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hiPSC MODELING OF INHERITED CARDIOMYOPATHIES

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Opinion Statement

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) represent a powerful new model system to study the basic mechanisms of inherited cardiomyopathies. hiPSC-CMs have been utilized to model several cardiovascular diseases, achieving the most success in the inherited arrhythmias, including long QT and Timothy syndromes (1,2) and arrhythmogenic right ventricular dysplasia (ARVD) (3). Recently, studies have applied hiPSC-CMs to the study of both dilated (DCM) (4) and hypertrophic (HCM) cardiomyopathies (5,6), providing new insights into basic mechanisms of disease. However, hiPSC-CMs do not recapitulate many of the structural and functional aspects of mature human cardiomyocytes, instead mirroring an immature, embryonic or fetal, phenotype. Thus, much work remains to better understand these differences as well as to develop methods to induce hiPSC-CMs into a fully mature phenotype. Despite these limitations, hiPSC-CMs represent the best current *in vitro* correlate of the human heart and an invaluable tool in the search for mechanisms underlying cardiomyopathy and for screening new pharmacologic therapies.

Keywords

Dilated Cardiomyopathy; Hypertrophic Cardiomyopathy; Arrhythmia; Stem Cells; Induced Pluripotent Stem Cells; Cardiomyocytes; Contractility; Development

Introduction

Recent groundbreaking advances in stem cell biology (7,8) have made it possible to generate human cardiomyocytes from cells isolated directly from patients with cardiomyopathies.

This new technology offers researchers a unique and unprecedented opportunity to study the mechanisms of cardiac disease *in vitro*, as well as to establish high-throughput platforms for screening drug efficacy and toxicity. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) provide distinct advantages over previous model systems.

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Conflict of Interest

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Compliance with Ethics Guidelines

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.

The majority of prior *in vitro* studies in this field have been performed on rodent cardiomyocytes which differ from human cardiomyocytes in many ways, including sarcomeric structure, expression of key contractile proteins, beat rate, electrical properties, and ion channel function, making it often difficult to extrapolate data from rodents to humans. This is especially problematic when trying to study human cardiomyopathies due to mutations in contractile proteins, such as β -myosin heavy chain (MHC), which are not highly expressed in the adult mouse or rat. Many drug discovery programs have failed because targets validated in animals proved to be non-predictive in humans (9). Attempts to study human cardiomyocytes have been limited due to the difficulty in isolating and propagating these cells in culture. Thus, most human tissue studies have been performed on samples obtained at the time of left ventricular assist device (LVAD) implantation or heart transplant. Unfortunately, these are representative of the very end stages of most cardiomyopathies, so that separating primary mechanisms induced by a particular gene variant from the secondary and tertiary mechanisms induced by chronic neurohormonal stimulation, adverse remodeling, and heart failure medications is often impossible. Thus, hiPSC-CMs arguably represent the best currently available *in vitro* correlate of the human heart (10).

Despite these advantages, concerns have persisted over the extent to which hiPSC-CMs recapitulate mature cardiomyocyte structure and function. This issue has been addressed, in part, by the recent successful application of hiPSC-CMs to understanding basic mechanisms of several cardiovascular diseases. This has been accomplished most successfully in the inherited arrhythmias, including long QT, and Timothy syndromes (1,2), and arrhythmogenic right ventricular dysplasia (ARVD) (3). These successes have been recently extended to models of dilated (DCM) (4) and hypertrophic (HCM) cardiomyopathies (5,6) providing new insights into basic mechanisms of disease and possible pharmacologic therapies. As our ability to develop hiPSC-CMs from patients increases, so has our ability to determine the molecular, structural and functional phenotype of these cells (Table I). hiPSC-CMs have also been utilized to develop large panels of patient-specific cells for testing both drug efficacy and cardiotoxicity (11). In combination, these pioneering studies have led to an enhanced understanding of the mechanisms of these diseases at the single myocyte level, not previously possible using human cells. We will review the limited number of hiPSC-CM studies that have been performed to model cardiomyopathies, suggest several criteria for moving this exciting field forward, and review our progress to date.

Hypertrophic cardiomyopathy

Fan and colleagues generated hiPSC-CMs from a ten-member family cohort with several individuals affected by hypertrophic cardiomyopathy (HCM) (5). These patients carried a missense mutation (Arg663His) in the MYH7 gene, encoding β -MHC. hiPSC-CMs from affected patients recapitulated several aspects of the HCM phenotype including cellular enlargement starting at 6 weeks after cardiac induction, contractile dysfunction in response to β -adrenergic agonists, and abnormal contractions suggestive of arrhythmia at the single-cell level. HCM cells also showed an increase in intracellular Ca^{2+} . Single cell patch clamp studies revealed frequent small depolarizations resembling the delayed after depolarizations (DADs) associated with arrhythmias, and these occurred with alterations in Ca^{2+} transients.

Of clinical interest, treatment with the Ca^{2+} channel blocker verapamil before the onset of hypertrophy (but not afterwards) normalized Ca^{2+} cycling and prevented the development of both arrhythmia and hypertrophy. Although Ca^{2+} channel blockers have not been effective in reducing hypertrophy in patients with well-developed HCM, they have not been tested in a preventive strategy in patients carrying the gene mutation who have not yet developed hypertrophy.

In another disease model, Carvajal-Vergara et al. generated hiPSC-CMs from two patients with LEOPARD syndrome, carrying a mutation (T468M) in *PTPN11*, encoding the SHP2 protein phosphatase (6). LEOPARD syndrome is characterized by skin lesions, hypertelorism, abnormal genitalia, deafness, pulmonary stenosis and hypertrophic cardiomyopathy. hiPSC-CMs from LEOPARD patients were larger than control cells and showed a greater degree of sarcomeric organization. Several signaling alterations were shown, including increased nuclear localization of the transcription factor NFATC4, and increased activation of the MEK1-ERK pathway, a known mediator of cell remodeling.

Dilated Cardiomyopathy

Sun and colleagues studied hiPSC-CMs derived from a members of a family with dilated cardiomyopathy (DCM) carrying a point mutation (R173W) in the gene encoding the sarcomeric protein cardiac troponin T (4). Compared to disease-free individuals in the same family, hiPSC-CMs derived from DCM patients showed altered regulation of Ca^{2+} transients, decreased contractility, and abnormal distribution of sarcomeric α -actinin. When stimulated with the combined α - and β -adrenergic agonist norepinephrine, DCM hiPSC-CMs showed reduced beating rate and compromised contraction, as well as a greater number of cells with abnormal α -actinin distribution. Treatment with the β 1-adrenergic blocker, metoprolol, improved contractile protein organization. Overexpression of the sarcoplasmic reticulum Ca^{2+} ATPase, *SERCA2A*, which is downregulated in patients with heart failure, reversed the decrease in contractile function, rescuing the phenotype.

Siu et al., derived hiPSC-CMs from a single member of a family with a mutation (R225X) in lamin A/C (*LMNA*) (12). Patients with lamin A/C mutations manifest dilated cardiomyopathy, often associated with conduction abnormalities. hiPSC-CMs from this patient showed morphologic changes, including a higher prevalence of nuclear bleb formation and micronucleation. Under field electrical stimulation, LMNA mutant hiPSC-CMs showed nuclear senescence and cellular apoptosis. When expression of LMNA was knocked down using short hairpin (sh)RNA, Siu was able to replicate these phenotypes in control cells. Pharmacological blockade of the ERK1/2 pathway attenuated the pro-apoptotic effects of field stimulation, suggesting a role for ERK signaling in mediating LMNA cardiomyopathy.

Tse and colleagues used hiPSC-CMs derived from a patient with dilated cardiomyopathy with an unknown genetic etiology in conjunction with whole exome sequencing to attempt to discover the causative mutation. They found a novel mutation (A285V in exon 4) in the intermediate filament protein desmin (*DES*) (13). hiPSC-CMs were then utilized to confirm that this mutation caused a cellular phenotype. Cells derived from the patient were compared

with hiPSC-CMs from a control line and to control cells that were transduced with the mutant gene. Patient-derived cells showed abnormalities of desmin co-localization with cardiac troponin-T, α -actinin and F-actin and malformation of the Z-discs. Seventy percent of A285V cells showed diffuse isolated aggregates of DES-positive protein. These abnormalities were recapitulated when the mutant A285V gene was transduced into wild-type cells. Functionally, A285V hiPSC-CMs showed decreased Ca^{2+} reuptake, slower spontaneous contractions, and failure to maintain a chronotropic response to the β -adrenergic agonist isoprotenerol. Thus, in this study, hiPSC-CMs were able to provide histologic and functional confirmation that the candidate gene variant detected by whole exome sequencing was responsible for the disease. This demonstrates another strength of using hiPSC-CMs, as the validity of a gene variant can be tested by inducing the identical mutation in a control cell line. However, in this report, the functional characterization of the hiPSC-CMS was limited, a not-infrequent issue in this nascent field.

Friedreich's Ataxia

Hick and colleagues derived hiPSCs from two patients with Friedreich's ataxia, an inherited neurodegenerative disease in which cardiac failure or dysrhythmia is the cause of death in more than half of patients (14). hiPSCs were then differentiated into both neurons and cardiomyocytes, the two cell types primarily affected. Friedreich's ataxia is caused by decreased expression of frataxin, a mitochondrial protein involved in iron-sulfur cluster biosynthesis, usually due to a GAA triplet repeat expansion in the first exon of the frataxin (*FXN*) gene. Both cell types showed a decrease in frataxin expression but no other biochemical phenotype. Although dysrhythmias are common in Friedreich's ataxia patients, hiPSC-CMs in this study showed no evidence of abnormal electrophysiology. However, both cardiomyocytes and neurons showed impairment of mitochondrial function, as evidenced by decreased membrane potential and increased mitochondrial degeneration. While an exciting observation, as of yet, no functional studies have been performed to determine the effect of these mitochondrial alterations on contractile properties in these cells.

Barth Syndrome

Dudek et al. performed studies on hiPSCs derived from three patients with Barth syndrome, an inherited dilated cardiomyopathy associated with skeletal myopathy, neutropenia, and growth retardation (15). Barth syndrome is secondary to a mutation in *TAZ*, the gene encoding the mitochondrial protein tafazzin, a phospholipid acyltransferase involved in remodeling of the mitochondrial membrane lipid cardiolipin. hiPSCs from Barth syndrome patients showed impaired remodeling of cardiolipin, and decreased basal mitochondrial oxygen consumption. This decrease in respiration was associated with structural changes in mitochondrial respiratory chain supercomplexes, and resulted in an increase in generation of reactive oxygen species (ROS). hiPSCs in this study were not differentiated into cardiomyocytes, so the extent to which these mitochondrial abnormalities would also be present in cardiomyocytes and their effects on cardiomyocyte function were not studied. Wang et al. took the next step and generated hiPSC-CMs from 2 patients with Barth syndrome and used "heart on a chip" technology to measure contractility. Barth patient cells

demonstrated sparse, irregular sarcomeric structure, decreased force development, and increased ROS despite normal whole-cell levels of ATP. Importantly, these abnormalities were reversed by reintroduction of the wildtype TAZ gene using genome editing (16).

Glycogen storage (Pompe) disease

Huang and colleagues derived iPSC-CMs from two patients with Pompe disease (glycogen storage disease type II), a hypertrophic cardiomyopathy caused by mutations in *GAA*, the gene encoding the lysosomal glycogen-degrading enzyme, acid α -glucosidase (17). Pompe hiPSC-CMs had low *GAA* activity, high glycogen content, altered respiration and ultrastructural abnormalities characteristic of Pompe pathology. Whole transcriptome analysis identified altered expression of genes involved in glycogen metabolism, lysosome and mitochondrial regulation. Of interest, this phenotype was rescued by treatment with recombinant human *GAA* and partially reversed by treatment with L-carnitine.

Limitations of hiPSC-CMs

Despite the success of these early applications, hiPSC-CMs do not mirror all aspects of adult cardiomyocytes and thus may not be appropriate models for all cardiovascular diseases. Although hiPSC-CMs express many cardiomyocyte-specific genes, such as troponin T, α -actinin and both α and β -myosin heavy chain, and contract spontaneously in culture, their phenotype is still markedly different from that of mature cardiomyocytes (Figure 1). hiPSC-CMs are typically round in shape, with myofilaments arranged at multiple angles, which may be a reflection of cardiomyocytes at an immature stage (embryonic or fetal) compared to the typically rod-shaped adult mammalian cardiomyocyte, which has highly ordered myofilaments. hiPSC-CMs also express several genes (connexin45, smooth muscle actin) that are more typical of immature cardiomyocytes. Applying our extensive knowledge of developmental cardiac biology will help to discriminate which functions in hiPSC-CMs are going to be similar to those in adult cells and which are going to be different. For example, there are marked differences in adrenergic signaling during cardiomyocyte development: in addition to increasing inotropy and chronotropy, β -adrenergic agonists induce cardiotoxic, pro-apoptotic signaling in adult cardiomyocytes (18). In contrast, β -adrenergic stimulation induces cardioprotective signaling in neonatal cardiomyocytes (19). It would be important to know these differences when testing new β -blocker drugs using hiPSC-CMs.

Following, we present a series of criteria that we believe should be applied to studies using hiPSC-CMs to model human cardiomyopathies in order to further refine their ability to recapitulate human cardiovascular disease.

Are all the parts there?

What is the expression level (both mRNA and protein) of key contractile, structural, bioenergetic, channel and regulatory proteins? How close is the cellular ultrastructure (mitochondria, lysosomes, endosomes, T-tubules, sarcoplasmic reticulum [SR] cell-cell junctions) to that of the mature cardiomyocyte? Are the close functional connections between cellular components (e.g. links between the SR and the mitochondria) present?

As mentioned above, current differentiation protocols generate cells that are highly immature and do not resemble the rod-shaped, highly organized and often binucleated cardiomyocytes found in an adult mammalian heart (Figure 1). In addition to the expression of contractile proteins, e.g. α - and β -MHC, actin, and troponin T, mRNA for genes involved in the regulation of Ca^{2+} transients are also expressed in hiPSC-CMs, including *NCX1*, *SERCA2A*, *RyR2*, *CSQ2*, *Cav1.2*, *IP3R2* and *PLN*. However, not all have been confirmed by simultaneous measurements of protein expression nor have they all been localized to their correct intracellular domains. Critical to the function of the mature cardiomyocyte is the mechanism of Ca^{2+} -induced Ca^{2+} release, which depends on a mature SR and T tubule system. In rat cardiomyocytes, T-tubules are absent at birth and begin to appear at 10 days after birth (20). So far, electron microscopic studies have failed to find evidence of well developed T tubules in hiPSC-CMs (21,22) nor expression of key components of T tubule formation (caveolin-3, amphiphysin-2 and junctophilin-2) in human embryonic stem cells (hESCs) (23)). However, there is some evidence suggesting that immature T tubules begin to develop as hiPSC-CMs mature (24).

Important to cell-cell communication, gap junction proteins have been visualized in hiPSC-CMs (25), although expression of some components, such as connexin45, suggests that they more resemble embryonic or fetal cardiomyocytes. Interestingly, connexin45 has been shown to upregulated in patients with heart failure (26). Ion channel gene expression in hiPSC-CMs has also been studied in depth, showing that hiPSC-CMs express *SCN5A*, *CACNA1C*, *CACNA1D*, *RCNQ1*, *KCNH2* (*HERG*), *HCN2*, *KCND3*, and *KCNJ2* (11,27), however, again the expression pattern appears to be more reminiscent of fetal-like cells.

Do the parts work normally?

Do ion channels regulate trans-membrane currents similar to that in adult cardiomyocytes? Do transmembrane receptors couple to their downstream effectors normally and regulate both chronotropy and inotropy? Do the myofilaments respond appropriately to alterations in intracellular Ca^{2+} ? Is cell-cell communication and cell-extracellular matrix communication intact? And finally, and perhaps most importantly for studies on the heart, are hiPSC-CMs able to do real work, i.e. to generate force under loaded conditions, not just shorten?

For studies of arrhythmia, there is accumulating data suggesting that the trans-membrane currents in hiPSC-CMs regulated by ion channels are similar enough to mature cardiomyocytes to support their utility in screening for drug-induced cardiac toxicity (28). Using single-cell patch clamp electrophysiology, hiPSC-CMs can be separated into cells displaying ventricle-like, atrial-like, or nodal (or pacemaker)-like action potentials, similar to those recorded in cells from 16 week human fetal hearts (29,30). Ventricular-like action potentials show a longer plateau phase compared to the more triangular shaped, atrial-like action potentials. Nodal-like cells show slower upstroke velocities and smaller amplitudes (28,30).

For studies of contractile function, cardiac contraction in mature cardiomyocytes is initiated by the release of Ca^{2+} from intracellular stores in response to an action potential, a process known as excitation-contraction coupling (ECC). Although no study has investigated in

detail all aspects of the ECC process of hiPSC-CMs, several pharmacological studies have suggested at least partially intact sarcoplasmic reticulum (SR) Ca^{2+} cycling through the ryanodine receptor (using caffeine as an inducer and ryanodine as an inhibitor of the receptor) and the SR Ca^{2+} ATPase SERCA (using the inhibitor thapsigargin). Studies have also showed Ca^{2+} -induced Ca^{2+} release, the main mechanism for increasing intracellular Ca^{2+} in the adult cardiomyocyte, which can be blocked by ryanodine (31). However, most of the intracellular Ca^{2+} transient in hiPSC-CMs appears to be dependent on entry through the L-type Ca^{2+} channel and only partly from SR stores, similar to that in immature cardiomyocytes (32,33). In addition, hiPSC-CMs show less synchronized Ca^{2+} transients (faster peripheral vs. slower central transients) compared to adult cardiomyocytes (34), again suggesting an immature alignment of functional proteins and structures such as the SR and T-tubules.

When it comes to regulation of contractility, the picture is less clear. Studies have shown that treatment with the β -adrenergic agonist isoproterenol increases beating rate (chronotropy) in hiPSC-CMs in a dose-dependent manner (35). This effect is blocked by the β -antagonist propranolol, as expected. In contrast, studies showing a change in contractile force (inotropy) have been more controversial. Isoproterenol has been shown to increase amplitude of contraction (31) and Ca^{2+} amplitude (36). Ryanodine, which blocks Ca^{2+} -induced Ca^{2+} release through the ryanodine receptor, decreases contraction amplitude, suggesting a possible Ca^{2+} -mediated inotropic response to isoproterenol in hiPSC-CMs. However, others have failed to demonstrate a positive inotropic response to isoproterenol (37) and one study showed a negative force-frequency relationship, the opposite of that seen in mature cardiomyocytes (31).

Do the parts respond normally to internal/external signals/stressors?

The intact heart has to respond rapidly to alterations in demand, from increasing chronotropy, inotropy and lusitropy in response to physiologic stressors such as exercise, to responding to pathologic stressors such as pressure or volume overload or ischemia. In this regard, how do hiPSC-CMs regulate inotropy, lusitropy and chronotropy? Do they respond to altered preload and afterload (the Frank-Starling relationship)? Are cell remodeling (e.g. hypertrophic) pathways intact? In the face of adverse stressors, do cell death pathways (apoptosis, autophagy) operate normally? Given the huge energetic needs of the myocardium, how are the processes of mitochondrial biogenesis (fission, fusion, mitophagy) regulated?

One of the limitations to measuring the regulation of hiPSC-CM contractility has been the difficulty in measuring force generation directly in isolated cells (38). Many studies showing increased “contractility” in hiPSC-CMs have relied on microscopic demonstration of changes in cell shortening, which are not necessarily reflective of altered force generation. Liu and colleagues showed that atomic force microscopy (AFM) was able to quantitate contractile forces of both single cells and clusters of hiPSC-CMs (39). They showed a dose-responsive, positive inotropic effect with norepinephrine which was inhibited by the β 1-adrenergic antagonist metoprolol. Cardiomyocytes derived from subjects with dilated cardiomyopathy showed decreased force and decreased cellular elasticity compared to

controls. However, AFM measurements are usually made perpendicular to the axis of the myofilaments and therefore may not fully represent the cell's force generation capacity. Others have utilized a two-point carbon fiber rod technique (38) to address this issue of directionality. Utilizing a totally different methodology, Taylor et al. used an axial technique for measuring the contractile forces of isolated hiPSC-CMs using arrays of elastomeric microposts (40). This platform avoids the extensive cell manipulation typical of other systems, such as AFM and two-point carbon fiber techniques, and is potentially adaptable for high-throughput analysis.

How much variability is there between different cell lines?

What is the variability between hiPSC-CM lines derived from the same patient? Between different patients with the same gene mutation? Between different patients with the same disease but carrying a different mutation?

Most of the studies cited above have utilized a small number of cell lines (1 to 3) derived from an equally small number of patients. Burridge et al. provide data suggesting that there is an acceptably low variability in cell lines derived from the same patient (intra-individual homogeneity) whereas cell lines derived from different patients are distinctly different (inter-individual heterogeneity) (41). White et al. has confirmed similar homogeneity in hiPSCs differentiated into endothelial cells (42). As the technology to produce hiPSC-CMs advances and the cost declines, the optimal number of cell lines required for confirmation of biologic differences will increase and studies will require greater numbers of patients with the same mutation as well as patients with the same disorder (e.g. dilated cardiomyopathy) with different mutations to confirm genotype-phenotype associations. Previous studies have mainly used the embryoid body method to differentiate hiPSCs into cardiomyocytes, which has a yield rate of only 5%–10% and produces a relatively immature phenotype. With rapid advances in differentiation protocols, yields of beating cardiomyocytes are now reaching as high as 95% (43). Thus, depending on the differentiation method and culture conditions, the maturity and phenotype of cardiomyocytes can be dramatically different.

What are the maturational changes that occur with increased time from cardiomyocyte induction?

Maturational changes in hiPSCs, as they differentiate from pluripotent stem cells to mesodermal cells to cardiomyocytes, have mostly been characterized by examining gene expression patterns (Table II). How well do these changes recapitulate the developmental changes that occur as the normal cardiomyocyte matures from embryonic to fetal to neonatal to adult form? How can these cells be induced to form a more mature phenotype? Or, are hiPSC-CMs permanently relegated to a very immature state?

At the pluripotent cell stage, hiPSCs appear to be very similar to human embryonic stem cells (hESCs) in terms of their morphology, global expression of genes and proteins, and potential to differentiate into various cell types. However, there are epigenetic variations between hiPSCs and hESCs that could influence their fidelity in modeling human cardiovascular diseases (44,45). As hiPSC-CMs mature, the structural organization of

myofibrils increases, and fewer cells retain any proliferative capacity. More mature intracellular structures become apparent, including more densely striated myofibrils, with organized sarcomeres bounded by Z lines, intercalated discs, gap junctions and desmosomes, and an early T tubule system (14,24). The majority of hiPSC-SMs at this stage are mononucleated although a few are binucleated. However, in cultures of differentiated hiPSC-CMs, it is not uncommon for cells at different stages of maturation to be identified, e.g. within the same area of an embryoid body (14), complicating any measurements of phenotype.

Researchers are currently exploring ways to induce greater maturation in hiPSC-CMs, using methods ranging from growing cells on patterning scaffolds, 3D cell alignment, electrical stimulation, cyclic stretch, co-culture with other cardiac cell types and alteration of growth media (46,47). Differentiated hiPSC-CMs can be cultured and matured *in vitro* for up to 150 days after induction and evidence is accumulating that prolonged culture can result in contractile and structural maturation (48). With longer time after cardiac induction, hiPSC-CMs show an increase in peak Ca^{2+} , faster rate of Ca^{2+} release, and increased Ca^{2+} SR stores (32,48). Coordinated intracellular Ca^{2+} signaling is an important hallmark of maturity, which comes with well-aligned SR and structurally mature T-tubules.

Summary

hiPSC-CMs represent a unique platform for cardiovascular researchers to study basic mechanisms of the cardiomyopathies using a human cell-based system. Their utility has already been demonstrated in studies of several genetic cardiomyopathies, where they have provided unique insights into disease mechanisms. However, the hiPSC-CMs that are available today still largely represent an immature version of the adult cardiomyocyte and thus may not be the best model for all cardiovascular diseases. Particular attention needs to be paid to developmental differences, e.g. those mediated through drug-targetable cell surface receptors, if these cells are to be used for drug screening. The good news is that research in this area is accelerating rapidly, and systems to induce greater maturation in hiPSC-CMs are being actively tested.

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Figure 1. Photomicrographs comparing (a) hiPSC-CM at 1 month after cardiac induction and (b) murine adult cardiomyocyte. Stained with anti-sarcomeric actinin antibody.

Table I

Methodologies for phenotyping hiPSC-cardiomyocytes.

hiPSC-CM characteristic	Methods
Cellular Structure	Light and electron microscopy
Gene expression and regulation	RNA-seq, quantitative PCR, CHIP (chromatin immunoprecipitation)
Protein expression	Immunofluorescence staining, Western immunoblot, FACS (fluorescent activated cell sorting), mass spectrometry
Electrophysiology	Whole-cell patch clamp, microelectrode arrays (MEA)
Contractility	Atomic force microscopy, two-point carbon fiber rods, micro-engineered force posts
Calcium transients	Whole cell fluorescent Ca^{2+} imaging
Reactive oxygen species	Cell ROX
Mitochondrial function	Oxygraph measurements, MitoSOX

Table II

Expression of genes marking the progression of hiPSCs from mesodermal precursors to hiPSC-CMs. Adapted from Dambrot et al (49).

Cell Type	Genes Expressed
Pluripotent stem cell (hiPSC)	<i>Oct4, Nanog, Sox2</i>
Early mesodermal cell	<i>Brachyury T, Fox C1, Dkk 1, MXL-1</i>
Cardiac committed mesoderm	<i>MESP1</i>
Cardiac progenitor cell	<i>Nkx2.5, Tbx5, Tbx20, Gata-4, Mef2c</i>
hiPSC-CM	<i>cTnT, α-actin, MHC, MLC2a, MLC2v</i>