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SPOTLIGHT REVIEW

Modelling sarcomeric cardiomyopathies in the dish: from human heart samples to iPSC cardiomyocytes

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One of the obstacles to a better understanding of the pathogenesis of human cardiomyopathies has been poor availability of heart-tissue samples at early stages of disease development. This has possibly changed by the advent of patient-derived induced pluripotent stem cell (hiPSC) from which cardiomyocytes can be derived *in vitro*. The main promise of hiPSC technology is that by capturing the effects of thousands of individual gene variants, the phenotype of differentiated derivatives of these cells will provide more information on a particular disease than simple genotyping. This article summarizes what is known about the 'human cardiomyopathy or heart failure phenotype *in vitro*', which constitutes the reference for modelling sarcomeric cardiomyopathies in hiPSC-derived cardiomyocytes. The current techniques for hiPSC generation and cardiac myocyte differentiation are briefly reviewed and the few published reports of hiPSC models of sarcomeric cardiomyopathies described. A discussion of promises and challenges of hiPSC-modelling of sarcomeric cardiomyopathies and individualized approaches is followed by a number of questions that, in the view of the authors, need to be answered before the true potential of this technology can be evaluated.

Keywords Induced pluripotent stem cells • Disease modeling • Sarcomeric cardiomyopathy • Heart Failure • Disease phenotype

This article is part of the Spotlight Issue on Sarcomeric cardiomyopathies: from bedside to bench and back.

1. What is a (human) cardiomyopathy phenotype in the dish?

The central assumption underlying the concept of modelling cardiomyopathies *in vitro* is that the phenotype seen in patients has its correlate in the pathology of isolated cardiac myocyte or heart muscle preparations, i.e. that it is based on an intrinsic cardiac myocyte pathology. This assumption may not be always true. For example, the initial myocyte function is essentially normal in infarcted or pressure-overloaded hearts; the problem lies outside the myocytes (coronaries, peripheral vasculature, valves). It is just that the work overload and/or neurohumoral stimulation can reach a level at which compensatory mechanisms fail and clinical symptoms (the syndrome called 'heart failure') occur. Thus, very likely many of the characteristics we know from human heart failure (HF) are secondary alterations and likely to disappear if cells are not exposed to such stressors. The situation is, however, different in sarcomeric cardiomyopathies on which this chapter focuses. Here the primary defect lies in the sarcomere itself (i.e. mutant sarcomeric protein) and everything else follows. The problem is that much less is known about cardiac myocyte function in human (sarcomeric) cardiomyopathies because they represent only a minor fraction of explanted human hearts and have not been studied systematically in relation to aetiology. An exception is hypertrophic cardiomyopathy (HCM) where surgical septum myectomy provides a unique opportunity to study living human heart samples. The following paragraph therefore summarizes characteristics of *in vitro* preparations from patients with terminal HF (mixed aetiology, mainly ischaemic and dilated, few HCM) and myectomy samples of patients with HCM.

1.1. Isolated heart muscle preparations with intact sarcolemma

Much of our current understanding of mechanisms of heart failure origins in work on trabeculae carneae, i.e. small diameter heart muscle strips excised from the right of left ventricle of explanted, terminally failing, or non-failing human hearts, an experiment first described by Sonnenblick *et al.*[1] in 1965. It was this preparation in which Bristow *et al.*[2] identified a reduced positive inotropic response to β-adrenergic

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stimulation of the failing heart, but also a normal maximal Ca^{2+}- or cardiac glycoside-stimulated force generation (at low stimulation rate). This (somewhat forgotten) finding was reproduced and extended by others in similar preparations,[3,4] indicating that mechanisms regulating force rather than those underlying maximal force generation capacity are affected in heart failure. Accordingly, myofilament Ca^{2+} sensitivity (as measured in skinned preparations, see below) is likely unaltered in terminal HF.[5] Other characteristics of the failing heart muscle were unravelled in the 90s: blunted or reversed force–frequency relation, i.e. lack of increase or even reduced force generation with increasing stimulation rate,[6] prolonged relaxation,[3] and increased diastolic tension with increasing stimulation rate and reduced post-rest-potentiation.[7] Another key regulatory mechanism of heart muscle, the length-dependent activation (Frank-Starling mechanism) has been either described as blunted[8] or unaltered.[9,10] Most functional abnormalities in failing human hearts pointed to altered Ca^{2+} handling and could be related to a decreased function of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a[11]) and increases in the sarcolemmal Na^{+}/Ca^{2+} exchanger.[12,13]

Multifold evidence suggests energy deficit to be another common alteration in heart failure and cardiomyopathies.[14] Whereas energetic alterations in heart failure are, in most cases, the consequence rather than the cause of failure,[14] this is likely different in sarcomeric cardiomyopathies. Lowered phosphocreatine–ATP ratios have been measured in HCM patients irrespective of the degree of hypertrophy,[15] pointing to a primary defect in HCM. A recent study confirmed and extended this conclusion by showing with [^{11}C]-acetate PET and CMR imaging that myocardial energy efficiency was lower in patients with HCM associated with mutations in MYH7 or MYBPC3 than healthy controls.[16] The *in vivo* findings in patients correlated with higher tension costs (ATP consumption per force development) measured in permeabilized muscle strips *in vitro*, indicating (i) that the defect in energetic efficiency of myofilament function is a primary alteration in sarcomere-positive HCM and (ii) can be faithfully analysed *in vitro*.

It is important to keep in mind that most of the alterations have been identified in preparations from patients with terminal heart failure, i.e. patients exposed to chronically increased catecholamine levels, with marked dilatation of the ventricular walls, maximal wall stress, and extremely reduced left-ventricular ejection fraction. It is therefore likely that much of the 'HF-specific myocyte phenotype' is in fact an adaptation to increased workload and humoral stimulation. For example, the force–frequency-relation was blunted in HCM samples from patients with decompensated HCM,[17] but unaltered in samples from compensated HCM patients undergoing septum myectomy in which maximal force development and response to β-adrenergic stimulation were preserved.[18]

1.2. Freshly isolated intact beating myocytes

The few studies on electrically stimulated beating human cardiomyocytes, freshly isolated either from (terminally failing) explanted human hearts or myectomy samples from (compensated) patients with obstructive HCM, principally supported the above data on muscle strips (*Table 1*). Important additional information comes from measurements of action potentials, ion currents, Ca^{2+} transients, Ca^{2+} sparks, and mitochondrial membrane potential and the redox state. Caveats of this approach include rather harsh conditions of enzymatic isolation (from heart muscle blocks or biopsies) and the unloaded conditions of contraction, i.e. the almost complete absence of workload. A peculiarity of HCM myectomy samples is the large degree of fibrosis,[19] a condition that notoriously impedes cell isolation. Since the degree of fibrosis is systematically lower in non-failing hearts, but also in HCM samples without sarcomeric gene mutation,[20] it is well possible that some of the reported differences between these groups (*Table 1*) in fact represent isolation artefacts rather than true differences in biology, e.g. enzymatic over-digestion and oxidation.

1.3. Skinned cells/heart preparations

A key parameter of cardiac contractile function is the response of myofilaments to Ca^{2+}. This can be experimentally assessed in a whole spectrum of preparations, ranging from muscle strips/cells in which the plasma membrane is perforated to make it freely permeable for Ca^{2+} (staphylococcus exotoxin or β-escin) to fully skinned muscle strips/cells or isolated myofibres hooked to nano-force transducers. The perforated membrane methods have the advantage that part of the signalling cascade remains intact, allowing the evaluation of agonist responses. Isolated skinned cells/myofibrils are more prone to experimental artefact, but can be isolated from frozen whole heart samples, which facilitates logistics. Force measurement can be combined with determination of ATP consumption, providing an index of energy cost.

Experiments in skinned human preparations have not revealed systematic alterations in myofilament Ca^{2+} sensitivity in terminal HF.[5] In contrast, passive stiffness was found to be increased in skinned cardiomyocytes from biopsies of patients with diastolic, but not systolic heart failure, likely a consequence of altered titin function.[36] Common, but not unequivocal findings in HCM samples are increased myofilament Ca^{2+} sensitivity,[34] reduced maximal force generation,[37] and faster cross-bridge relaxation kinetics of isolated myofibrils[57] associated with higher energy consumption and tension costs.[58]

1.4. Actin–myosin sliding assays

Even further reduction in complexity is achieved by assays that allow video-optical control of fluorescently labelled actin filaments sliding on myosin preparations that have been fixed on cover slides.[59] Maximal myosin motility was found to be unchanged in mitral valve HF[39] or slightly reduced in terminal HF,[40] again suggesting that the principal components of the contractile apparatus are unaffected or only mildly affected in secondary forms of HF. In contrast, several abnormalities were described in myosin preparations of HCM patients with mutations in *MYH7*. For example, homozygous V606M mutations were associated with increased actin sliding motility.[40] Similarly, homozygous R403W mutations in *MYH7* were associated with a small increase in actin-sliding velocity, but a more than two-fold increase in ATP consumption, indicating inefficient energy utilization and increased tension costs associated with this mutation.[41] On the other hand, actin-sliding velocity was normal on myosin preparations from an unselected cohort of HCM myectomy samples,[40] indicating that altered myosin properties are not a general feature of HCM.

1.5. Biochemical assays and gene expression

Whereas decreased myofibrillar ATPase activity has been identified as one of the earliest alterations in HF,[60] maximal actomyosin ATPase activity was found unchanged in HF,[39] suggesting that primary myosin properties are unchanged in common forms of HF, but that the assembly or post-translational modifications differ. Indeed, numerous HF- and HCM-associated alterations have been described in the phosphorylation state (e.g. decreased cMyBP-C, myosin light chain-2, troponin I phosphorylation) and isoform composition of myofilament proteins (increase in the atrial isoform of myosin light chain-1 in the

Table 1 Commonly used *in vitro* preparations of cardiac samples for the analysis of a pathology-related phenotype

In vitro preparation	Readout parameters	Advantages/Disadvantages	Pathology in human terminal HF or HCM (myectomy or explanted hearts)
Electrically stimulated muscle strip in organ bath (generally isometric)	- Twitch force	- Relatively intact	- Unchanged max. force in HF (low frequency)[2–4]
	- Twitch kinetics (time-to-peak [TTP] and time-to-relaxation [TTR])	- Simple	- Unchanged FLR in HF[8–10]
	- Diastolic tension	- Integrated readout	- Subsensitivity to catecholamines in HF[2–4]
	- Force–length relation (FLR)	- Amenable to full concentration–response curves	- Prolonged TTP in HF[3,21]
	- Force–frequency relation (FFR)	- Requires immediate exp. (difficult logistics for human material)	- Prolonged TTR in HF, HCM[3,18]
	- Post-rest-potentiation (PRP)	- Handling artefacts	- Prolonged Ca^{2+} transient in HF, HCM[3,21]
	- Action potential (APD, sharp microlectrodes, voltage-sensitive dyes)	- Core ischaemia	- Increased cross-bridge force–time integral in HF[22]
	- Ca handling		- Blunted FFR in HF[6,17]
	- Heat/oxygen consumption		- Unchanged FFR (HCM myectomy)[18]
	- Pharmacology		- Blunted PRP in HCM[23]
			- Unchanged PRP (HCM myectomy)[18]
			- Increased diastolic tension in HF, HCM[12,18,21]
Freshly isolated electrically stimulated cardiomyocytes	- Cell or sarcomere shortening (unloaded)	- Intact cardiac myocyte	- Unchanged max. shortening at low frequency in HF[24,25]
	- Peak shortening	- Amenable to measurements of subcellular functions	- Hypertrophy in HF[26]
	- Shortening kinetics (TTP, TTR)	- Isolation artefacts (particularly from human heart)	- Decreased shortening response to catecholamines in HF[25]
	- Diastolic sarcomere length	- Loss of 3D context	- but increased kinetic response to catecholamines in HF[27]
	- Ca^{2+} transients	- Unloaded contraction	- Prolonged TTR in HF[25]
	- Ca^{2+} sparks	- Limited work	- Prolonged Ca^{2+} transient in HF[28,29] and HCM (myectomy)[18]
	- Redox potential	- Unstable over time = short time window of exp.	- Decreased SR Ca^{2+} content in HF[30]
	- Mitochondrial parameters		- Prolonged APD[18,29], decreased K-currents[18,31,32] and increased Na-current[18] in HF and HCM (myectomy)
	- Pharmacology		- Increased PRP[33]
	- APD (sharp microlectrodes, patch clamp, voltage-sensitive dyes)		- Loss of T-tubules[26]
	- Ion currents (patch clamp)		
Skinned preparations (muscle strips, cells, myofibres)	- Myofilament Ca^{2+} sensitivity (pCa-force)	- Direct access to myofilament function	- Normal Ca^{2+} sensitivity in HF[5]
	- Maximal force	- Simple logistics (analysis from frozen samples)	- Altered Ca^{2+} sensitivity in HCM[34,35]
	- Contraction kinetics	- Preparation artefacts	- Altered passive tension in HF[36]
	- ATP consumption	- Unphysiological condition	- Normalized function after phosphorylation[34]
	- Myofilament response to phosphorylation/oxidation		- Decreased maximal force in HCM[37,38]
	- Passive tension		- Decreased length-dependent activation in HCM[34]
Myosin preparations for actin-sliding assays	- Sliding velocity (unloaded, actinin-loaded)	- Direct assessment of actin–myosin interactions	- Normal[39] or slightly decreased[40] sliding velocity in HF
	- ATP consumption	- Simple logistics	- Slightly increased sliding velocity, but doubled increase in ATP consumption in R403W β-MHC[41]

Continued

Table 1 *Continued*

In vitro preparation	Readout parameters	Advantages/Disadvantages	Pathology in human terminal HF or HCM (myectomy or explanted hearts)
		- Confounding presence of regulatory proteins	- Overproportional increase in ATP consumption in V606M β-MHC[41]
		- Preparation artefacts	
		- Unphysiological	
Tissue/cell homogenates	- Protein activity, concentration, isoforms, phosphorylation, oxidation. . .	- Simple logistics	- Down-regulation of β1-adrenoceptors[2]
	- mRNA/miR concentration	- Mechanistic insights	- Reduced SERCA activity,[3] mRNA, and, inconsistently, protein[3,11], Inhibitor-1[42]
		- Confounding cell mixtures (70% non-myocytes)	- Up-regulation of Giα[43,44], GRK2[45], phosphatase 1[46], CaMKII[47], NCX[13]
		- Unclear representation of *in vivo* state	- Hypophosphorylation of PLB[48], TnI[48], cMyBP-C[48–50], MLC-2[40,51]
			- Hyperphosphorylation of RyR2[52], hyperactivity of LTCC[53]
			- ANP[54], BNP[55], sACT[56] up

The table lists typical readout parameters, advantages, and disadvantages and a selection of pathologies described in samples from human terminal heart failure (HF) compared with non-failing controls and myectomy samples from patients with HCM. Abbreviations are explained at first entry.

ventricle. . .,) as well as in the gene expression (e.g. down-regulation of β-adrenoceptors or SR Ca^{2+} ATPase, SERCA) and phosphorylation state of proteins involved in cardiac excitation–contraction–coupling (*Table 1*). As for the HF-associated alterations in contractile function, many molecular HF-abnormalities have also been identified in HCM myectomy samples, suggesting that they are secondary to cardiac stress rather than primary causes of disease.

2. Making hiPSC and hiPSC-derived cardiomyocytes

A novel source of cardiomyocytes for modelling disease is human pluripotent stem cells (hPSC). These may either be human embryonic stem cells (hESC) in which mutations for particular cardiac diseases are introduced by gene targeting or human induced pluripotent stem cells (hiPSC) derived from patients with the disease.[61] The generation of hiPSC from somatic tissue (*Figure 1*) has been reviewed extensively elsewhere,[62–64] but broadly the approaches can be divided into two main categories depending on whether or not the reprogramming genes integrate into the genome of the target cell. Most widely used 'integrating' methods use either retroviral or lentiviral vectors to deliver the reprogramming genes; these are efficient, but may integrate into relevant genes of importance to the derivative cells. So they are falling in use in favour of non-integrating Sendai virus or episomal vectors. In both cases, combinations of 'reprogramming genes' are introduced into cells from somatic tissue growing in culture. These cells may be dermal fibroblasts, keratinocytes or blood, but also dental pulp or kidney cells shed into urine are now being used. Initially, the transcription factors OCT4, SOX2, KLF4, and cMYC[61] or OCT4, SOX2, NANOG, and Lin2[65] were identified as crucial reprogramming genes, but multiple other combinations of fewer factors, in combination with miRNA or small molecules have also been described. In all cases, the reprogramming genes induce endogenous pluripotency genes, remove the somatic cell gene expression profile and are then silenced. Cells reprogrammed in this way acquire all of the characteristics of pluripotent hESC. Like hESC, they can self-renew indefinitely, express characteristic transcription factors (e.g. OCT4, NANOG, SOX2), cell-surface proteins (SSEA3, SSEA4, and a glycoprotein recognized by antibody TRA-1–60) and differentiate *in vitro* and *in vivo* (as teratomas) into derivatives of the three embryonic germ layers: ectoderm (neural), endoderm (pancreas, liver, lung), and mesoderm (cardiomyocytes, vascular endothelial cells).

Making cardiomyocytes for experimental use requires that the pluripotent stem cells first differentiate to the mesodermal germ layer. All protocols for cardiomyocyte differentiation rely on the premise that recapitulating development signals in the embryo that first direct mesodermal fate and then pattern the nascent mesoderm to cardiogenic mesoderm will result in the formation of bona fide cardiomyocytes. The various ways to do this have been recently reviewed,[66,67] but can be divided broadly into two approaches: (i) aggregation-based methods, in which the undifferentiated cells are forced together as clumps (also known as 'embryoid bodies'), and (ii) monolayer-based methods, in which the cells remain attached to a culture substrate or extracellular matrix protein.[68] In both cases, the cells are first exposed to a sequence of mesoderm differentiation-inducing molecules, usually bone morphogenetic protein (BMP), activin (as a substitute for nodal signalling), and wnts or wnt activators such as CHIR for approximately 3 days, then an inhibitor of wnt signalling (Dkk1) is added to pattern the mesoderm. First beating cardiomyocytes are generally observed on Day 7 after induction of differentiation, reaching a maximum after 12–14 days. The aggregation-based methods have the advantage of scalability, in that aggregates may be grown in bioreactors in suspension to large numbers. This method can result in several hundreds of million cardiomyocytes fairly easily and is preferred industrially. In addition, cardiomyocytes can be easily selected to purity based on incorporation of an antibiotic resistance gene coupled to a cardiomyocyte promoter. The monolayer method is lower scale, but has the advantage of allowing

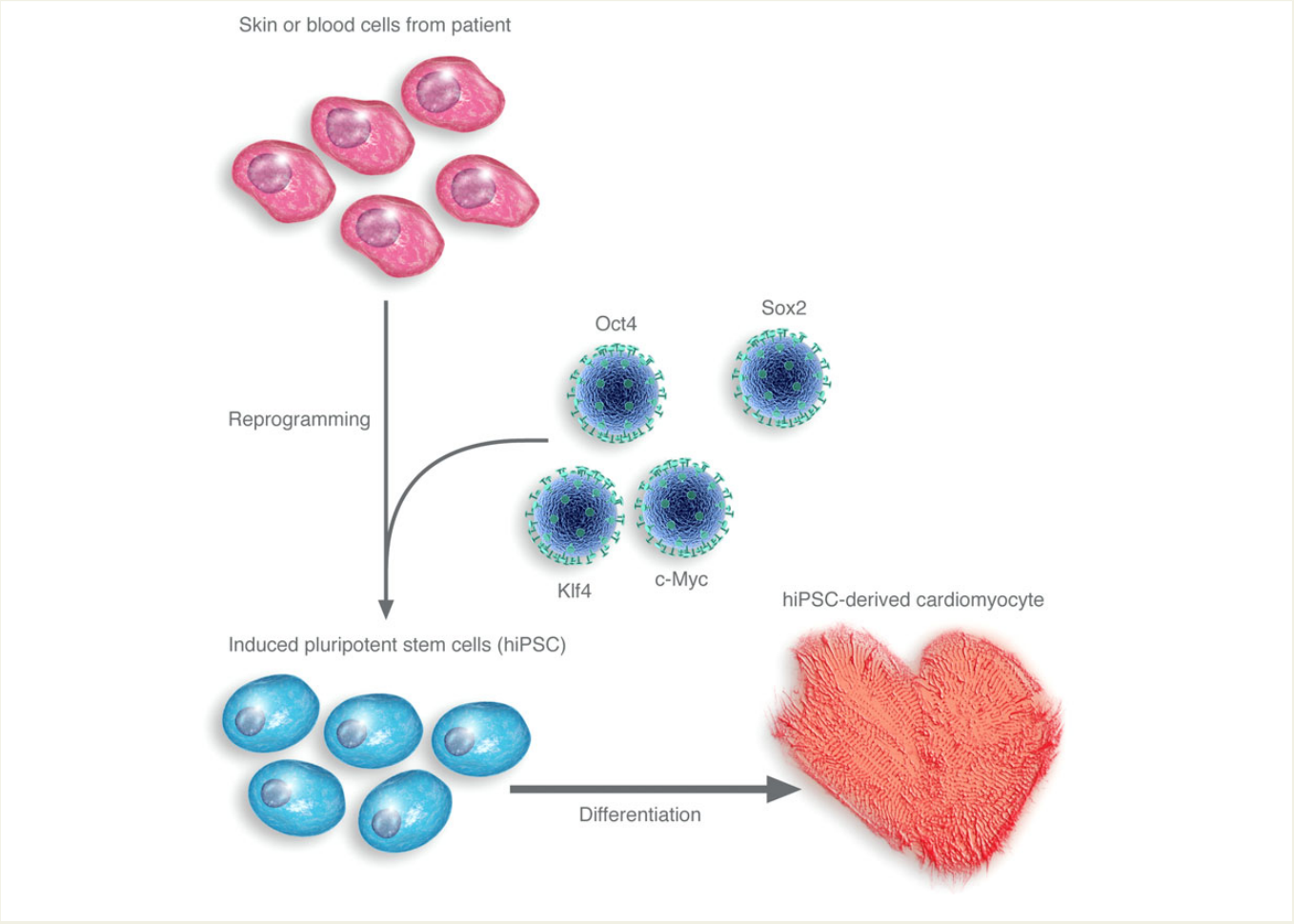

Figure 1 Principle of making cardiomyocytes from somatic cells.

the individual cells to be monitored and remain accessible to various stimuli during the differentiation process itself. While there has been no direct comparison of monolayer vs. bioreactor methods for cardiomyocyte differentiation using the same line in parallel experiments, monolayer methods are presently regarded as having the highest efficiency, the bioreactor methods better scalability.

These protocols have been developed over a number of years and each new formulation has generally resulted in improved differentiation efficiencies. In the very earliest protocols developed for hESC, only 10–30% of cell aggregates actually began beating synchronously and the proportion of individual cardiomyocytes within beating aggregates was unknown.[69] In later protocols using defined growth factors under defined and optimized conditions, 70–90% cardiac differentiation efficiencies have been reported, rising to >99% if the cardiomyocytes are selected and purified. Selection methods have included the use of mitochondrial dyes (but these may introduce some toxicity) and tittered metabolic substrates (but these may also affect cell function). Least invasive are antibody selection methods based on SIRPA and VE-CAM epitopes early during differentiation.[66,67,70] In general, despite increasing use of defined reagents, there has remained a certain amount of variability between the efficiencies with which individual cell lines differentiate. Although the better the protocol, the smaller these differences in efficiency,[71] it has been proposed that the differences may be because individual lines produce their own growth factors in variable amounts, making their sensitivity to exogenous growth or inhibitory factors different.[72] These differences have become increasingly evident as an increasing number of hiPSC lines have been produced: despite the similarity of these lines in terms of pluripotency many groups find the differentiation of hiPSC to cardiomyocytes more challenging than hESC although many hiPSC lines do differentiate just as efficiently as hESC. Minor genetic differences between the lines may account for this variability, which does not seem to be the result of a specific mutation in cardiac genes.[73]

3. Published hiPSC-CM models of inherited heart disease

The hiPSC technology has been used to model a number of inherited heart diseases, with a total of 39 publications to date. *Figure 2* shows the breakdown by inherited disease. Most reports investigated inherited channelopathies that cause either long QT syndrome (LQTS[74–87]) or catecholaminergic polymorphic ventricular tachycardia (CPVT[88–94]) in patients with structurally normal hearts. The remaining studies investigated various forms of inherited cardiomyopathies due to mutations in mitochondrial proteins (Friedreich's ataxia,[95–97] Barth syndrome,[98] carnitine palmitoyltransferase II deficency[99]), in desmosomal proteins associated with arrhythmogenic right ventricular cardiomyopathy (ARVC[100–103]), and other rare genetic syndromes associated with

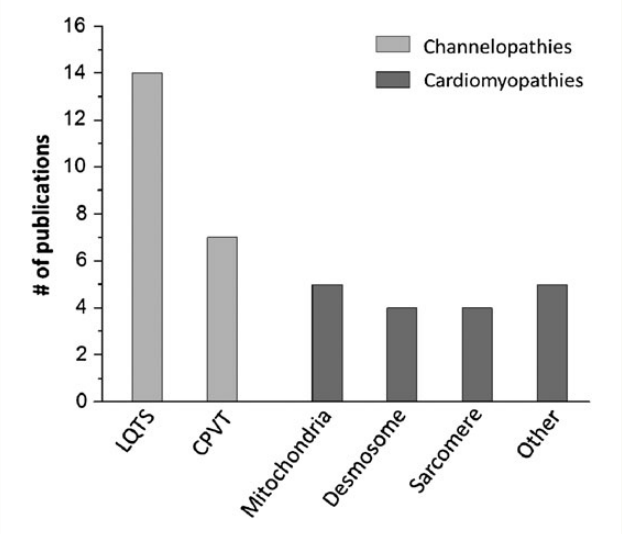

Figure 2 Number of publications reporting hiPSC-CM models of inherited heart diseases (PubMed accessed 18 September 2014). LQTS, long QT syndrome; CPVT, catecholaminergic polymorphic ventricular tachycardia.

cardiomyopathy (LEOPARD syndrome,[104] Pompe disease,[105–107] laminopathies,[106,107] intermediate filament mutations[108]). Currently, there are only four reports of hiPSC models of sarcomeric cardiomyopathies, the focus of this spotlight issue. Two extensive studies from the Wu lab published in 2012[109] and 2013,[110] and two more limited studies published online in the fall of 2014.[111,112] The findings of those four studies are compared in *Table 2*.

How do the phenotypes of the four iPSC models compare with that of 'real' human cardiomyopathy in the dish described in *Table 1*? Let us first consider the two most recent studies that describe the phenotype of hiPSC-CM generated from patients with HCM. The major finding in both studies was that all HCM hiPSC-CM lines exhibited cellular hypertrophy and disorganized sarcomeres. In the report by Tanaka *et al.*,[112] endothelin 1 enhanced this phenotype in hiPSC-CM generated from 3 HCM patients, where the underlying mutation (cMyBP-C deletion, *Table 2*) was known only in one subject, whereas the other two HCM patients were negative for sarcomeric mutations. While the effect of endothelin 1 is provocative (validation studies using neonatal myocytes from cMyBP-C heterozygous mice also exhibited a hypertrophic response to endothelin 1), it remains unclear what mechanism was responsible for the common hypertrophic phenotype in the HCM hiPSC-CM that carry different mutations. Maybe even more puzzling is a result from the other report: *MYH7-R442G* hiPSC-CM exhibited massive AP prolongation and a profound up-regulation of Na and Ca currents,[111] which has not been reported in human myocytes harvested from HCM patients (*Table 1*). A plausible explanation for these discrepancies is that, in both studies, the investigators used as controls hiPSC-CM that were previously generated from unrelated healthy subjects as part of other published studies (*Table 2*). Such an indirect comparison can give rise to differences in AP duration that are unrelated to any cardiomyopathy phenotype (see also 6 below).[74] Hence, the relevance of the findings for HCM pathophysiology remains unclear.

In contrast, both reports by the Wu lab used as controls hiPSC-CM that were generated from mutation-negative family members. The first report describes the hiPSC cardiomyocyte phenotype from patients with DCM associated with a troponin T mutation.[109] Compared to hiPSC-CM generated from three unaffected family members, hiPSC-CM generated from four carriers of the TnT mutation all exhibited more disorganized sarcomere ultrastructure, reduced contractility by atomic force microscopy (AFM) and impaired SR Ca handling, all of which are hallmarks of myocytes isolated from explanted hearts from DCM patients (*Table 1*). On the other hand, cell size and cardiac action potential were not different from mutation-negative hiPSC-CM controls, which is different from that of myocytes isolated from DCM hearts. It is possible that culture conditions were not optimal, since serum supplement in the culture medium can mask hypertrophic phenotype of hiPSC-CM.[113] Since no comparison to human heart tissue from the same patient was made, it remains unanswered if that is specific to the mutation, or unique to the iPSC-CM. DCM hiPSC-CM were also more sensitive to β-adrenergic stimulation, which could be blocked by β-adrenergic receptor antagonists (β-blocker). Given that β-blockers had the biggest effect when β-agonists were supplied in the media, the relevance of this finding is uncertain. The authors went on to show that gene-transfer of SERCA partially rescued the Ca handling defect of the DCM iPSC-CM, analogous to what has been described in various DCM animal models and humans.

The second report examined iPSC-CM generated from a family of 10 individuals, five of which carried a missense mutation in β-myosin heavy chain (*MYH7*) previously associated with HCM,[110] the other five individuals were mutation-negative. The clinical phenotype of the five mutation carriers was variable: only the index patients exhibited classic asymmetric hypertrophy, two individuals had only mild hypertrophy and the youngest two individuals (aged 10 and 14) had no evidence of cardiac hypertrophy. Despite the variable penetrance in patients, hiPSC-CM from all five mutation carriers were significantly larger than those generated from the five controls. HCM iPSC-CM expressed hypertrophic markers, had elevated diastolic Ca and impaired Ca handling, which was responsible for an increased incidence of delayed afterdepolarizations and triggered beats (*Table 2*). Based on the finding that overexpression of mutant β-myosin in hESC-derived myocytes recapitulated the Ca handling abnormalities of the HCM hiPSC-CM, and that cellular hypertrophy and Ca-triggered arrhythmias were prevented by L-type Ca channel blockers, the authors concluded that elevated intracellular Ca was a central mechanism in HCM, and that their findings 'validate iPSC technology as a method to understand how sarcomeric mutations cause the development of HCM and to identify new therapeutic targets for the disease'.[110] While we agree that the report nicely illustrates the promises of hiPSC technology, together the four reports also raise questions on how to best use hiPSC in sarcomeric cardiomyopathy research, both of which will be discussed in more detail in the next section.

4. Promises of hiPSCs in modelling sarcomeric cardiomyopathies

As patient-derived *in vitro* models, hiPSC technology promises a number of specific advantages:

(1) *Testing the pathological significance of a gene mutation and establishing its causality.* A major challenge faced by doctors caring for patients and family members with suspected sarcomeric cardiomyopathies is how to establish causality of a specific gene mutation, especially with incomplete penetrance and asymptomatic young patients so

Table 2 Published hiPSC-CM models of sarcomeric cardiomyopathies

Sarcomere gene mutation	Human disease	Number of mutation negative/positive family member hiPSC lines	hiPSC-CM phenotype	*In vitro* assays employed
Cardiac Troponin T *TNNT2-R173W*[109]	DCM	3/4 multiple lines from each subject	Sarcomere disorganization	EP by MEA of EBs and patch clamp single-cell AP
			Impaired Ca transients and contractility	Contractility by AFM and video edge detection
			Impaired SR Ca uptake	Ca handling
			Enhanced susceptibility to inotropic stress and prolonged strain	Cell size
			No change in AP	Drug testing
			No cell hypertrophy	
β-Myosin Heavy Chain *MYH7-R663H*[110]	HCM	5/5 multiple lines from each subject	Cell hypertrophy with increased	Single-cell EP
			ANF expression, multi-nucleated cells, disordered sarcomeres, and nuclear NFAT	Contractility by video edge detection
			Hypercontractility	Ca handling
			No change in AP duration	Cell size
			Delayed afterdepolarizations	Expression of hypertrophic genes
			Reduced SR Ca content, increased resting Ca	Drug testing
			Enhanced hypertrophy in response to inotropic stress	
β-Myosin Heavy Chain *MYH7-R442G*[111]	HCM	0/1 2 control lines from unrelated donors	Cell hypertrophy with disordered sarcomeres and nuclear NFAT	Whole transcriptome sequencing
			Massive AP prolongation	Single-cell AP
			Increased Ca, Na, and Ito currents	Patch clamp analysis of ion channels
			Elevated diastolic Ca	Ca handling
			Irregular spontaneous beating	Cell size
				Drug testing
cMyBP-C *MYBPC3-999-1004del* 2 HCM of unknown cause[112]	HCM	0/1 2 HCM lines from sarcomere mutation negative patients, 3 control lines from unrelated donors	Cell hypertrophy with disordered sarcomeres, exacerbated by endothelin 1. 'Disordered' contractility. Similar endothelin 1 response in neonatal mouse cMyBP-C$^{\pm}$ myocytes	Contractility by video analysis
				Cell morphology by EM
				Drug testing

cMyBP-C, cardiac myosin binding protein C; AP, action potential; NFAT, nuclear factor of activated T-cells; EP, electrophysiology; MEA, multi-electrode array.

typical of HCM. For example, the Wu lab reported that the hiPSC cellular phenotype tracked completely with mutation carrier status, both for the HCM and the DCM model. The causality of the mutation can be further examined by correcting the putative mutation with gene-editing approaches [e.g. transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeats (CRISPRs) Cas9 systems] and thereby generate genotype-matched iPSC control cell lines.[114]

(2) *Determine proximate disease mechanisms caused by sarcomeric mutation.* HiPSC could be an enabling technology that allows testing of hypotheses generated from studies in humans (*Table 1*) and/or transgenic animal models. For example, energetic deficit hypothesis, Ca sensitivity hypothesis, autophagy hypothesis, loss of Frank-Starling hypothesis discussed elsewhere in this issue could all be tested in hiPSC-models for different mutations.

(3) *Discovery of new disease mechanisms.* HiPSC-CM models could help us discover new cellular mechanism caused by gene mutations. For example, the Wu lab concluded that elevated cytosolic Ca was the underlying cellular mechanism responsible for the hypertrophy and arrhythmia phenotype in the HCM cells. It remains to be seen whether this conclusion is valid and applies to other HCM-linked sarcomeric mutations.

(4) *Validation of genetic modifiers using gene-editing approaches.* Gene-editing approaches using CRISPRs or TALENs coupled with expression studies and/or proteomics can be used to not only establish causality of disease-causing mutations, but also to validate the effect of genetic modifiers.

(5) *Cellular phenotyping to aid risk stratification and prognosis.* If the cellular phenotype is robust and clinical correlation can be established, one could envision using the hiPSC cellular phenotype as novel tool for risk stratification and prognosis. This would help address a major

challenge to doctors caring for patients carrying sarcomeric mutations and their family members, as discussed elsewhere in this issue of the Journal. However, for this to work, prospective, long-term iPSC phenotype/clinical phenotype correlation studies would have to be done in a large population of mutation carriers to establish the natural history and prognosis of sarcomeric cardiomyopathies.

(6) *Testing of patient or mutation-specific drug/gene therapies.* One of the most widely advertised promises of the hiPSC technology is the ability to test efficacy of drug or gene therapy in a mutation-specific or patient-specific fashion. The Wu lab has demonstrated such testing with clinically-available drugs used in patients with HCM or DCM. However, the Ca channel blocker identified as effective in the HCM hiPSC model—verapamil—failed to provide objective benefit to HCM patients in a double-blinded clinical trial.[115] Future, more systematic studies will have to determine the validity of this approach.

(7) *Use for drug discovery and development.* Once hiPSC lines from multiple patients with sarcomeric mutations become available, those could be used for large-scale high-throughput screens for drug discovery.[116] Alternatively, one could perform efficacy studies of a lead compound in disease hiPSC-CM of multiple genetic backgrounds, in essence conducting a 'clinical trial in the dish'.

(8) *Drug safety testing.* One of the most widely advocated uses of hiPSC is for drug safety testing. Here the hiPSC could be used as a source of human myocytes for safety pharmacology, or even for testing drug safety in a disease or mutation-specific fashion.[117] Again, the validity of these approaches remains untested.

5. HiPSC-CM approaches

As outlined in *Table 1*, a human '*in vitro* HF phenotype' has been relatively well defined in a whole array of *in vitro* preparations, ranging from homogenates for the molecular data to intact trabeculae. Not all of these technologies have been applied to hiPSC-CM as yet, but no principal limitation exists. HiPSC-CM have been subjected to measurements of action potentials (both microelectrodes and fluorescent dye-based), ion currents (patch-clamping), Ca^{2+} transients (fluorescent dyes), cell shortening (video-based edge detection), sarcomeric structure and orientation and cell size (immunofluorescent staining), and AFM. Multi-electrode arrays integrated in cell culture dishes are frequently applied for the evaluation of stem cell-derived field potentials ('*in vitro* ECG') and detect repolarization-prolonging drug effects with good sensitivity.[118] It should be noted, though, that the hiPSC-CM assays are all based on cells cultured for weeks (embryoid body or monolayer), whereas none of the data from human HF and HCM samples (*Table 1*) is.

One possibility to narrow this gap is to generate three-dimensional engineered heart constructs. Different formats have been developed over the past 20 years, both for cardiac repair and as experimental test beds (reviewed in Hirt *et al.*[119]). The hydrogel (collagen I, matrigel, fibrin, and mixtures thereof)-based methods are probably best suited for hiPSC disease modelling. They come in different flavours (rings, strips, biowire, networks), but the principals are the same as described in the original publication.[120] A suspension of cells in a liquid matrix material is poured into casting forms and forms a 3D, cell-containing hydrogel that acquires the form of the casting mold. Importantly, the gel needs to be anchored to a support structure to expose the remodelling cell–hydrogel block to mechanical strain. The geometry of the casting mold and the integration of mechanical supports have seen intense development. Whereas the original methods (lattices,[120] rings,[121] Flexcell[122]) required a two-step procedure (casting and manual transfer in organ baths for force measurements), newer methods[123,124] integrate flexible silicone posts in the casting molds which allows the growing heart tissue to develop around and fix to two posts and start to deflect them when coherent beating starts. In this format, CM form spontaneously beating, force-generating heart muscles strips that perform contractile work against an elastic load (silicone posts; *Figure 3*). The 24-well-fibrin-engineered heart tissue (EHT) technology, applied to rat, mouse, and hPSC-derived cardiac myocytes,[124–126] is simple and robust and, when coupled with an automated video-based analysis system, provides a high-content readout of contractile function. Measurements can be done repeatedly under sterile, steady-state conditions without the need for manual handling. The most radical simplification and miniaturization is a 200-well-format in which tissues self-organize around posts, omitting casting of individual EHTs.[123] The higher throughput potential and the simplicity of the system are attractive, but the applicability of the system to drug screening and disease modelling remains to be demonstrated. Another technology produces 3D networks by pouring a cell–hydrogel mixture into silicone molds with arrays of mesoscopic posts.[127] Using EHTs, we and others faithfully recapitulated key abnormalities of *MYBPC3* knockout[122,128] and targeted knockin mice.[128] Of note, the differences between genotypes were discrete and still well detected. Even more, a pathological phenotype (increased sensitivity to external calcium concentrations) was robustly seen in EHTs from heterozygous mice, which develop diastolic dysfunction, but no hypertrophy.[129] The data indicate high sensitivity of the *in vitro* system. The 3D systems work with pluripotent stem cell-derived cardiomyocytes[125,126,130] and comparisons of different patient-derived hiPSC-EHTs are underway.

The '*in vitro* HF phenotype' refers to terminal stages of the multifaceted syndrome HF, and specificities of an (early) human DCM phenotype characteristics are unknown. Somewhat surprisingly, key HF characteristics were also found in myectomy samples from patients with HCM who clearly exhibit a different clinical picture. This observation may indicate that the HF phenotype develops relatively early in response to cardiac stress, that it is rather non-specific and/or, at least in part, an artefact introduced by the isolation procedures from fibrotic myectomy samples. In any case, we still have a very incomplete picture of the real mechanisms involved in specific cardiomyopathies. The only firm statement that can be made today is that, for the vast majority of familial cases, sarcomeric gene mutations (or mutations in genes with indirect effects on sarcomeric proteins such as RB20[131]) are the underlying cause of the disease and that they have almost certainly direct consequences on intrinsic cardiac myocyte properties. Likely, however, both gain- and loss-of-function alterations are disease-causing as exemplified by increases and decreases in myofilament Ca^{2+} sensitivity associated with HCM and DCM,[132] respectively. In other words, it is quite possible that any (genetically determined and thereby fixed) deviation from 'normal function' causes cardiomyopathy if it exceeds a certain severity. Such deviations could be detected in patient-derived hiPSC-CM and the type of assays described earlier (*Table 1*). Doing such evaluation on an individual basis could in fact represent an important step forward in individualized risk prediction, drug testing, and therapy. However, critical issues and challenges need to be resolved as outlined in the following sections.

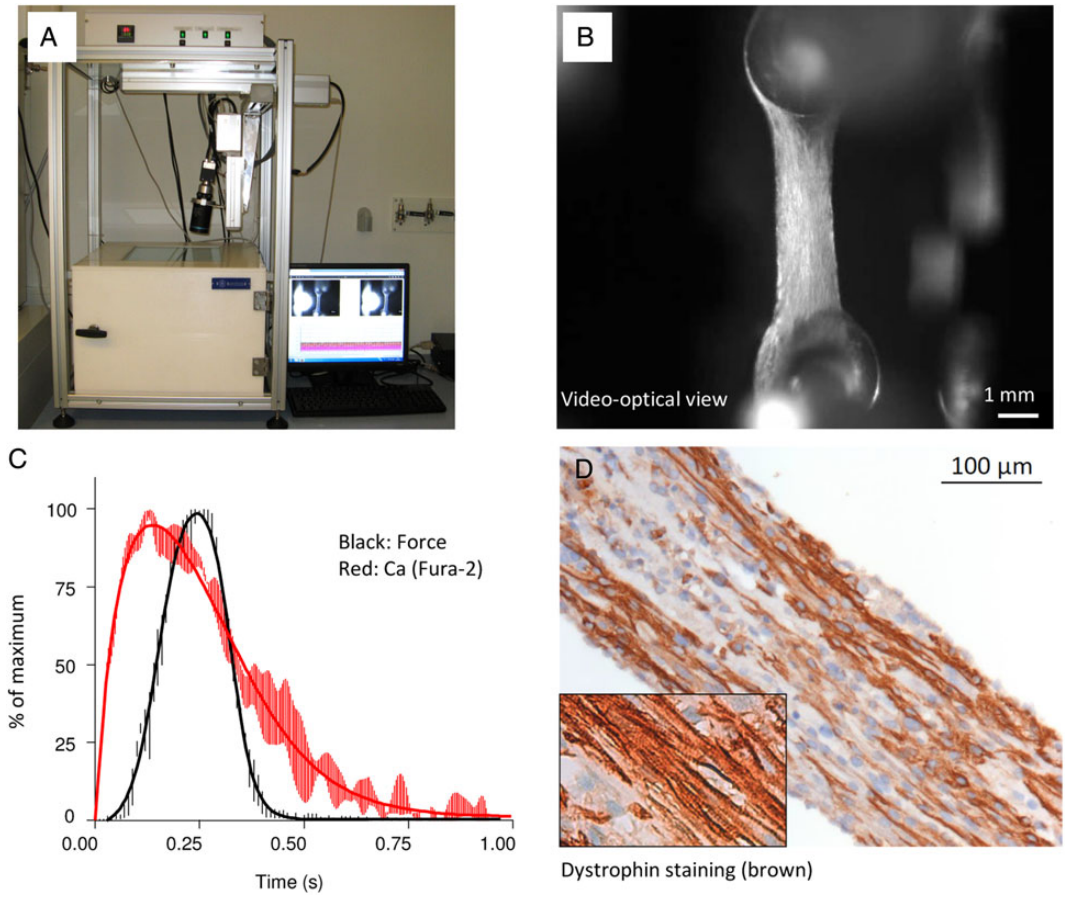

Figure 3 Fibrin-based engineered heart tissue (EHT) as an automated, high content readout of functional parameters of hiPSC-derived cardiomyocytes in a three-dimensional heart muscle construct. (*A*) Setup to measure spontaneous or electrically stimulated contractile activity of EHT cultured around elastic silicone posts in a 24-well format over extended periods. Note a temperature-, gas-, and humidity-controlled incubation chamber and a PC-controlled video camera with XYZ drive above. (*B*) Image of a hiPSC-EHT between two silicone posts as viewed by the video camera. Note the muscular structure. (*C*) Overlay of averaged contraction peak (black) and Ca^{2+} transient (red), normalized to their respective maxima. From Stoehr *et al.*[126] (*D*) Dystrophin-stained heart muscle structure of hiPSC EHT. Note longitudinal orientation and cross-striation. From Hirt *et al.*[133]

6. Challenges and bottlenecks of the hiPSC-CM approach

One of the key shortcomings of the field concerns the lack of systematic studies to define 'normal values +/- SD' for the parameters summarized in *Table 1*. Only this would allow the detection of abnormalities in individual patients or groups of patients with cardiomyopathy or, possibly, even other forms of heart failure (e.g. heart failure with preserved ejection fraction, HFpEF). Defining meaningful normal values with reasonable SD (similar to current clinical chemistry parameters) of hiPSC-derived cardiomyocytes from healthy people requires experimental procedures that are highly efficient, robust, and standardized to an extent that they can be reproduced in many laboratories worldwide. We are not at this point yet (discussed earlier). In contrast to the direct measurement of, e.g. sodium levels in blood serum, the generation and assessment of hiPSC-derived cardiomyocytes involves numerous time-consuming and technically demanding manual handling steps with still imperfect efficacy. Moreover, the approaches used to reprogram somatic cells and then create cardiomyocytes are fundamental interventions into the biology of the cell and incompletely understood. Thus, accumulation of experimental variability at each of the many steps of this approach is considerable and likely accounts for the current situation that differences between baseline values of 'controls' are often larger than those between mutated samples and controls (e.g. action potential duration, compare *Fig. 7B,D*[74]). This emphasizes the importance using isogenic controls where possible that only differ in the gene mutation of interest.

Assays are generally defined by their sensitivity and specificity, both are currently unknown for the current tests employed for hiPSC disease modelling. It remains to be shown that the effect size of heterozygous mutations (the standard in autosomal dominant diseases) on the parameters listed in *Table 1* is larger than the experimental variability. Specificity will be similarly important. Does an abnormality of hiPSC-CM from one person reflect true biological abnormality or a technical artefact that has either been introduced during the numerous rounds of making hiPSC and CM or even earlier. An additional problem that is increasingly recognized is that even normal cells (fibroblasts, but also neurons) exhibit a surprising degree of large copy number variations and even aneuploidy.[134] Thus, clonally derived hiPSC may not necessarily reflect 'the' genome of the corresponding person. Systematic

experiments in larger series of patient-derived hiPSC-CM with experimenters blinded to the genotype or group assignment are warranted. Such an approach should also include interlaboratory tests.

HiPSC-CM are immature when compared adult heart CMs (for comprehensive review see Yang *et al.*[135]). This could create the situation that proteins which are affected in certain cardiomyopathies are either not expressed at all or at abnormal levels or in an environment that differs substantially from that in the normal, mature heart. All methods described in (iii) result in cardiomyocytes with action potentials of lower than normal amplitude, disorganized sarcomeres and low forces of contraction. Although they are often described as atrial-, ventricular-, or pacemaker-like based on the shape of their action potentials, this is usually difficult and not accurate since they are immature. For example, myofilaments, sarcoplasmic reticulum, and mitochondria all exhibit smaller than normal volume fractions,[136] myofilaments are poorly oriented, t-tubules are lacking, cells predominantly utilize glucose rather than fatty acids, and show little inotropic response to β-adrenergic stimulation. Is it reasonable to assume that, under such conditions, heterozygous mutations in RyR2 (CPVT) or PLB (DCM) are really faithfully detectable? Many groups work therefore on means to improve maturation. One simple measure may be time of culture as impressively demonstrated by Lundy *et al.*[137] Here, culture of isolated hiPSC-CM for >3 months led to substantial increases in multinucleation, sarcomere organization and orientation as well as cell size. Further optimization likely requires the combination of 3D tissue formation,[124,125] co-culture,[130] humoral factors,[138,139] geometric patterning,[140] and electrical or mechanical stimulation.[141]

3D engineered heart constructs clearly promote maturation, but do not solve all issues and have a number of shortcomings themselves. (i) The advantage of being more complex, heart-muscle-like entails the potential disadvantage of cellular heterogeneity and undefined cell–matrix (often non-human) interactions. This complicates the establishment of cause-effect relationships. Whereas many data suggest that the presence of non-myocytes (stromal cells, endothelial cells) improves the quality of 3D *in vitro* tissues[130,138] 24-well fibrin-EHTs can be readily produced from >99% pure hiPSC-derived cardiomyocytes (CDI, Axiogenesis) with excellent alignment, force generation, and pharmacological response profiles similar to EHTs from 70 to 80% hiPSC-cardiomyocyte preparations (unpublished data). This shows that the presence of non-myocytes in EHTs is not a conditio sine qua non. (ii) Reproducibility and robustness may be expected to decrease with the increased complexity of 3D constructs. Large data sets from hiPSC-CM are not

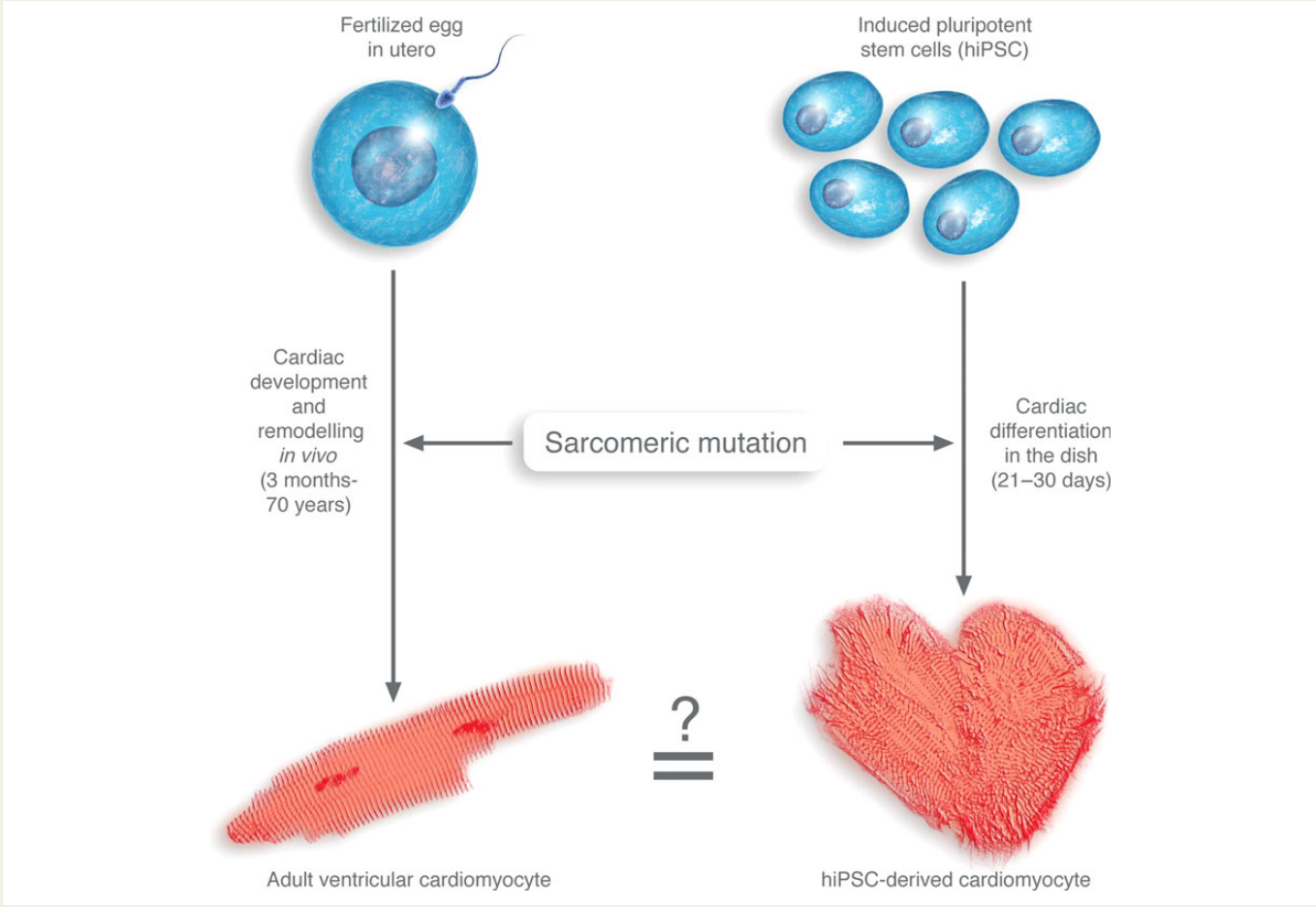

Figure 4 The inherent assumption of using hiPSC for modelling inherited sarcomeric cardiomyopathies is that the effect of the sarcomeric mutations during cardiac development *in vivo* is the same as its effect on cardiac induction in the dish, which cannot be valid. Only certain aspects of disease pathology will be approachable by the hiPSC technology.

published yet, but in our hands the by far largest source of variability is cardiomyocyte differentiation efficiency. With homogenous cell batches, the variability between EHTs of one batch and different batches is small. It actually appears that the EHTs format reduces rather than increases variability, maybe by integrating the function of many thousands of (individually different) cardiomyocytes. (iii) Classical microelectrode and patch clamp analyses are difficult to obtain from whole EHTs (possibly due to the relatively high fraction of extracellular matrix), and isolating cardiomyocytes from EHTs requires relatively harsh experimental conditions, calling in question the validity of the data. We and others therefore work on methods to determine membrane potential and intracellular Na^+ and Ca^{2+} concentrations[126,142] with fluorescent dyes.

Time may not be considered sufficiently. Patients with HCM or DCM generally grow into adulthood without major symptoms or even with normal heart function. Can we expect (immature) iPSC-CM to develop a disease phenotype in the dish over an observation period of a few days or weeks? A critical factor maybe mechanical load. HiPSC-CM are normally not loaded (in embryoid bodies) or fixed to the stiff surface of plastic dishes, are not oriented and perform little or no contractile work. In contrast, cardiomyocytes in a real heart are strictly oriented (anisotropically organized) and contract auxotonically, i.e. undergo phasic periods of isometric and isotonic force development. Advanced *in vitro* models orient cells and/or modify surface stiffness[143–145] or impose mechanical load on auxotonically contracting EHT.[146] Such approaches may be able to systematically unmask a disease phenotype not present under baseline conditions.

In view of these considerations, one could question the validity of the *in vitro* hiPSC phenotype for modelling the cellular pathophysiology caused by sarcomeric mutations. All four published hiPSC-CM models of sarcomeric cardiomyopathies exhibited robust cellular phenotypes (*Table 2*). A major question for the field is how to interpret this finding. Does the hiPSC-CM phenotype truly reflect the phenotype of the native myocytes in the patient, or does it reflect the effect of the gene mutation on cardiac differentiation and *ex vivo* culturing (*Figure 4*)? To address this issue, studies directly comparing hiPSC-CM with myocytes isolated from patient hearts, ideally from young patients prior to onset of macroscopic disease would be needed, but are close to impossible to conduct. Such studies have so far only been done using mouse models of channelopathies and, as discussed earlier, of *MYBPC3*-related HCM. For Na channel mutations, the hiPSC-CM indeed recapitulated typical electrophysiological disease features found in ventricular myocytes examined immediately after isolation.[80] On the other hand, hiPSC-CM lacking the major sarcoplasmic reticulum Ca binding protein calsequestrin, which in humans causes a severe form of CPVT, were immature, had impaired ultrastructure, and pronged action potentials,[93] none of which are features of CPVT caused by loss of calsequestrin in humans.[147] A likely explanation is that calsequestrin levels determine the maturity of hiPSC-derived myocytes in culture based on experiments in ESC-derived CM.[148] Hence, this may be an example where the hiPSC phenotype is driven by the interaction between the mutation and the *in vitro* culture conditions in the dish rather than reflecting the effect of the mutation during normal cardiac development in humans. Based on the published reports of HCM hiPSC models, results for sarcomeric mutations maybe somewhere in the middle; all HCM hiPSC models regardless of the underlying mutation exhibit cellular hypertrophy in a matter of weeks (*Table 2*), whereas it takes decades for patient to develop cardiac hypertrophy. Paradoxically, while the published hiPSC-CM models do not provide much new insight into underlying disease mechanisms or therapy of sarcomeric cardiomyopathies, this accelerated hypertrophic response in the dish could prove valuable for using hiPSC as prognostic tools to aid risk stratification in the future.

7. Summary and conclusion

The seminal discovery of ways to induce pluripotency in somatic cells and the enormous progress in deriving cardiomyocytes from these stem cells at high numbers and with increasing robustness have opened a new research field with great potential for cardiovascular research. This encompasses applications in preclinical cardiac toxicology, drug testing, disease modeling, and individualized risk prediction. To realize the full potential of hiPSC-derived cardiomyocytes, great care will nevertheless be required in applying the technology wisely and ensuring the outcomes are not over-interpreted. It will need careful consideration of the challenges and limitations of the procedures and cells as discussed above, the development of automated approaches using robotics for increased accuracy and throughput, blinded comparisons of statistically meaningful numbers and interlaboratory tests. If such precautions are followed, hiPSC-derived cardiomyocytes have a real chance of moving the field forward and, almost 25 years after the discovery of the first HCM disease gene,[149] to provide a better understanding of the pathophysiology of sarcomeric cardiomyopathies and their individualized treatment.

Conflict of interest: C.L.M. is co-founder of Pluriomics BV.

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