thus potential differences in derivative iPSC-CMs, to protocols for directed differentiation and characterization of iPSC-CMs. Only if studies use iPSC-CM populations of comparable purity (CMs vs non-CMs), subtype (atrial, ventricular, nodal), and maturity, robust and eventually more clinically relevant results can be acquired.

Additionally, to increase rigidity of cardiac disease modelling studies, not only multiple patients as donors of cells to generate iPSC lines, but also multiple cell lines from each reprogramming[201], should be included to account for variability between individual patients with different genetic background and comorbidities, and between different clonal lines with

potentially different cellular characteristics arising from stochastic reprogramming events, especially regarding epigenetics[202].

For modelling more complex diseases involving multiple cell types and specific spatial organization, like ARVC, which is characterized by fibro-fatty depositions inside the myocardial tissue and crosstalk between different cell types[89], an increase in model complexity from a homogeneous 2D cell layer to 3D structures with multiple cell types will be needed[203,204]. These can be potentially generated by the same iPSC line, leading to patient specific myocardial tissue engineering, but also could use a mixture of different backgrounds, e.g. diseased and healthy. Recently, the necessity of complex models was shown for a specific type of DCM linked to mutated titin, with engineered heart tissues (EHT), composed as 3D structures of iPSC-CMs and stromal cells in a collagen type I / fibrinogen matrix, showing the typical contractile deficient phenotype, whereas the phenotype was absent in the 2D iPSC-CM model[110].

Finally, especially if standardization issues have been addressed, as many iPSC lines and iPSC-CMs from various disease specific backgrounds should be collected, validated, stored and made accessible to researchers[205,206] to build a comprehensive and cohesive body of knowledge on inherited cardiac disease. This will lead to improved disease models and generation of a critical amount of reproducible and robust data to draw clinically relevant conclusions, which would otherwise rely on animal and first-in-man studies, without having assessed the fundamental disease mechanisms and potential new therapies in a valid human, cardiac disease specific modelling system, as represented by iPSC-CMs.

Conclusion

Patient-specific iPSC-CM models have demonstrated the ability to facilitate the study of many aspects of the clinical disease phenotype and are particularly useful in elucidating the molecular mechanisms of certain cardiac diseases. However, major differences between the complex *in vivo* architecture and pathophysiology and simplified *in vitro* conditions can result in limitations for recapitulating functional mechanisms. It is evident that the use of iPSC-CMs for disease modelling requires a set of criteria for thorough characterization, which we have listed in Table 1. Additionally, future optimization of iPSC-CM maturation, functional tissue engineering and culture conditions should lead to the establishment of more predictive iPSC-CM disease models that more closely mimic the disease. Finally, a consensual multi-dimensional approach to standardize generation of iPSC, differentiation to iPSC-CM, functional characterization of iPSC-CM and to define valid controls is necessary to generate more robust and reproducible iPSC-CM disease models.

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Legends:

Figure 1. Methods used to generate iPSC-CM models. A) Cell source. B) Reprogramming method. C) Differentiation method. D) Proportion of iPSC cell lines generated per disease. Data were retrieved from Supplementary Table 1.

Figure 2. Subcellular localization of cardiac disease-associated proteins studied using iPSC-CM models.

Table 1. Key characteristics of iPSC-CM maturation and related recommended measurements to assess these characteristics.