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REGENERATIVE MEDICINE (SM WU, SECTION EDITOR)

Modeling Nonischemic Genetic Cardiomyopathies Using Induced Pluripotent Stem Cells

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Abstract

Purpose of Review The advent of induced pluripotent stem cells (iPSC) has paved the way for new in vitro models of human cardiomyopathy. Herein, we will review existing models of disease as well as strengths and limitations of the system.

Recent Findings Preclinical studies have now demonstrated that iPSCs generated from patients with both acquired or heritable genetic diseases retain properties of the disease in vitro and can be used as a model to study novel therapeutics. iPSCs can be differentiated in vitro into the cardiomyocyte lineage into cells resembling adult ventricular myocytes that retain properties of cardiovascular disease from their respective donor. iPSC pluripotency allows for them to be frozen, stored, and continually used to generate iPSC-derived myocytes for future experiments without need for invasive procedures or repeat myocyte isolations to obtain animal or human cardiac tissues.

Summary While not without their limitations, iPSC models offer new ways for studying patient-specific cardiomyopathies. iPSCs offer a high-throughput avenue for drug development, modeling of disease pathophysiology in vitro, and enabling experimental repair strategies without need for invasive procedures to obtain cardiac tissues.

Keywords Induced pluripotent stem cell (iPSC) · Genetic cardiomyopathy · Lysosomal storage disorders · Infiltrative cardiac disorders · COVID-19 myocarditis · Gene editing

Introduction

Induced pluripotent stem cells (iPSC) have become an invaluable tool for studying diseases of various organ systems [1–6]. In the past, there has been very limited access to cardiac tissues for research, obtainable only through open heart surgeries or endomyocardial biopsy, which are invasive and can potentially increase harm to the patient. Through a simple cheek swab, blood draw, or skin biopsy, scientists can obtain somatic cells and then induce their pluripotency to become stem cells in vitro. These iPSCs can then be differentiated into any tissue-specific lineage for further study depending on the environment in which they are cultured. In 2007, James Thomson (University of Wisconsin) and Shinya Yamanaka (Kyoto University) both published their methods for generating iPSCs. Thomson used the four genes *Oct4*, *Sox2*, *NANOG*, and *LIN28* while Yamanaka used *Oct4*, *Sox2*, *Klf4*, and *c-Myc* [7, 8]. Methods for differentiation of iPSCs into cardiac lineages subsequently allowed for generation of iPSC-derived cardiomyocytes (iPSC-CMs) used to study cardiovascular disease in vitro [9–15]. This has accelerated cardiovascular research in myriad ways. While the initial focus was on using iPSC for regenerative therapy, more recent work suggests their utility for disease modeling, the first study of which was on LEOPARD syndrome [16]. Herein, we will focus on cardiac disease modeling using iPSC, including both acquired and inherited disorders.

Various genetic mutations of cardiomyocytes have been associated with arrhythmogenic right ventricular

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cardiomyopathy/dysplasia (ARVC/D), familial-dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), noncompaction cardiomyopathy (NCCM), and restrictive cardiomyopathy (RCM). These genetic diseases are quite heterogeneous, even within each phenotype. Systemic disorders including lysosomal storage disorders and infiltrative disorders such as hemochromatosis have also been shown to negatively affect cardiac function. Finally, iatrogenic, infectious, and even behavioral etiologies have been implicated. iPSC studies over the last decade have delineated and recapitulated many of these pathological features (Table 1).

Arrhythmogenic Cardiomyopathies

Arrhythmogenic right ventricular cardiomyopathy is a rare, inherited cardiac disorder in which progressive heart failure and arrhythmias result from fibrofatty infiltration of myocardium. It is associated with mutations to desmosomal, adherens junction, and gap junction proteins, though more than half of ARVC patients will not have an identified causative genetic mutation [18–21, 23–25, 37, 67–70]. Variants in five cardiac desmosomal genes account for more than 60% of cases of inherited ARVC: plakoglobin (*JUP*), plakophilin-2 (*PKP2*), desmoglein-2 (*DSG2*), desmoplakin (*DSP*), and desmocollin-2 (*DSC2*) [23]. Many studies have validated that iPSC-CMs generated from fibroblasts isolated from ARVC patients can recapitulate disease characteristics in vitro [67–70], and these cells been used to elucidate pathomechanisms of fibrofatty infiltration, conduction abnormalities, and cardiomyocyte death. Novel mutations have also been identified that may be involved in disease pathogenesis. One novel mutation is N116S in desmin (*DES*), one of the key intermediate filament proteins, which results in abnormal intermediate filament organization and abnormal desmin aggregates [22].

When compared to control cells, iPSC-CMs from ARVC patients demonstrate cytoplasmic lipid droplets, increased pleomorphism, irregular Z bands, and altered calcium handling [17, 26]. Cardiac conduction experiments have previously been hindered by the cytotoxicity of fluorescence imaging and the low spatial resolution and signal-to-noise ratio of microelectrode arrays [19]. iPSC-CMs overcome these limitations to provide more accurate conduction velocity mapping. Studies with *PKP2* knockdown iPSC-CMs showed low conduction velocities, recapitulating the arrhythmogenic properties of a mutated *PKP2* [19]. In *DSG2* deletion variants, iPSC experiments validated the established abnormalities of ARVC: cytoskeletal disorganization and disarray, increased cytokine expression and inflammation, and aberrant electrophysiology secondary to alterations in calcium handling [23].

iPSC models have shown that ARVC iPSC-CMs exhibit a rate of apoptosis three times higher than controls. This may be mediated by reduced wingless-related integration site (*WNT*) ligands, allowing for degradation of catenin β1 that is protective against apoptosis [17, 71, 72]. iPSC models of the WNT/β-catenin and RhoA-ROCK pathways showed that the concomitant inactivity of both pathways is necessary for a significant increase in peroxisome proliferator-activated receptor-γ (PPAR-γ) expression, which may lead to fibrofatty infiltration of the myocardium [24, 25]. Other implicated pathways include Hippo-Yes-associated protein (*YAP*) and transforming growth factor-β (TGF-β) [17].

iPSC studies also revealed a novel frameshift mutation in obscurin (*OBSCN*), a gene that has been frequently associated with HCM, DCM, and NCCM, but not previously in ARVC [26]. In ARVC-derived iPSC-CMs, the frameshift mutation resulted in decreased expression and disrupted localization of ankyrin1.5, hypothesized to increase calcium currents and PPAR-γ expression which may mediate arrhythmogenesis and fibrofatty infiltration [24–26].

Dilated Cardiomyopathy

Familial-dilated cardiomyopathy is characterized by eccentric hypertrophy and progressive systolic dysfunction. One-third to one-half of cases are associated with genetic mutations [27], most commonly involving titin (*TTN*) [27, 28]. Many other structural and sarcomeric proteins have been implicated in the pathogenesis of DCM. iPSC-CM studies have focused on *TTN*, lamin (*LMNA/C)*, BCL2-associated athanogene 3 (*BAG3*), and cardiac troponin T2 (*TNNT2*).

Titin, the largest protein in the human body, helps to maintain structural integrity of the myocyte and sarcomeres, and its mutations are thought to be highly destabilizing. In patient-derived DCM iPSC-CMs, truncations in *TTN* resulted in impaired contractile function [28]. In addition to these baseline deficits, there is an exaggeration of symptoms in response to mechanical and adrenergic stress. In studies with mutated *TTN* iPSC-CMs, administration of positive inotropes resulted in exacerbation of symptoms [73].

LMNA, a nuclear envelope scaffolding protein with critical regulatory roles on gene expression through its effects on stabilization of chromatin, is another gene associated with DCM [29]. *LMNA* is invaluable to nuclear structure and cell function, and its mutations cause many degenerative diseases such as premature aging syndrome (Hutchinson-Gilford Progeria Syndrome) [31]. One particular *LMNA* mutation studied using iPSC was the K117 frameshift, and electrophysiological studies demonstrated disrupted calcium homeostasis that leads to an arrhythmogenic phenotype commonly seen in the laminopathies. Calcium mishandling in this study was shown to be mediated by dysregulation of platelet-derived growth factor (PDGF)-signaling pathway, and treatment experiments with PDGF inhibitors such as sunitinib were found to rescue the iPSC-CMs from arrhythmia [29]. While these therapies could be repurposed in clinical practice, they may

Table 1 Cardiomyopathy-causing genetic mutations and their consequences, as evidenced in iPSC studies

Cardiomyopathy	Implicated gene (protein)	Mutation	iPSC study findings
Arrhythmogenic	*PKP2* (plakophilin 2)	c.2484C > T[17]	Reduced catenin-β1 activity with aberrant lipogenesis and increased PPARγ expression
		1760delT[18] V587Afs*655[18]	Normal desmosomal plakophilin protein reduced by 50%, disrupting intercalated discs
		Knockout[19]	Low conduction velocities and arrhythmogenesis
	JUP (plakoglobin)	Various[17,18,20,21]	Abnormal cell-cell adhesion and communication
	DES (desmin)	N116S[22]	Abnormal cytoplasmic desmin accumulation
	DSG2 (desmoglein-2)	2358delA[23]	Cytoskeletal disorganization and disarray, increased cytokine expression and inflammation, and aberrant electrophysiology secondary to alterations in calcium handling
	WNT (family of secreted glycoproteins)	Various[17]	Reduced WNT-catenin-β1 signaling contributes to apoptotic cardiomyocyte loss
		Various[24]	Increased PPAR-γ expression with concomitant loss of RhoA ROCK pathway
	RhoA-ROCK pathway	Various[24,25]	Increased PPAR-γ expression with concomitant loss of WNT-β1 catenin pathway, leading to fibrofatty replacement of the myocardium
	OBSCN (obscurin)	Frameshift[26]	Decreased expression of anchoring protein ankyrin1.5
Dilated	*TTN* (titin)	Various[27,28]	Reduced contractility and impaired response to mechanical and β-adrenergic stress; pathogenic for familial DCM; most common mutation
	LMNA (lamin A/C)	K117fs[29]	Abnormal activation of the platelet-derived growth factor (PDGF) pathway leading to aberrant calcium handling
		Various[30]	Disruptions in nuclear structure, aberrant cell function, and degenerative disease
		R225X[31] Frameshift[31]	Increased nuclear senescence and hypersensitivity to external stress leading to apoptosis
	BAG3 (bag cochaperone 3)	R447H[32] Various[33]	Disrupted chaperone system and impaired myofibril maintenance
	TNNT2 (cardiac troponin T)	R173W[34]	Aberrant calcium handling and abnormal sarcomeric α-actinin distribution
	SPEG (striated muscle enriched protein kinase)	E1680K[35]	Disruptions in SERCA2a; causes early-onset fulminant DCM

Table 1 (continued)

Cardiomyopathy	Implicated gene (protein)	Mutation	iPSC study findings
Hypertrophic	*MYH7* (β myosin heavy chain)	R663H[36]	Elevated intracellular calcium and contractile arrhythmias
	MYBPC3 (myosin binding protein C)	Various[37,38]	Abnormal calcium handling; increased calcium-independent cross-bridge cycling; contractile defects
		R943X[39] R1073P[39] Frameshift[40] 1358-1359insC[41]	Constitutive expression of nonsense-mediated decay (NMD) pathway, resulting in degradation of mRNA transcripts of *MYBPC3* and other pathways
	MYH7, *MYL2* (myosin light chain 2), *MYL3*	Various[42]	Disrupt IHMs (interacting - head motifs) that play a crucial role ATP binding, calcium binding, and energetics of the cell
	ALPK3 (α-kinase 3)	W1264X[37,43]	Sarcomeric disarray and abnormal intercalated discs
Noncompaction	*ACTC1* (cardiac actin)	E99K[44]	Abnormal contractility, increased calcium sensitivity, arrhythmogenesis
	TBX20 (T-box transcription factor 20)	Y317X[45] T262M[45]	Dysregulated TGF-β signaling, reduced cardiac transcription factors, decreased cell proliferation, poor myocardial development
	PRDM16 (transcription coregulator PR domain containing 16)	Exon 9 truncation[45]	Downstream effector of *TBX20*; causes dysregulated TGF-β signaling leading to reduced cell proliferation and poor myocardial development
	DES	A337P[46]	Desmin aggregates form with abnormal intermediate filament structure
	DSP (desmoplakin)	L1348X[46]	Disruption in linkage of desmosomes with intermediate filaments; most often seen in the setting of Carvajal syndrome
	TPM1 (tropomyosin)	R178H[47]	Weaker cardiomyocyte contractile function; energetics of the cardiomyocyte negatively affected
Restrictive	*DES*	Y122H[48] Y122C[48]	Abnormal cytoplasmic desmin aggregates; pathogenic for RCM
Lysosomal storage disorders	Fabry disease	X-linked; deficiency in α-galactosidase A[49,50]	Accumulation of glycosphingolipids in the heart, brain, and kidneys; progressive degeneration and dysfunction
	Danon disease	X-linked mutation; deficiency in lysosome-associated membrane-protein-2 (*LAMP*) [51,52]	Increased anaerobic metabolism; stress-prone cells, arrhythmias, early senescence, and fibrosis
	Pompe disease	Autosomal recessive loss of α-glucosidase[53,54]	Glycogen accumulation that leads to myopathies and hypertrophic cardiomyopathy
Infiltrative disorders	Hemochromatosis	C282Y[55]	Iron overload resulting in increased reactive oxygen species that lead to mitochondrial dysfunction, DNA damage, membrane depolarization, and disrupted calcium kinetics
	Amyloidosis	V122I[56]	Mutated transthyretin deposits in the extracellular matrix of the myocardium
		L55P[56,57]	Cardiac amyloidosis and amyloid polyneuropathy[56]; increased apoptosis in cardiac cells[57]

Table 1 (continued)

Cardiomyopathy	Implicated gene (protein)	Mutation	iPSC study findings
Acquired	Doxorubicin toxicity[58]	-	Increased susceptibility to cardiotoxicity of doxorubicin treatment in breast cancer patients
	Digoxin toxicity[59,60]	-	Decreased Na^+ amplitude, increased Ca^{2+} amplitude, shortened field potential duration, arrhythmogenesis
	Tobacco/vaping[61–63]	-	Increased reactive oxygen species, decreased myocyte function, cell death
	COVID-19 myocarditis[64–66]	-	SARS-CoV-2 direct damage of cardiomyocytes, leading to impaired electrophysiology, contraction, and apoptosis

Fs frameshift, *del* deletion, *ins* insertion

be hindered by the cardiotoxicity associated with many tyrosine kinase inhibitors [74]. Other DCM-associated mutations was that of an autosomal dominant nonsense R225X in exon 4 of *LMNA* discovered in a family cohort with three generations of dilated cardiomyopathy [31]. iPSC-CMs with this mutation, along with another distinct frameshift mutation, showed reduced levels of lamin A/C and high nuclear senescence [31]. Furthermore, they exhibited hypersensitivity to external stress; electrical stimulation led to more senescent cells and, imminently, apoptosis. Rapamycin treatment has proved beneficial in laminopathies that cause premature aging syndrome [31]. However, in this study, rapamycin treatment of *LMNA* iPSC-CMs were not successful in preventing electrical stimulation-induced apoptosis [31], highlighting the heterogeneity of these mutations in their response to treatment. Whereas drug therapies cannot currently provide curative treatment for cardiomyopathies, gene editing techniques can. In other studies of *LMNA* mutations, helper-dependent adenoviral vectors (HDAdVs) were found to safely correct mutations in many regions of the *LMNA* gene locus without any off-target genetic changes [30].

Another gene associated with DCM is *BAG3*, which plays a crucial role in protein organization and myocyte stability in both heart and skeletal muscle [32]. R447H and knockout mutations of *BAG3* resulted in muscle dysfunction, through chaperone system dysfunction (*BAG3-HSC70*) with consequent impaired myofiber maintenance. In a separate study on *BAG3* in an isogenic cell line of iPSC-CMs, heterozygous mutations were shown to reduce protein levels by 50% [33]. These iPSC-CMs exhibited dysfunctional electrophysiology, similar to *BAG3* knockouts, highlighting the necessity for tight regulation of *BAG3*. Furthermore, these studies confirmed *BAG3*'s role in protecting cardiomyocytes from the cardiotoxic effects of proteasome inhibitors like bortezomib [33]. Hence, iPSC offers a platform for refining these drugs to minimize their cardiotoxicity.

TNNT2 mutations have been previously identified in cardiac tissue from DCM patients [34, 75] and are known to cause DCM in mouse models [76]. In 2012, Sun et al. generated iPSC-CMs from a family with a history of DCM carrying the point mutation R173W in *TNNT2* [34]. This resulted in the characteristic features of DCM. The DCM iPSC-CMs exhibited more variability in the sarcomeric organization, such as more disorganized Z-lines. As with the *TTN* mutation, stimulation of *TNNT2* DCM iPSC-CMs with positive inotropes recapitulated heart failure physiology in vitro: reduced rate of contraction, compromised contractility, and an increase in the number of cells with abnormal sarcomeric organization [34]. Treatment of iPSC-CMs from patients with DCM with β antagonists reduced the number of DCM iPSC-CMs with disorganized sarcomeres, reduced

chronotropy, and increased global calcium transients, all contributing to improved contractility [34].

iPSC models such as these provide a rapid throughput system to allow for future reparative genetic therapies targeting the underlying mutations in hopes of correcting the aberrant pathology at the molecular level. Adenoviral transduction of the sarcoplasmic endoplasmic reticulum calcium ATPase (*SERCA2a*) gene in these *TNNT2* DCM iPSC-CMs resulted in overexpression of SERCA, which led to changes in gene expression in approximately 191 genes to levels similar to those of controls [34]. Calcium handling is affected in many mutation profiles of DCM. One recent DCM iPSC study implicated a novel autosomal recessive E1680K mutation in the striated muscle enriched protein kinase (*SPEG*) gene, a gene that has been implicated in centronuclear myopathies [35].

Hypertrophic Cardiomyopathies

Hypertrophic cardiomyopathy, the most common genetic heart disease, is an autosomal dominant group of conditions characterized by hypertrophy of the ventricular myocardium without secondary causes such as hypertension. Patients present with symptoms of progressive heart failure, arrhythmia, and sudden cardiac death. Like ARVC, HCM is frequently genotype negative in up to 30–40% of patients [77]. Over half of patients with HCM have mutations in myosin heavy chain 7 (*MYH7*) or myosin binding protein C3 (*MYBPC3*) [77].

HCM mutations have been well-described over the past two decades, but the actual pathways that lead to the phenotype of myocyte hypertrophy and ventricular arrhythmia have remained elusive. Lan et al. studied iPSC-CMs derived from a family cohort with HCM carrying the missense mutation R663H in *MYH7* and demonstrated upregulation of genes involved in pathologic hypertrophy and increased arrhythmogenicity [36]. Other studies on HCM-derived iPSC-CMs have shown β agonist exaggeration and calcium blocking alleviation of the hypertrophic phenotype and diastolic dysfunction [78].

MYBPC3 is a very commonly implicated gene in HCM patients. Mutations such as R943X and R1073P often result in premature stop codons, resulting in reduced mRNA expression levels with unaffected protein levels, challenging the previously suggested theory that haploinsufficiency caused the HCM phenotype [39]. Investigation with iPSC-CMs revealed that the *MYBPC3* premature stop codons constitutively activated the nonsense-mediated decay (*NMD*) pathway, which results in the destruction of mRNAs of various signaling pathways, resulting in an HCM signature on the transcriptome. Inhibition of this pathway in iPSC-CMs alleviates the HCM phenotype and pins *NMD* as a potential therapeutic target [39]. Several studies have recapitulated this phenotype of *MYBPC3* cardiomyopathy in iPSC-CM models and have demonstrated how gene transfer with wild-type *MYBPC3* can correct the aberrant phenotype [39–41]. This work supported the development of adeno-associated virus-9 (AAV-9) based gene therapy for *MYBPC3* HCM in preclinical animal models of HCM [79].

In addition to the structural, electrical, and calcium abnormalities of HCM, the energetics of cardiomyocytes have also been implicated in preceding the morphological phenotype. Encoded by *MYH7*, *MYL2*, or *MYL3*, interacting - head motifs (IHM), which allow for paired myosin molecules to engage in a super relaxed state, are critical to efficient myosin function and ATP consumption [42]. In iPSC-CM models of HCM, dysfunction of IHM led to an increase in myosin molecules in a disordered relaxed state (DRX) and demonstrated a 5-fold increase in energy usage. HCM patients with variants of unknown significance (VUS) in IHM genes that shift towards DRX have been shown to have increased rates of heart failure and atrial fibrillation [42].

Losses of function mutations in α-kinase 3 (*ALPK3*), such as nonsense W1264X, have recapitulated the HCM phenotype [43]. However, haploinsufficiency in these isogenic iPSC-CMs was not sufficient to cause disease; biallelic loss of function was necessary, which was true of many other heterozygous mutations of *ALPK3*. Transmission electron microscopy of these mutations revealed disorganized sarcomeres and abnormal intercalated discs [43].

Noncompaction Cardiomyopathies

Noncompaction cardiomyopathy is a consequence of abnormal myocardial morphogenesis during fetal development that results in spongy rather than solid heart muscle with hypertrabeculation [80]. Phenotypes can be mild or asymptomatic in some, and as such have a wide range of age at presentation from the neonatal period to late adulthood [45, 81]. Many mutations linked to NCCM overlap with those of other cardiomyopathies, including *MYH7*, *MYBPC3*, *DES*, *DSP*, or tropomyosin 1 (*TPM1*) [80]. iPSC-CMs have recapitulated findings of NCCM in vitro and have also identified novel pathways implicated in noncompaction such as the T-box transcription factor 20 (TBX20) and PR domain containing 16 (PRDM16) signaling pathway [45].

iPSC models have demonstrated their effects on cardiac actin (*ACTC1*), resulting in abnormal contractility, increased calcium sensitivity, and arrhythmogenicity [44, 47]. iPSC-CMs derived from NCCM patients carry a mutation in the essential T-box transcription factor 20 (*TBX20*) [45]. One of its downstream targets is *PRDM16*, which suppresses TGF-β signaling and prevents proper myocardial proliferation and development. *PRDM16* mutations have been previously shown to cause NCCM seen in zebrafish experiments [82].

iPSC models corroborated these findings when they showed that all NCCM iPSC-CMs exhibited downregulation of *PRDM16* [45]. Since then, mutations in *PRDM16* have been accepted as pathogenic of NCCM, such as exon 9 truncation [45]. This specific pathway may be a key regulatory cascade for normal development of the heart's trabecular layer. Progenitor NCCM cells may not express enough protein to differentiate and mature into the cardiac lineage and they also exhibit up to 50% slower growth in the presence of growth factors [45]. While treatment can involve TGF-β receptor-1 inhibitors to prevent dysregulation in activating pathways, curative strategies would entail gene editing; CRISPR-Cas9 gene editing has been performed in iPSC-CMs to correct the *TBX20* mutation [45]. Both of these strategies reversed the NCCM phenotype in iPSC studies [45].

The *DES* gene has been implicated in a wide range of myopathies and cardiomyopathies. In a study of an NCCM family, iPSC-CM transfection experiments of the A337P mutation in *DES* revealed significant desmin aggregates, supporting its pathogenicity in NCCM [46]. Less commonly affected is the structural protein *DSP*, which links desmosomes to the intermediate filaments. In the same family cohort, the novel missense mutation L1348X was discovered [46]. Interestingly, pathogenic mutations of *DSP* have often been seen in conjunction with wooly hair and palmoplantar keratoderma, a syndrome known as Carvajal syndrome [46].

A missense mutation R178H was also discovered in *TPM1* [47]. In iPSC experiments testing contractile force, the muscle produced less force at any calcium concentration. These mutant iPSC-CMs exhibited disrupted calcium sensitivity as well as disrupted binding of actin to myosin during contraction, the latter of which suggests altered energetics within cardiomyocytes. The pathomechanisms of tropomyosin mutation thus include disrupted calcium signaling, contraction deficits, and energetic disturbances.

Restrictive Cardiomyopathies

While restrictive cardiomyopathy can be acquired through diseases such as hemochromatosis or amyloidosis, the literature has reported it more often as idiopathic [83]. It makes up less than 5% of genetic cardiomyopathy cases and confers a poor prognosis, especially in a childhood-onset [27, 83]. It is characterized by impaired ventricular relaxation with preserved systolic function with normal to near normal myocardial thickness [83, 84].

The genetic etiology of RCM remains poorly described and understood, and lack of consistent genetic RCM animal models has made this disease challenging to study in vivo [37]. iPSC models have been used to study it, but to a much lesser extent than other cardiomyopathies. It was actually considered an acquired cardiomyopathy until a D190H mutation of the cardiac troponin I (*TNNI3*) was identified in a familial cohort with RCM [27, 83]. Genotyping studies since then have shown that most mutated genes associated with heritable RCM encode sarcomere or cytoskeleton proteins, including cardiac troponin T (*TNNT2*), *MYH7*, *TPM1*, myosin light chain 2 (*MYL2*), *MYL3*, *TTN*, and even *DES* [27]. Notably, many of the associated genes are not unique to RCM, which results in shared features with some of the other cardiomyopathies. This would explain why, for instance, myofilament hypersensitivity to cytoplasmic calcium is a shared feature between RCM and HCM [84]. In transgenic mice experiments, the crossing of two mutations, RCM-associated *TNNI3* R193H (hypersensitivity) and N-terminal truncated *TNNI3* (decreased sensitivity), corrected the impaired relaxation phenotype [84]. Lusitropy could thus be a potential therapeutic strategy for RCM patients.

One RCM-associated gene studied using the iPSC paradigm was *DES* [48]. The missense mutation Y122H in *DES* was incidentally discovered in a 19-year-old male with cardiac abnormalities using next-generation sequencing [48]. In transfection experiments with RCM iPSC-CMs, the *DES* mutation led to abnormal cytoplasmic desmin aggregates, similar to those seen with mutated *DES* in NCCM. This particular mutation was actually classified as a pathogenic mutation for RCM according to criteria set by the American College of Medical Genetics [48, 85]. Unfortunately, desminopathies and sarcomeric abnormalities have no available therapies [86].

Lysosomal Storage Disorders

iPSCs have also served as representative models for the lysosomal storage disorders that affect the heart such as Fabry disease, Danon disease, and Pompe disease [87, 88]. Fabry disease is an X-linked lysosomal storage disorder defined by deficiency in α-galactosidase A that leads to accumulation of glycosphingolipids such as globotriaosylceramide (GL-3) in the heart, brain, and kidneys, leading to their progressive degeneration [49]. One iPSC study demonstrated that substrate reduction therapy via glucosylceramide synthase inhibition was able to clear GL-3 in cardiomyocytes [50].

Danon disease is an X-linked disorder caused by deficiency in the lysosome-associated membrane-protein-2 (*LAMP-2*) [51]. Patients can present with intellectual disability, retinopathies, myopathies, and cardiomyopathies that result in arrhythmias and fibrosis [51, 52]. Studies of Danon iPSC-CMs showed that the *LAMP-2* deficiency results in a stress-prone cardiomyocyte with early senescence and fibrosis that inevitably results in degeneration of the heart [51]. Damaged mitochondria accumulated in iPSC-CMs and demonstrated diminished respiratory capacity [52], leading to a rerouting from aerobic to anaerobic metabolism in order

to maintain the NAD+/NADH ratio necessary for the energy demands of a contracting cardiomyocyte [51].

Pompe disease is an autosomal recessive disorder characterized by complete loss of α-glucosidase (GAA) activity that leads to glycogen accumulation and subsequently myopathies and cardiomyopathies [53]. iPSC-CMs showed no contractile dysfunction but were able to reveal a previously unknown abnormality in *LAMP* hypoglycosylation, a protein processing defect similar to that seen in congenital disorders of glycosylation that cause HCM [53]. Mitochondrial dysfunction was also identified, and L-carnitine therapy was able to improve the dysfunctional cellular respiration [54]. Recombinant human GAA rescued the iPSC-CMs from the Pompe phenotype by reducing glycogen accumulation [54].

Infiltrative Cardiomyopathies

Hemochromatosis can be an acquired or hereditary (C282Y missense mutation) systemic infiltrative disorder of iron overload [55]. iPSC studies have recapitulated the many deleterious pathological features of this disorder. Iron overload in cardiomyocytes results in mitochondrial dysfunction, DNA damage, and membrane depolarization due to the generation of reactive oxygen species (ROS) [55]. Iron's disruption of SERCA results in aberrant calcium kinetics, including a prolonged calcium decay and reduced calcium clearance from the cytosol. These abnormalities contribute to the prolonged action potential duration, arrhythmogenesis, and contractile dysfunction that are characteristic of cardiac hemochromatosis. Divalent metal transporter 1 (*DMT1*), the membrane protein responsible for iron transport into cells, has been implicated previously in the pathogenesis of hemochromatosis [89]. Ebselen, an anti-inflammatory and antioxidant organoselenium compound, is already under investigation for its protection against myocardial ischemia-reperfusion injury [90] and against hearing loss [91]. In studies of iron-overloaded iPSC-CMs, it inhibited *DMT1* and successfully rescued the iPSC-CMs [55].

Amyloidosis is a group of systemic disorders where aggregates of misfolded protein deposit in the extracellular matrix of tissues, leading to dysfunction that is often related to mass effect. Hereditary transthyretin (*ATTR*) amyloidosis has been associated with many mutations, with certain mutations characterized by predilections to specific organs [56]. Patients with the V122I mutation, for example, express cardiomyopathy, whereas the L55P mutation results in cardiac amyloidosis and polyneuropathy [56]. Furthermore, the L55P mutation was associated with higher rates of apoptosis [57]. Due to the systemic and age-related nature of the disease process, animal models have fallen short of accurately recapitulating the pathogenesis of *ATTR* amyloidosis [56]. The advent of iPSCs has now allowed us to reproduce these features. *ATTR* iPSC-CMs were studied in parallel to hepatocyte-like cells (HLCs) to understand the liver's role in the pathogenesis [92]. In mutant HLCs that expressed the mutant *TTR* protein, 92 genes showed differential expression, many of which have been previously shown to be upregulated during endoplasmic reticulum stress and result in proteostasis [92]. The most notable genes included transferrin and genes of the unfolded protein response (UPR), such as activating transcription factor 6 (*ATF6*). In vitro fibril formation assays showed that increased levels of transferrin reduced formation of amyloid fibrils by 60%, highlighting its potential role as a chaperone for mutated *TTR* [92]. Inducing *ATF6* resulted in increased proteostasis and reduced secretion of mutated *TTR* [92]. *ATF6* activation has exhibited remarkable therapeutic value in other contexts such as cardiac ischemia and stroke, and future studies will elucidate its efficacy and safety in vivo [93].

Acquired Cardiomyopathies

Screening for drug toxicity is an important area of research using the iPSC models. Doxorubicin cardiomyopathy was one of the first toxicity models to be studied using iPSC-CMs. Wu and colleagues demonstrated that iPSC-CMs from two patient populations—those with breast cancer and those without—exhibited different cardiotoxicity thresholds to doxorubicin, with cells from breast cancer patients demonstrating increased susceptibility to injury [58]. These early studies demonstrated how iPSCs can be used to model patient-specific diseases in vitro without the need for invasive cardiac biopsies or surgeries. In genome-wide screenings of iPSC-CMs with CRISPR-Cas9, cell surface transporters *SLCO1A2* and *SLCO1B3* were implicated in doxorubicin uptake into cardiomyocytes, unmasking potential targets to minimize the cardiotoxic damage of doxorubicin [94].

The cytotoxic effects of cardiac glycosides (e.g., digoxin) via inflammasome activation are well-established in the literature [95, 96]. iPSC-CMs have allowed us to recapitulate many of these features: the down-sloping ST-segment depressions, cardiomyocyte beat arrest associated with shortening of corrected field potential duration (FPDc), and arrhythmias [59]. Administration of digoxin was reported to reduce the Na^{+} amplitude and increase the Ca^{2+} amplitude; the latter is closely related to QT shortening, enhanced contractility, and arrhythmias, highlighting how iPSC-CMs faithfully represent the cardiac pharmacological response [60].

When iPSC-CMs were used to compare the toxic effects of cigarette smoke extract (CSE) and electronic cigarette extract (ECE) on cardiomyocytes, the findings revealed that although the CSE solution contained a six-fold higher

nicotine concentration than ECE, the toxic effects of both solutions were actually comparable: high ROS, decreased cardiomyocyte function, slower beating, and higher cell death [61]. Vaping liquids were found to have negative effects on beating frequency and field potential durations of iPSC-CMs [63]. Notably, similar studies in iPSC endothelial cells echoed these smoking extract findings, delineating cytotoxicity, increased ROS, and increased caspase activity as contributing factors to overall endothelial cell dysfunction [62].

During the coronavirus disease 2019 (COVID-19) global pandemic, there were widespread reports of viral myocarditis; however, the mechanism by which severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes myocarditis remains unclear. iPSC-CMs are susceptible to SARS-CoV-2 infection in vitro. After SARS-CoV-2 invades iPSC-CMs by binding angiotensin-converting enzyme II receptor (*ACE2*), the subsequent viral replication and cytotoxic effects result in impaired electrophysiological properties and contractility [65•], as well as reduced myocyte contraction and apoptosis [64, 65•]. Other studies showed that SARS-CoV-2 infected iPSC-CMs have increased brain natriuretic peptide (*BNP*) expression and upregulation of proinflammatory cytokines and chemokines such as chemokine C-X-C motif ligand (*CXCL1*), *CXCL2*, interleukin-6 (IL-6), IL-8, and tumor necrosis factor (*TNF*) [66]. Whether direct viral infection is the true mechanism of COVID-19, myocarditis remains unclear, especially as growing reports of myocarditis after vaccination with SARS-CoV-2 mRNA vaccines suggest an autoimmune mechanism rather than direct cytotoxic effect [97–99]. While iPSC-CMs remain a useful tool for studying direct cardiotoxicity of SARS-CoV-2 in vitro, this model does not mimic the multisystem effects of systemic COVID-19 and may not reveal the true mechanism underlying COVID-19 myocarditis.

Limitations and Current Advances

Human iPSC models have become an invaluable tool for cellular modeling that has allowed us to minimize use of animals for preclinical models and avoid invasive procedures for isolating cardiac tissues from human subjects. While promising, iPSC models are not without limitations. Cardiomyocytes function as part of a very complex network of cells, making up only 25–35% of myocardial tissue [100], and their appropriate function depends on interaction with many different neighboring cell types including fibroblasts, vascular cells, inflammatory cells, and proteins of the extracellular matrix.

iPSC-CMs are also functionally immature, more closely resembling fetal CMs in terms of morphology, gene expression, and function. iPSC-CMs are smaller and morphologically pleomorphic with less well-organized sarcomeres, resulting in an altered membrane capacitance, rate of action potential depolarization, and maximum contractile force [9, 101]. iPSC-CMs also lack the polarity in structure compared to adult CMs; iPSC-CMs express connexin-43 gap junctions uniformly across the cell membrane, whereas in adult cardiac tissue, these connexins are concentrated at the intercalated discs [102]. Functionally, there are noticeable differences in the contractions and electrical properties [103], as iPSC-CMs, like fetal CMs, lack mature T-tubules.

Many strategies are under investigation to overcome these deficits including use of engineered heart tissue or use of mechanical substrates to improve iPSC differentiation. Embryoid body co-culturing promotes an environment similar to the embryonic heart, positively influencing electrophysiological maturity [104] and improving iPSC differentiation [105–107]. Culturing iPSC-CMs on extracellular matrix derivatives may also improve function and phenotype [105, 106, 108••]. Recent advances in 3D bioprinting may also allow for in vitro engineering of functional myocardial tissue derived from iPSCs [109].

Despite these limitations, iPSCs and iPSC-CMs remain a useful tool for exploring pathomechanisms of genetic and acquired cardiomyopathies, and act as an effective tool for high throughput screening of novel therapeutics. As iPSC technology evolves to improve differentiation and maturation, it will allow the generation of functional in situ cardiac tissue for more accurate assessment of novel cardiac therapies.

Abbreviations iPSC: induced pluripotent stem cells; CM: cardiomyocyte; ARVC/D: arrhythmogenic right ventricular cardiomyopathy; DCM: familial dilated cardiomyopathy; HCM: hypertrophic cardiomyopathy; NCCM: noncompaction cardiomyopathy; RCM: restrictive cardiomyopathy; TGF: transforming growth factor; PDGF: platelet-derived growth factor; HDAdVs: helper-dependent adenoviral vectors; SERCA/SERCA2a: sarcoplasmic endoplasmic reticulum calcium ATPase; NMD: nonsense-mediated decay; AAV: adeno-associated virus; IHM: interacting - head motif; ROS: reactive oxygen species; TTR: transthyretin; HLC: hepatocyte-like cell; UPR: unfolded protein response

Declarations

Conflict of Interest Tarek Khedro has no conflicts of interest. Jason M. Duran JMD is a paid consultant for Lexeo Therapeutics (New York, NY). He also reports US Non-Provisional Application No. 14/900,809. Eric D. Adler reports consulting for Abiomed, Novartis, Abbott, Ionis Pharmaceuticals, Sana Biotechnology, Medtronic, Lexeo Pharmaceuticals, Cytokinetics, and Endotronics. He also reports Papillion Therapeutics (Founder and Board Member) and ResQ Pharmaceuticals (Scientific Advisory Board); he is a Shareholder for Rocket Pharmaceuticals; and he was an expert witness for AstraZeneca.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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• Of importance
•• Of major importance

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