Inherited arrhythmia syndromes

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Chapter 2

Immaturity of stem-cell derived cardiomyocytes: fatal flaw or soluble problem?

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ABSTRACT

Cardiomyocytes from human pluripotent stem cells (hPSC-CMs) are increasingly used to model cardiac disease, test drug efficacy and for safety pharmacology. Nevertheless, a major hurdle to more extensive use is their immaturity and similarity to fetal rather than adult cardiomyocytes. Here, we provide an overview of the strategies currently being used to increase maturation in culture that include, prolongation of time in culture, exposure to electrical stimulation, application of mechanical strain, growth in 3 dimensional tissue configuration, addition of non-cardiomyocytes, use of hormones and small molecules and alteration of the extracellular environment. By comparing the outcomes of these studies, we identify the approaches most likely to improve functional maturation of hPSC-CMs in terms of their electrophysiology and excitation-contraction coupling.
INTRODUCTION

Human pluripotent stem cells (hPSCs) are capable of prolonged cell division and differentiation into any somatic cell-type including cardiomyocytes. Cardiac diseases are still the most prevalent cause of death in the Western world\(^1,2\) and hPSCs are proving particularly useful in understanding molecular mechanisms underlying many cardiac afflictions and identifying individual drug sensitivities. Potential applications of hPSC-derived cardiomyocytes (hPSC-CMs) range from tissue replacement in heart failure after myocardial infarction to basic science on heart development, cardiac disease modelling, drug discovery and cardiac safety pharmacology\(^3\). Although promising, tissue replacement for the heart is still quite far from daily clinical practice\(^4\), however interest in using hPSC-CMs in the pharmaceutical context is more immediate since toxic side effects on the heart often limit implementation of valuable drug therapies. Moreover, the ability to reprogram adult somatic cells into a pluripotent state as induced pluripotent stem cells (iPS cells or iPSCs)\(^5,6\), coupled with advances in methods for genetic engineering\(^7\) now make it possible to study patient- and disease-specific cardiac cells carrying specific (disease related) mutations or polymorphisms\(^3\), facilitating insight into disease mechanisms.

hPSC-CMs however remain immature in culture and resemble heart cells of mid-gestation human foetuses. They show disorganized sarcomeres, small forces of contraction and small action potentials compared with adult working cardiomyocytes. This limits their use in studying human cardiac physiology and pathology in the laboratory, while in the context of tissue repair, the immature properties of hPSC-CMs may cause lethal arrhythmias after transplantation in the heart\(^8\). Inducing hPSC-CM to a more adult state would significantly increase their value. Various strategies for this have already been described\(^9\). Here, we provide an overview of these studies that have included 1) increasing time in culture\(^10–14\); 2) applying electrical stimulation to cause continuous contractile and electrical activity\(^15–19\); 3) adding chemicals or small molecules\(^20–22\); 4) applying mechanical stretch\(^23,24\); 5) co-culture with non-cardiac cells\(^25\); 6) growth as 3-dimensional (3D) tissues\(^18,22,26\); and 7) adjusting composition, stiffness and topography of extracellular environment\(^27–29\). These strategies mainly mimic cardiac physiology, however artificial methods in which the expression of key genes is modified have been applied as well\(^15,30,31\). The discussed approaches have often been identified in isolated and cultured rodent cardiomyocytes, in which the reversion from a mature to an immature state is a common phenomenon\(^12\). Where relevant, results of these studies are discussed as well. Before considering maturation strategies, however, we first describe how cardiomyocytes are derived in culture from hPSCs followed by the distinguishing features of mature (adult) and immature (fetal) cardiomyocytes that will allow benchmarking of maturation state of the derivative hPSC-CMs.
DISTINGUISHING FEATURES OF IMMATURE AND MATURE CARDIOMYOCYTES

Morphology
Adult cardiomyocytes are elongated and rod-shaped with an aspect ratio (length to width) of 5:1, while fetal cardiomyocytes are typically round- or polygonal-shaped. As human cardiomyocytes mature, they increase in size due to physiological hypertrophy. Early hPSC-CMs (10–15 days after onset of differentiation) are mostly round and small, although rod-shaped cells have been described. Later (> 50 days in culture), hPSC-CMs become more elongated although they remain smaller than adult cardiomyocytes. In addition, adult cardiomyocytes in the heart are longitudinally aligned, facilitating fast electrical conduction and efficient muscle contraction via connecting intercalated discs. However, fetal cardiomyocytes and monolayers of hPSC-CMs are chaotically organized. The number of nuclei per cell also distinguishes adult and fetal cardiomyocytes: 25–30% of adult cardiomyocytes in the heart are binucleated independent of age, while fetal cardiomyocytes are almost exclusively mononucleated. Ploidy does increase during maturation, and may be a better marker of maturity. hPSC-CMs are largely mononucleated, showing binucleation only sporadically.

Sarcomere
The sarcomere is the contractile unit of cardiomyocytes that consists of proteins that form thick and thin filaments. By electron microscopy, adult cardiomyocytes show highly organized and aligned sarcomeres with different landmarks corresponding to different functional units: Z-discs mark sarcomere borders, I-, H- and A-bands different areas of thick, thin and overlapping filaments and M-bands form the central line. In the human fetal heart, this organization develops gradually during gestation, with Z-discs and I-bands forming first, followed by H-, A- and M-bands. This process of sarcomerogenesis also occurs in maturing hPSC-CMs, however in most cells only Z-discs and I-bands are formed. Sarcomere length, the distance between two Z-discs, is another indicator of cardiomyocyte maturation: adult cardiomyocytes contain considerably longer sarcomeres than hPSC-CMs and fetal cardiomyocytes. Proteins assembled in the sarcomeres are also differentially expressed in immature and mature cardiomyocytes: both myosin heavy chain (MHC) and myosin light chain (MLC) protein isoforms change during maturation. In the adult ventricle, it is mainly the β-MHC (encoded by MYH7) that is expressed, with very low levels of the atrial isoform α-MHC (encoded by MYH6). This abundance of β-MHC over α-MHC is already clear in early fetal stages and shows a further increase at later gestational ages. Interestingly, the ratio of β-MHC/α-MHC in hPSC-CMs is different from fetal and adult ventricular cardiomyocytes and changes little over time. This may be because hPSC-CMs are often a mixture of atrial and ventricular cardiomyocytes. The two predominant isoforms of MLC are MLC2a and
Immaturity of stem-cell derived cardiomyocytes

MLC2v, encoded by the genes MYL7 and MYL2. Again, an isoform switch occurs during maturation. MLC2a, the predominant isoform in adult atria, is expressed in human fetal ventricle although gradually decreases with gestational age, when MLC2v is the isoform almost exclusively expressed. In hPSC-CMS, both isoforms are expressed. Again, a mixture of cardiomyocyte subtype may give rise to the fact that a predominance of one over the other is not clearly observed. Titin, a sarcomeric protein with many spliced isoforms, anchors between the Z-discs and the M-lines of the sarcomere and plays a crucial role in sarcomeric elasticity. In fetal cardiomyocytes and hPSC-CMs, it is mainly the N2BA isoform that is expressed, whilst in adult cardiomyocytes, N2B is the most abundant isoform.

Electrophysiology

The cardiac action potential (AP) governs cardiomyocyte electrical behaviour. AP characteristics are unique for each cardiomyocyte subtype (atrial, ventricular, pacemaker, Purkinje). The sum of the activity of various depolarizing and repolarizing currents through different ion channels is responsible for the cardiac AP (Figure 1B). Adult and fetal cardiomyocytes differ in the availability of ion channels on their cells which results in different AP profiles. The AP is initiated by the large, rapid influx of Na⁺ (I_{Na}) through Na⁺ channels, resulting in fast depolarization of the membrane; this is called the AP upstroke or phase 0. Subsequently, there is a brief repolarizing phase (phase 1), resulting from efflux of K⁺ caused by activation of the transient outward potassium current (I_{To1}). Next, inward flow of Ca²⁺ (I_{Ca,L}) through L-type calcium channels leads to the plateau phase (phase 2). Finally, the membrane repolarizes to its original state due to activation of the rapid and slow delayed rectifier K⁺ channels (conducting the I_{Kr} and I_{Ks} currents respectively) in phase 3 of the AP. Adult ventricular and atrial cardiomyocytes also exhibit phase 4 in which the resting membrane potential (RMP) does not change. This is due to the rectifying K⁺ current (I_{K1}), which stabilizes the membrane potential at the reversal potential of K⁺. In hPSC-CMs, I_{K1}, if even detected, is only a small fraction compared to adult cardiomyocytes and KCNJ2 mRNA expression (encoding the α-subunit of the channel) is correspondingly low. Accordingly, the RMP is less negative in hPSC-CMs compared to adult cardiomyocytes (-50 to -60 mV versus -85 mV). Moreover, low or absent I_{K1}, together with the pacemaker current (funny current, I_{f}), which is very low in adult ventricular cardiomyocytes, causes gradual diastolic depolarization and consequent spontaneous contractile activity in hPSC-CMs. Because of the more depolarized state of hPSC-CMs, functional availability of Na⁺ is reduced, resulting in a slow upstroke of the AP, although low expression of sodium channels (encoded by SCN5A) in early hPSC-CMs might contribute to this as well. Similarly, I_{To1} is inactivated at more positive membrane potentials, leading to less pronounced phase 1 repolarization. Although the latter demonstrates similar expression and current density levels...
in hPSC-CMs and adult cardiomyocytes, $I_{to1}$ does increase during development and is also considered a sign of maturation. The other main repolarizing currents, $I_{Kr}$ and $I_{Ks}$, are present in hPSC-CMs at similar levels to adult cardiomyocytes. To date, current densities in human fetal cardiomyocytes have not been reported but data in other animals suggest that $I_{Ks}$ increases during development.

Figure 1. Qualitative comparison of morphological (A), electrophysiological (B), and calcium-handling-related (C) features of mature (adult) and immature [fetal or human pluripotent stem cell (hPSC)-derived human cardiomyocytes]. A. Microscopic image of an adult human atrial cardiomyocyte stained with anti-a-actinin (green) and anti-myosin light chain-2a (red) and a human embryonic stem-cell derived cardiomyocyte stained with anti-cardiac Troponin I (green) and anti-a-actinin (red). Scale bars are 100 mm and 25 mm for the left and right panel, respectively. B. Typical action potential shapes of adult ventricular and immature cardiomyocytes and their accompanying ion currents. Note the less negative resting membrane potential, slow upstroke, and small amplitude in immature cardiomyocytes compared with the adult cardiomyocytes, which coincide with lower densities of $I_{K1}$, $I_{Na}$, and $I_{to1}$. The gene symbols corresponding to the ion currents are stated in parenthesis. C. Illustrative scheme representing $Ca^{2+}$ influx and extrusion in adult and immature cardiomyocytes. Inset shows representative $Ca^{2+}$ transients.
Two types of calcium channels are present in the human heart: L-type and T-type. While the functional presence of the T-type calcium current ($I_{T, Ca}$) is typical of fetal cardiomyocytes, atrial and ventricular cardiomyocytes of the adult heart do not exhibit $I_{T, Ca}$. Here, $I_{T, Ca}$ is restricted to the conduction system. For hPSC-CMs, the presence of $I_{T, Ca}$ has been debated: $I_{T, Ca}$ was reported in a subset of hPSC-CMs in one study, while in another, the current was not detected. By contrast, $I_{Ca,L}$ is observed at similar densities in both hPSC-CMs and adult cardiomyocytes, although exact values have varied between hPSC-CMs.

Conduction velocity

Given the reduced availability of sodium channels, propagation of the electrical signal, to which $I_{Na}$ is a major contributor, is relatively slow in monolayers of hPSC-CM (10–20 cm/s compared to 60 cm/s in adult human left ventricle). However, for a high conduction velocity, other contributors are of importance. Density and composition of gap junctions, for example, through which the electrical signal is conducted, is also critical. With respect to the gap junctions, fetal, hPSC-CMs and adult cardiomyocytes do not show clear differences in the expression of connexin 43 ($GJA1$), which forms gap junctions in the ventricle. Conduction velocity is however also determined by the localization of gap junctions and sodium channels. In adult cardiomyocytes, gap junctions and sodium channels accumulate at the intercalated discs located at the short edges of two neighbouring cells, resulting in a faster conduction in the longitudinal direction compared to transversal. However, in fetal cardiomyocytes and hPSC-CMs, gap junctions are distributed around the circumference of the cells on all sides of the membrane, rather than at the ends. Finally, cell size, which is positively correlated with conduction velocity, may be an important factor contributing to slow conduction velocity in hPSC-CMs. Of note, other factors not related to cell maturation, such as fibrosis and non-cardiomyocyte cell populations, also impact conduction velocity, therefore this parameter should be taken into consideration when using this as a marker of maturation.

Calcium handling and Excitation-contraction coupling

Following an AP, influx of $Ca^{2+}$ via L-type channels triggers the release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) through ryanodine receptor (RyR) channels, a process known as $Ca^{2+}$-induced- $Ca^{2+}$ release. For relaxation, $Ca^{2+}$ is pumped back into the SR via sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) and is extruded from the cell via the $Na^{+}$-$Ca^{2+}$ exchanger (NCX). This $Ca^{2+}$ transient modulates cardiac contraction. $Ca^{2+}$ release and extrusion is efficient in adult cardiomyocytes, however in fetal cardiomyocytes and hPSC-CMs kinetics are slow and amplitudes small (Figure 1C). In hPSC-CMs, $Ca^{2+}$ intrusion may even be completely dependent on sarcolemmal influx (through L-type calcium channels) and not through release from the SR, although...
SR-mediated Ca\(^{2+}\) release have been described in most studies\(^{75-77,80,81}\). Amplitudes of Ca\(^{2+}\) transients also decrease with pacing frequency in hPSC-CMs in contrast to adult cardiomyocytes, in which they increase\(^{79,82}\). Different components of the Ca\(^{2+}\) handling system are missing in fetal cardiomyocytes and hPSC-CMs. One of the most important are transverse tubules (T-tubules), invaginations in the membrane where L-type Ca\(^{2+}\) channels are concentrated near RyR channels. In adult cardiomyocytes, this compartmentalizes Ca\(^{2+}\), leading to fast excitation-contraction coupling (ECC), and synchronized contraction in multiple sarcomeres\(^{83}\). Because T-tubules are absent and expression of Ca\(^{2+}\) handling proteins low in hPSC-CMs and fetal cardiomyocytes, ECC is slow. Although the expression of SERCA (encoded by \textit{ATP2A2}) is generally high in hPSC-CMs and fetal cardiomyocytes (although lower than adult ventricular cardiomyocytes)\(^{76,78}\), other important proteins involved in mediating uptake and release of Ca\(^{2+}\) from the SR, such as calsequestrin (\textit{CASQ2}), ryanodine receptor type 2 (\textit{RyR2}) and phospholamban (\textit{PLN}) are expressed at low levels, or are even absent\(^{53,84}\). As for NCX channels, expression increases during human development, after which it decreases postnatally\(^{85}\). Expression of NCX in hPSC-CMs and adult cardiomyocytes is comparable although current density does increase over time in hPSC-CMs, as in fetal development\(^{65}\).

Immaturity of hPSC-CM is also reflected in their small contraction forces. Active forces generated in 3D hPSC-CM tissue constructs are \(\sim 0.1-0.5\) mN/mm\(^2\)\(^{224,86}\), compared to 10–50 mN/mm\(^2\) in adult ventricular cardiomyocytes\(^{87,88}\). Passive forces (resulting from myofibrillar compliance) also increase with maturation and in adult cardiomyocytes increase with increasing beating frequency (positive force-frequency relationship)\(^{88}\). Due to insufficiencies in Ca\(^{2+}\) release and uptake, there is a negative force-frequency relationship in fetal cardiomyocytes and hPSC-CMs\(^{49}\).

**Adrenergic stimulation**

Fetal cardiomyocytes show chronotropic responses (increased beating frequency) in response to beta-adrenergic stimulation (norepinephrine) early in development\(^{90}\). This is also observed in hPSC-CMs\(^{91}\). The effects of this on contractile force (inotropy) and relaxation rate (lusitropy) are inconsistent, some studies showing a response upon beta-adrenergic stimulation, others none\(^{91-93}\). If present though, inotropic and lusitropic responses are not as robust as in adult cardiomyocytes\(^{92}\).

Response to alpha adrenergic stimulation, which in the human heart plays a role in physiological hypertrophy\(^{94}\), is described for both hESC-CMs\(^{95,96}\) and hiPSC-CMs\(^{95}\). Interestingly, a robust hypertrophic response upon alpha-adrenergic stimulation is evoked in hESC-CMs\(^{95,96}\), in contrast with hiPSC-CMs, where no such response takes place\(^{95}\). In comparison with adult and fetal human cardiomyocytes, both hESC-CMs and hiPSC-CM lack expression of the main cardiac alpha-adrenergic receptor ADRA1A, suggesting that downstream signalling proteins are differentially functional in both cell types.
Metabolism

Energy demand in the beating heart is high at rest and increases to even more dramatic levels during physical activity. Energy production pathways are therefore of utmost significance for working cardiomyocytes. Reflecting this, during development in vivo the mitochondria-to-cell volume ratio of a cardiomyocyte increases to a value of 20–30%\(^{67}\). In addition to the increasing density and organization of mitochondria with maturation, evidence suggests that with heart development substrate utilization shifts from glucose and lactate in the fetal heart, to primarily fatty acids in the adult heart\(^{98,99}\). Mitochondrial biogenesis has been shown to increase over time in hESC-derived cardiomyocytes, mimicking heart development, and this is driven primarily by the transcriptional co-activator PGC-1α\(^{100}\). In terms of the bioenergetic profile of hPSC-CMs, it seems that even in these immature cells oxidative phosphorylation accounts for the majority of ATP production. In terms of substrate utilization, one report has suggested that under baseline conditions beta-oxidation is only a minor contributor to respiration, whereas when energy demand was raised with stimulatory factors it became more prominent\(^{101}\). Therefore it remains possible that under the right physiological conditions PSC-derived cardiomyocytes do substantially utilize fatty acids. Maturation-related events such as the regulation of genes important for fatty acid uptake or oxidation may play an important role in determining this activity however.

PROMOTING MATURATION OF HPSC-CMS IN CULTURE

Having considered the salient features of cardiomyocyte maturation, we now discuss how these might be enhanced in culture. A summary of the published functional and electrophysiological outcomes is given in Table 1.

Time in culture

Intuitively it was expected that maturity would increase with time in culture. Sartiani et al\(^{10}\) compared current densities and kinetics of several ion channels and the expression of their corresponding genes in cardiomyocytes kept for 15–30 days or for 55–110 days in culture. \(I_{K1}\) and \(I_{to}\) current densities clearly increased over time, whereas the kinetics of \(I_f\) activation were slower in cardiomyocytes during prolonged culture. \(I_{Ca,L}\) and \(I_{Ko}\) densities were unchanged. By contrast, Ivashchenko et al reported increased \(I_{Ca,L}\) current density and upregulation of the corresponding gene CACNA1C, after 37 days compared with 80 days\(^{12}\). Furthermore, increased \(I_{Na}\) current density, upregulation of GJA1 (connexin-43), SCN5A and KCNJ2 and increased MYH6/MYH7 gene expression ratio were observed over time, although a decrease would have been expected for the MYH6/MYH7 ratio. Otsuji et al\(^{14}\) reported similar effects on \(I_{Ca,L}\) and \(I_{Na}\) current densities in hESC-CMs at 28 days
### Table 1. Features of Morphology, Gene Expression, and Function that Are Altered During Pluripotent Stem-Cell-Derived Cardiomyocyte Maturation in Related Studies

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Condition</th>
<th>Observed effects</th>
<th>Gene expression</th>
<th>Function</th>
<th>Electrophysiology</th>
<th>Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time in culture</td>
<td>55–110 days in culture</td>
<td>.</td>
<td>HCN1, HCN2, HCN4↓</td>
<td>.</td>
<td>V_{max}↑, Frequency I_{K}↑, I_{F}↑, rate of activation</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>37–80 days in culture</td>
<td>.</td>
<td>NPPA, NPPB↓ SCN5A, GJA1, CACNA1C, KCNJ2↑ Ratio MYH6/MYH7↑</td>
<td>.</td>
<td>I_{Cal}↑, I_{Na}↑</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>80–100 days in culture</td>
<td>Cell area ↑ More elongated cells Multinucleation ↑ Sarcomere length ↑ organized and aligned myofibrils. Aligned Z-disks, A, I-bands H-zone present MYH6, MYH7, GJA1, HCN4↑ SERCA2↑</td>
<td>Release and decay rate↑ Time to peak↓ Time to 50% decay↓</td>
<td>V_{max}↑, APA↑, MDP↓, Spontaneous beating rate↓</td>
<td>.</td>
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<tr>
<td></td>
<td>28–231 days in culture</td>
<td>.</td>
<td>SCN5A, SCN1B, CACNA1C↑ CACNA1H↓</td>
<td>.</td>
<td>I_{Cal}↑, I_{Na}↑</td>
<td>V_{max}↑</td>
</tr>
<tr>
<td></td>
<td>360 days in culture</td>
<td>Cell area ↑ 40% increase of MLC2v positive cells A, H, I, Z- and M-bands LRRC39, MYOM1, MYOM2↑ (M-band genes), cTnT, MYH6, MYH7, MYL2↑, GJA1↓</td>
<td>.</td>
<td>Spontaneous beating rate↓</td>
<td>.</td>
<td></td>
</tr>
<tr>
<td>Electrical stimulation</td>
<td>1 Hz for 4 days</td>
<td>More elongated cells Improved organization SERCA2, MYL2↑ Release and decay rate↑, amplitude↑, SR store↑</td>
<td>.</td>
<td>No change in MDP, beating rate, upstroke velocity, APD90%↑</td>
<td>.</td>
<td></td>
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<tr>
<td>Increasing frequency up to 6Hz for 7 days</td>
<td>.</td>
<td>More I-bands, H-zones and (at 6 Hz) desmosomes</td>
<td>.</td>
<td>Release and decay rate↑, amplitude↑, SR store↑ ↑, excitation threshold↑, CV↑</td>
<td>.</td>
<td></td>
</tr>
</tbody>
</table>

- ↑: Increase
- ↓: Decrease
- .: Not specified
Table 1. Features of Morphology, Gene Expression, and Function that Are Altered During Pluripotent Stem-Cell-Derived Cardiomyocyte Maturation in Related Studies (continued)

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<tr>
<th>Strategy</th>
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<th>Sarcomere structure</th>
<th>Gene expression</th>
<th>Function</th>
<th>Calcium transient</th>
<th>Electrophysiology</th>
<th>Contraction</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Improved organization, Sarcomere length ↑</strong></td>
<td></td>
<td>Improved organization</td>
<td>KCNJ2, CSQ, JCT, Trdn, Cav3 ↑ Amp2, MYH6, MYH7, MYL2 ↑ SERCA2 ↑</td>
<td>Amplitude ↑</td>
<td>Stable and ↓ RMP</td>
<td>..</td>
<td></td>
</tr>
<tr>
<td>Mechanical load</td>
<td>Increasing static stretch for 14 days¹²</td>
<td>Improved organization, Sarcomere length ↑</td>
<td></td>
<td>Improved organization</td>
<td>Ratio MYL2/MYL7 ↑</td>
<td>..</td>
<td>..</td>
<td>Passive + active force ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclic stretch, 1–3 Hz for 3 days¹¹⁴</td>
<td>Improved alignment, Improved organization</td>
<td></td>
<td>..</td>
<td>..</td>
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<tr>
<td></td>
<td>Cyclic stretch, 1.25Hz for 3 days²³</td>
<td>More Z-bands, improved alignment</td>
<td></td>
<td>Improved organization</td>
<td>KCNJ2 ↑</td>
<td>Decay rate ↑</td>
<td>..</td>
<td>..</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Static stretch and cyclic stretch, 1Hz for 4 days²⁴</td>
<td>Cell area ↑, improved cell alignment</td>
<td></td>
<td>More intercalated discs</td>
<td>Improved sarcomere alignment, ↑ titin intensity</td>
<td>..</td>
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<td></td>
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<tr>
<td>Chemical compounds</td>
<td></td>
<td></td>
<td></td>
<td>Tissue of murine iPS-CMs¹²²</td>
<td>More intercalated discs</td>
<td>Improved sarcomere alignment, ↑ titin intensity</td>
<td>..</td>
<td>Passive and active force ↑</td>
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<tr>
<td></td>
<td>Ascorbic acid</td>
<td>Improved organization</td>
<td></td>
<td>Improved organization sarcomere</td>
<td>SERCA2, RYR2, PLN ↑ (also on Western Blot)</td>
<td>Release and decay rate ↑, amplitude ↑</td>
<td>AP characteristics unchanged</td>
<td>..</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Murine iPS-CMs²²</td>
<td>Improved organization</td>
<td></td>
<td>Improved organization sarcomere</td>
<td>SERCA2, RYR2 ↑</td>
<td>V_max ↑</td>
<td>More negative MDP</td>
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<td></td>
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<tr>
<td></td>
<td>Murine ESC-CMs, T₃ supplement for 7 days¹³⁰</td>
<td></td>
<td></td>
<td>Improved organization sarcomere</td>
<td>SERCA2, RYR2 ↑</td>
<td>V_max ↑</td>
<td>More negative MDP</td>
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<tbody>
<tr>
<td></td>
<td>IPS-CMs, T3 supplement for 7 days(^\text{19})</td>
<td>Cell area ↑, circularity ↓</td>
<td>Sarcomere length ↑</td>
<td>SERCA2, ratio MYH6/ MYH7 ↑</td>
<td>Release and decay rate ↑, (V_{\text{max}}) ↑</td>
<td>..</td>
<td>..</td>
<td>Active force ↑</td>
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<tr>
<td></td>
<td>Neuregulin-1β murine iPS-CMs treated for 14 days(^\text{20})</td>
<td></td>
<td></td>
<td>MYH6, MYL3, RYR2, SERCA2 ↑ PDK4, CD36 ↑, SLC2A1, SLC2A4 ↓</td>
<td>V(_{\text{max}}) ↑, MDP ↓, Frequency ↑ APA ↑ APD90% ↓</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3D tissues</td>
<td>3D tissues in casting mold(^\text{18})</td>
<td>↑ number of rod-shaped cells, improved alignment</td>
<td>..</td>
<td>KCNJ2, MYH6 ↓</td>
<td>..</td>
<td>MDP ↓, (V_{\text{max}}) ↑, (I_{\text{m}}) ↑ (at -100 mV)</td>
<td>..</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3D tissue in mold with staggered posts(^\text{26})</td>
<td>..</td>
<td>Sarcomere length ↑</td>
<td>CASQ2, SERCA2↑</td>
<td>..</td>
<td>CV ↑</td>
<td>..</td>
<td></td>
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<tr>
<td></td>
<td>Pure CMs co-cultured with unspecified non-CMs(^\text{25})</td>
<td>..</td>
<td>..</td>
<td>SCN5A, CACNA1G, KCNJ12 ↑</td>
<td>..</td>
<td>MDP ↓, (V_{\text{max}}) ↑, APA ↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cardiac microtissues of NKX2.5+ hESC-CMs with fibroblasts (CD90+) in a 75:25 ratio(^\text{127})</td>
<td>Improved cell alignment</td>
<td>Improved sarcomere alignment, sarcomere length ↑, Z-disks, H-zone present</td>
<td>ANF, NPPB, MYL7, MYL2 ↑ ratio MYH7/ MYH6 ↑</td>
<td>..</td>
<td>CV ↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracellular substrates</td>
<td>Modified stiffness (4.4–99.7 kPa)(^\text{28})</td>
<td>Substrate stiffness No morphologic changes</td>
<td>..</td>
<td>..</td>
<td>..</td>
<td>..</td>
<td>Force ↑ in higher stiffness</td>
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</table>
Table 1. Features of Morphology, Gene Expression, and Function that Are Altered During Pluripotent Stem-Cell-Derived Cardiomyocyte Maturation in Related Studies (continued)

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Condition</th>
<th>Observed effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Morphology/Structure</strong></td>
<td>Improved sarcomeric alignment in grooves with small widths</td>
<td>Peak amplitude ↓, time to peak ↓, decay rate ↑, % caffeine responsive cells</td>
</tr>
<tr>
<td><strong>Sarcomere structure</strong></td>
<td>Improved alignment</td>
<td>% of quiescent cells ↑, Stable and ↓ RMP</td>
</tr>
<tr>
<td><strong>Gene expression</strong></td>
<td>KCNJ2 ↑ MYH6, MYH7, MYL7, MYL2 ↓</td>
<td>% of quiescent cells ↑, Stable and ↓ RMP</td>
</tr>
<tr>
<td><strong>Function</strong></td>
<td>Transient peak amplitude ↑, upstroke velocity ↑, decay velocity ↑, I_{Ca,L} density unchanged</td>
<td>% of quiescent cells ↑, Stable and ↓ RMP</td>
</tr>
</tbody>
</table>

Observed effects of each approach are in comparison to the control culture condition without the proposed strategy. Data are from hPSC-CMs, unless stated otherwise. CV, conduction velocity; MDP, maximum diastolic potential; RMP, resting membrane potential; AP, action potential; V_{max}, maximum upstroke velocity; APD_{90}, AP duration at 90% of repolarization; APA, AP amplitude ↑ increase; ↓ decrease; hPSC-CMs, human pluripotent stem cell-derived cardiomyocytes.
compared with 231 days. The expression of sodium channel genes, SCN5A and SCN1B (encoding the beta-subunit of the sodium channel) were clearly upregulated along with CACNA1C. The upstroke velocity of the AP was thus correspondingly increased. However, no increase in the $I_{K1}$ current density was observed and no upregulation of its alpha-subunit gene KCNJ2. Lundy et al\textsuperscript{11} also examined the effect of long-term culture and primarily focused on morphological maturation. hPSC-CMs were plated on two different substrates in succession and designated as early (20–40 days in culture) or late stage (80–100 days in culture). Multinucleation, sarcomere length, cell size and elongated shape all increased and MYH7, MYH6, GJA1 and SERCA2 gene expression was upregulated over time in culture. Although not expected, HCN4 (the gene primarily underlying the pacemaker current, $I_f$) was also upregulated. AP upstroke velocity and amplitude were increased and RMP became more negative in late versus early hPSC-CMs. Lastly, the Ca\textsuperscript{2+} handling of late stage cardiomyocytes were characterized by higher upstroke and decay velocities, although the Ca\textsuperscript{2+} transient amplitude was unchanged. In another study focusing primarily on the ultrastructural phenotype, Kamakura et al\textsuperscript{13} reported gradual changes in sarcomere organisation in hiPSC-CMs maintained over a 1-year in culture. 30 day hiPSC-CMs showed only Z- and I-bands, while cardiomyocytes between 30 and 90 days developed sarcomeres that included Z-, I- and A-bands. Impressively, 360-day hPSC-CMs also showed the presence of M-bands. However, there was considerable variability among the cells analysed. In summary, some evidence suggests maturation of hPSC-CM over time in culture but even if prolonged, an adult cardiomyocyte phenotype is never acquired.

**Electrical stimulation**

hPSC-CMs usually exhibit spontaneous contractility. However, this electrical activity is usually irregular, and the frequency gradually decreases over time in culture\textsuperscript{11}. In isolated and cultured rodent cells electrical stimulation is known to improve cell-to-cell coupling and alignment\textsuperscript{17}. Remodelling of these cells (“de-differentiation”) to an immature phenotype in culture is prevented (and partially reversed) by electrical stimulation. Several studies have shown beneficial effects of electrical stimulation on hPSC-CM maturation evidenced by increased $I_{K1}$ and consequently lower RMP\textsuperscript{15,18}, improved Ca\textsuperscript{2+} handling (increase in amplitude, upstroke and decay rate of the Ca\textsuperscript{2+} transient)\textsuperscript{15,16}, increased contractile forces\textsuperscript{19} and a higher degree of sarcomeric organization\textsuperscript{15,16,18,19}. Most studies used a physiological pulse rate of 1 Hz but higher stimulus frequencies (1.5–2 Hz) generate a more robust response\textsuperscript{19}. In one study, the pacing frequency was gradually increased\textsuperscript{18}, which led to a more robust response than stimulation at lower frequencies. In general, the mechanisms underlying increased maturation by electrical stimulation are indirect, as pacing generates cyclic mechanical stretch leading to cardiomyocyte remodelling. However, the effects could also be direct: for example, electrical
activity was shown to alter gene transcription to a more mature profile in the presence of the contraction inhibitor blebbistatin\textsuperscript{102}. It has been suggested that intracellular generation of reactive oxygen species (ROS) upon electrical stimulation may contribute to increased maturation\textsuperscript{103}. High amounts of ROS are generally considered detrimental but limited amounts of ROS can function as intracellular second messengers and determine cellular differentiation and maturation fate\textsuperscript{104}. Although the production of ROS has been demonstrated\textsuperscript{103}, there are no studies describing direct effects on maturation or specific signalling pathways. Moreover, it is unclear whether the amount of ROS generated, especially in the case of continuous pacing, actually stimulates maturation or does the reverse. The latter might require antioxidants in the culture medium or continuous perfusion of the culture medium.

\textbf{Mechanical strain}  

The heart continuously undergoes mechanical stress, the result of hemodynamic load (cyclic stretch), physical interaction with extracellular matrix (static stretch), and laminar blood flow (shear stress)\textsuperscript{105}. It is believed that while undergoing mechanical stretch, cells are forced to change shape through transduction of the mechanical forces via the cytoskeleton and align in the direction of the applied traction, altering their gene expression as a result\textsuperscript{105,106}. Structural and functional changes follow\textsuperscript{107,108}, including induced expression of vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF-a) and insulin-like growth factor (IGF-1)\textsuperscript{109,110}. This occurs in cultured cells\textsuperscript{111,112} and in the intact heart\textsuperscript{113} making mechanical stress an obvious approach that may be beneficial for maturation of hPSC-CMs. To mimic cardiac hemodynamic load, cyclic stretch has been applied in several studies, demonstrating an increased rate of maturation in hPSC-CMs\textsuperscript{22–24,114,115}. In both hESC-\textsuperscript{23,24} and hiPSC- derived CMs\textsuperscript{24}, cyclic stretch and strain at rates of 1 to 2 Hz promote maturation measured structurally (cell elongation, higher degree of sarcomeric organization, higher density of gap junctions) and functionally (faster Ca\textsuperscript{2+} transients). The functional effects on Ca\textsuperscript{2+} handling are reflected in upregulation of \textit{RYR2} and \textit{SERCA2}. Mihic et al\textsuperscript{23} also showed increased expression of ion channel genes, including \textit{KCNJ2}, which could lead to increased \textit{I_{K1}} and more negative membrane potentials, although this was not assessed. An alternative method is to gradually increase static stretch\textsuperscript{22}. This was reported to improve cellular and sarcomeric organization compared with cyclic stretch or increased passive and active forces. However, other functional parameters of maturation were not assessed in this study.
Figure 2. Illustrative representation of all suggested strategies applied to favor maturation of cardiomyocytes from human pluripotent stem cells, that is: prolonged time in culture, electrical stimulation, addition of chemical compounds, provision of mechanical strain, addition of non-cardiac cells, growth in a three-dimensional structure, adjustment of extracellular environment, and artificial introduction or modification of key genes involved with cardiac maturation.

Chemically induced maturation

Among the most promising reagents to date identified as inducing maturation of hPSC-CM are thyroid hormones, which are known to play a crucial role in cardiac development\textsuperscript{116}. For example, reduction in levels of triiodothyronine (T\textsubscript{3}) during development induced by thyroidectomy of pregnant females results in fewer binucleated cardiomyocytes in the fetal heart and lower $\text{SERCA2}$ expression, which is rescued by T\textsubscript{3} replacement\textsuperscript{117}. Furthermore, T\textsubscript{3} converts the sarcomeric protein titin from the fetal N2BA- to the adult N2B isoform in cultured embryonic rat cardiomyocytes\textsuperscript{21}. Moreover, reduced expression of $\text{SERCA2}$ and restricted conversion of $\beta$-MHC to $\alpha$-MHC is observed during development of hypothyroid mice\textsuperscript{118}. Although $\alpha$-MHC downregulation would normally be expected during maturation, increased $\alpha$-MHC/$\beta$-MHC ratio and higher $\text{SERCA}$ expression is observed in hPSC-CMs, where T\textsubscript{3} also reduces expression of the fetal genes $\text{NPPB}$ (encoding brain natriuretic peptide, BNP) and $\text{HCN4}$$\textsuperscript{12,119}$. Structurally, T\textsubscript{3} was reported to increase cardiomyocyte size, induce cell elongation and increase sarcomere length\textsuperscript{119}. In this study, Ca\textsuperscript{2+} handling and contractility also showed remarkable improvements, as evidenced by increased Ca\textsuperscript{2+} transient upstroke and decay velocities. With respect to ion channels, only mouse ESC-CMs have been studied to date. Here, addition
Immaturity of stem-cell derived cardiomyocytes

of T3 resulted in more negative RMPs, accompanied by increased expression of KCNJ2. Moreover, much as in hPSC-CMs described earlier, Ca\(^{2+}\) handling improved, reflecting changes in expression of SERCA2 and RyR2\(^{120}\).

Ascorbic acid, which stimulates cardiomyocyte differentiation\(^{121}\), has also been shown to enhance maturation in mouse iPSC-CMs. Treated cells showed improved calcium handling at baseline and increased responsiveness to β-adrenergic and muscarinic stimulation\(^{122}\). In hiPSC-CMs, ascorbic acid enhances contraction forces and sarcomeric organization and leads to increased intercalated disc formation, titin expression, collagen deposition and metabolic activity\(^{22}\). Ascorbic acid promotes collagen-synthesis through the MEK-ERK1/2-pathway, which stimulates proliferation of cardiac progenitor cells\(^{122}\). Both the composition and amount of extracellular matrix as well as the differentiation efficiency can influence the degree of maturation\(^{123}\).

Neuregulin-1β (NRG-1β), which agonizes the Notch signaling pathway and plays a role in cardiomyocyte subtype specification, might also promote cardiomyocyte maturation. NRG-1β has been shown to promote hESC-CM differentiation towards ventricular-like cells\(^{124}\) while its inhibition leads to a nodal-cell phenotype. In mouse iPS-CMs, NRG-1β increases AP upstroke velocities, reduces the RMP and upregulates MYH6, RYR2, MYL3 and SERCA2A as well as genes involved in fatty acids metabolism (PDK4, CD36)\(^{20}\). Genes involved in glycolysis are downregulated (SLC2A1, SLC2A4).

Co-culture with non-cardiomyocytes

Kim et al\(^{25}\) provided among the earliest evidence that co-culture with non-cardiomyocytes improved electrophysiological characteristics and calcium handling properties of hESC-CMs. Using a puromycin resistance cassette in the promoter region of α-MHC to select pure cardiomyocyte populations from differentiating hESC followed by remixing, the cardiomyocytes grown as spheroids without other cell types present were more immature than those from spheroids containing non-cardiomyocytes: AP upstroke velocities and amplitudes were lower and RMPs less negative. Overall though, the AP characteristics remained relatively immature compared with other studies. The study did not elucidate the cell specificity of these non-cardiomyocytes. Ou et al\(^{125}\), on the other hand used rat embryonic cardiac fibroblasts co-cultured with mouse ESCs and found both an increase in the efficiency of the cardiomyocyte differentiation and in the organization of sarcomeric structures with clear gap junction patterns. The expression of the GJA1 gene was also increased in this co-culture system compared with mouse ESC-derived cardiomyocytes that were not co-cultured. Similar findings were presented by Blin et al\(^{126}\) in a study where cardiac progenitor cells derived from a primate (Rhesus) embryonic pluripotent line were co-cultured with human atrial cardiomyocytes and cardiac fibroblasts. The resulting cardiomyocytes had increased sarcomere size and organization and expressed connexin 43, MLC2v (80% of the cells) and β-MHC (50%
of the cells) at their membrane. Thavandiran et al went a step further by evaluating the impact of different hESC-CMs to cardiac fibroblast co-culture ratios, in the morphology, gene expression, sarcomere structure and conduction properties of engineered heart tissue structures designated as cardiac micro wires (CMW)\textsuperscript{127}. It was observed that the ratio of 75:25 hESC-CMs to cardiac fibroblasts yielded cardiac micro-tissues whose ANF (Atrial natriuretic factor), BNP, MYL7, MYL2 and MYH7/MH6 expression was the highest compared to other conditions. Z-disks and H bands were present in these tissue structures and most impressively they displayed conduction velocities comparable to those of an adult human heart.

3-dimensional (3D) culture
Cardiomyocytes tend to form 3D structures over time in culture, a phenomenon that is also observed in differentiating hPSC-CMs\textsuperscript{128}. This has been the basis constructing cardiac tissues with a predetermined 3D structure using either primary cardiomyocytes\textsuperscript{129–131} or more recently hPSC-CMs\textsuperscript{17–19,22–26,86,127,132–134}. The most common approach is to cast moulds, which determine the 3D form. Hydrogels of collagen type I are placed in these moulds with cardiomyocytes and attached between two anchor points which can move cyclically back-and-forth to induce remodelling and alignment of the cardiomyocytes in myocardial conduits\textsuperscript{22,24}. Other techniques include circular casting molds\textsuperscript{19,134}, porous sponges\textsuperscript{17,132} and stacked cardiac sheets\textsuperscript{133}.

Although the original goal of this “tissue engineering” was to create tissue replacement for the heart, it soon became clear that growth in 3D conduits affects phenotype and is a better mimic of real myocardium than culture on plastic\textsuperscript{135}. Moreover, 3D tissues allow easy measurement of contractile forces.

Most information on the effects of 3D growth has been derived using cardiomyocytes from chicken embryos\textsuperscript{129} and rodents\textsuperscript{130,131}. As stated earlier, primary cardiomyocytes dedifferentiate (remodel) in culture but loss of their sarcomeric organization and contractile force (basal and following beta-adrenergic stimulation) is attenuated in 3D\textsuperscript{130}. Prolonged culture under these conditions almost caused terminal differentiation of neonatal rat ventricular myocytes\textsuperscript{136}.

3D tissue engineering of hPSC-CMs has been reported\textsuperscript{18,19,22–26,86,132–134}, although not all of these studies made the direct comparison with 2D models in terms of maturation rate. Moreover, 3D cultures are often combined with other strategies that promote maturation, such as mechanical stretch or electrical stimulation. Where directly compared with 2D monolayers, results have been unambiguous. One recent study showed gene expression favouring a more mature phenotype in 3D including downregulation of fetal genes NPPA, NPPB, MYH6, upregulation of KCNJ2, increased $I_K$, density, more negative RMPs, higher AP upstroke velocities and less automaticity\textsuperscript{18}. Moreover, 3D tissue hPSC-CMs had larger surface areas and lower proliferation rates, again indicating increased
maturity. A second study demonstrated increased sarcomere length, faster conduction velocities, increased expression of the Ca$^{2+}$ handling genes SERCA2 and CASQ2 and increased β-MHC/α-MHC and MLC2v/MLC2a ratios. Of note, although the addition of isoproterenol had inotropic effects, relaxation rate was not increased, suggesting that Ca$^{2+}$ handling was not functionally mature.

The benefits of 3D culture on cardiomyocytes could partially result from increased mechanical stretch. In most formats, the tissue is attached to two static rods. As the cardiomyocytes are spontaneously active, the static rods provide the mechanical load upon each contraction and thereby force the cells to align properly. As it is clear that extracellular architecture (both on macro- and on micro-scale) affects cellular function and fate robustly, it is not possible to attribute the observed effect exclusively to mechanical load. As discussed below, adjustments of the extracellular microstructure in culture is also considered a promising approach to increase maturity of hPSC-CMs.

**Extracellular substrates**

Cell culture substrates are not inert. Maturity of hPSC-CMs can be induced through the substrate by: 1) coating with extracellular matrix proteins that can act directly as messenger molecules, either by biochemical or mechanical signalling; 2) changing/modulating the intrinsic elasticity or stiffness of the substrate, providing mechanical load not present when cells are cultured on rigid glass slides; 3) changing/modulating surface topography, both at the nano- and micro-scale, providing mechanical cues and forcing cardiomyocytes to align and elongate.

The effect of extracellular matrix in signalling was demonstrated in a study in which Matrigel, a mixture of laminin, collagen IV and proteoglycan, was added on top of differentiating hPSC-CMs. This extracellular matrix “sandwich” resulted not only in higher efficiencies of cardiac differentiation, but also promoted cardiomyocyte maturation. Electrophysiologically, the resulting cardiomyocytes were comparable to those from late stage aggregate cultures (embryoid bodies), suggesting their increased maturity. The rationale behind this approach is that cardiomyocytes could acquire a more realistic phenotype in an extracellular environment that closely resembled the native human heart. Here, the extracellular matrix consists of laminin, fibronectin, collagen and proteoglycans synthesized by cardiac fibroblasts. This mixture (also known as ‘cardiogel’) can be artificially generated in culture. Although beneficial effects are observed in cultured ventricular rat cells, similar effects have not been demonstrated in hPSC-CMs. Only one study evaluated the effects of cardiogel on mouse ESC-CMs by direct comparison with Matrigel, but no clear benefit on maturation was observed.

In the heart, the extracellular matrix is a dynamic, compliant structure, entirely different from rigid glass or plastic surfaces used in standard tissue culture. By adjusting culture substrate hardness (and not only the chemical composition of the extracellular
matrix per se), similar to that in real heart tissue, it was predicted that maturation would be induced. This has been explored in different cell types, usually by means of polyacrylamide hydrogels of varying stiffness. In most studies using cardiomyocytes other than hPSC-CMs, substrate stiffness in the physiological range (Young’s modulus 10–20 kPa), resulted in well-organized myofibrillar structures, high twitch power and calcium amplitudes and morphologically elongated cells, although one study using adult rat ventricular myocytes demonstrated optimal sarcomeric structure and calcium handling in soft (7 kPa) or very stiff (255 kPa) substrates. For hPSC-CMs, however, results are less clear. In one study, increasing stiffness up to 100 kPa led to increased contraction forces in hiPSC-CM, in line with the Frank-Starling principle. Cell morphology and sarcomeric organization on the other hand, clearly affected by substrate stiffness in other cell types, remained immature as in standard conditions. In this study however functional characterization by electrophysiology and Ca²⁺ handling was not carried out. Another study showed that morphology was affected in hESC-CMs by increasing stiffness, demonstrating more spreading and the formation of myofibrillar stress fibers. These stress fibers are also observed in neonatal rat cardiomyocytes at a stiffness higher than physiologic values and are detrimental for sarcomeric function.

By forcing the cells to grow on a topographically predetermined substrate, e.g. micro-grooved patterns with widths of several micrometers, cardiomyocytes align in that pattern and adjust their shape with typical length-to-width ratios as observed in mature ventricular cardiomyocytes. The resulting cell-cell and cell-matrix adhesions, which also physiologically resemble those of the native heart tissue, influence functional phenotype, as observed for example in neonatal rat cardiomyocytes, where aligned cells show polarized distribution of proteins N-cadherin and connexin43 in intercalated discs. Indeed, also in hPSC-CMs, this strategy resulted in improved maturation status, as evidenced by faster calcium kinetics and increased active force.

**Ectopic expression or direct regulation of genes associated with cardiomyocyte maturation**

Another option to induce maturation is to express ectopically key genes normally present in adult cardiomyocytes to see whether they actually drive functional maturation in culture. One such gene is KCNJ2 that encodes the alpha subunit of Kir2.1 channel (Iₖ1) and determines the RMP. Since Iₖ1 is the main current that determines the RMP of the cardiac AP, Lieu et al. used adenovirus to transfer Kir2.1 to embryonic bodies of hESC during cardiac differentiation. The resulting cardiomyocytes showed decreased spontaneous activity and hyperpolarized RMP values (-80 mV vs -60 mV in controls). However, Ca²⁺ handling was not improved (slow kinetics, small calcium transient amplitudes) and the expression levels of key sarcomeric proteins were even significantly decreased compared with controls. In a similar approach, Liu et al. forcibly expressed
calsequestrin in hESC-CMs. The Ca$^{2+}$ transient amplitudes were significantly increased and kinetics improved (higher upstroke and higher decay velocities). These outcomes were not linked to increased $I_{Ca,L}$ nor to cell hypertrophy, leading to the speculation that if the expression of other proteins involved in Ca$^{2+}$ handling such as junctin, triadin, RyR2 and SERCA2 were increased, the cells might mature even further. Other strategies might also include the use of micro-RNAs. Fu et al.$^{152}$ observed that lentiviral-mediated transduction of miR-1 caused the RMP of hESC-CMs to become significantly more negative compared to controls. Also, there was an improvement in the Ca$^{2+}$ transient amplitude and kinetics, and upregulation in the expression of other ion channel genes with the exception of HCN4. However, upstroke velocities remained as controls.

Transcriptional activity is in part determined by epigenetic state; this encompasses histone modifications and DNA methylation and is highly dynamic during cardiac differentiation.$^{153}$ One study demonstrated that by temporarily adding the histone deacetylase inhibitor valproic acid to hESC-CMs, the epigenetic state of the promoters of Ca$^{2+}$ handling and ion channel encoding genes changed, resulting in increased gene expression, as well as increased cell size, resembling hypertrophic growth.$^{31}$ Since epigenetic regulation is finely tuned, both repressive and stimulatory effects may result from histone modification. It is therefore doubtful whether it would be possible to alter transcriptional activity specifically by histone deacetylase inhibitors to affect only those genes involved in maturation.

Interfering with $I_{K1}$ function to drive the RMP of hPSC-CMs to more negative values has proven more challenging than expected. An alternative in silico approach using dynamic patch clamp has recently been developed and utilized to address this issue. Dynamic clamp integrates a computer modelled current recording to live cells upon patch clamp and allows the impact of this particular current on the overall AP output of the cell to be assessed in real time. Using a variation of this approach, Bett et al.$^{154}$ artificially injected $I_{K1}$ current to paced hiPSC-CMs and observed tremendous hyperpolarization of the RMP as well as an increase in the amplitude and upstroke velocity of the AP, resembling values highly similar to adult ventricular cardiomyocytes.

**CONCLUDING REMARKS: PAST, PRESENT, FUTURE**

Despite their limited maturation, hPSC-CMs are already proving useful for safety pharmacology, as models of cardiac disease and drug screening. The US Food and Drug Administration (FDA) proposed in a directive in 2013 that it intended within 2 years to require new drugs to be tested for their effects on all ion channels in human cardiomyocytes. hiPSC-CMs are the cell type of choice for this; despite their immaturity, the FDA considered they were already near appropriate for this purpose. hiPSC lines have
been generated from patients affected by cardiac diseases that until recently could not be studied appropriately in existing animal models because of species differences in cardiac physiology\textsuperscript{155}. Efforts are now focused on enhancing the functional characteristics of hPSC-CMs in order to improve their accuracy and robustness as models. To date, inducing maturation (and mimicking aging) of hPSC-CMs is still the most challenging aspect in the field. The approaches described in this review that address this issue have a common underlying rational: mimic the biochemical cues that drive heart development \textit{in vivo} and simulate the cardiac microenvironment. Certain features of the adult cardiomyocyte phenotype have, however, never been reproduced in culture, such as the presence of T-tubules, key structures of the excitation-contraction coupling mechanism in mature cardiomyocytes important for normal Ca\textsuperscript{2+} handling. Furthermore, although the organization of sarcomeric structure in single-hPSC-CMs may increase over time in culture, it is still considered poor compared with an adult cardiomyocyte. M-bands have been noted by Kamakura \textit{et al} but these were not ubiquitous (only in 10\% of cells)\textsuperscript{13} and only after very long term culture (360 days). Long differentiation and maturation protocols are unlikely to be practical.. At the genomic level, expression of key sarcomeric genes may be ectopically upregulated, but levels are still considerably lower than in the adult heart. Heterogeneous maturation, reflected by different degrees of sarcomeric organization and variability of electrophysiological parameters, is another issue that has not been solved in studies to date.

Despite the limitations, studies on hPSC-CM maturation have revealed that there are conditions under which hPSC-CMs lower their spontaneous beating rate, hyperpolarize the RMP and increase the force of contraction. hPSC-CMs that no longer spontaneously contract and have adopted prominent ventricular- or atrial-like features may have a more mature electrophysiological state. However, the absence of triggered electrical activity in these cells in culture may lead to the downregulation of sarcomeric proteins and proteins related to the contractile machinery, which could be counterproductive for maturation in the long run\textsuperscript{13}. In addition to the absence of continuous electrical stimulation, other factors might contribute to cell immaturity. These include the absence of haemodynamic workload (mechanical strain) but also the lack of adjacent non-cardiomyocytes that could act via paracrine and humoral signals \textit{in vivo}. Since each of the features of maturity may be independently regulated, strategies focusing on one particular aspect of the structural, electrophysiological, or functional phenotype alone may be insufficient to improve overall cardiomyocyte maturity. Combined approaches that impact multiple parameters at different levels simultaneously could be more effective in achieving this goal. For example, in the study of Nunes \textit{et al}\textsuperscript{18}, a 3D culture system with adaptable mechanical properties was used in combination with electrical stimulation and in the presence of non-cardiomyocyte cells. The resulting cardiomyocytes had considerably improved structural, functional and electrophysiological properties.
Another important observation is that, although the expression of key maturity markers (such as sarcomeric proteins or ion channels) may be considerably upregulated using a particular method, this does not always coincide with functional maturation. Gene expression may be used as an additional readout, but ultimately it is the functional/electrophysiological phenotype of hPSC-CMs that defines a successful maturation method.

It should be noted that much of the discussion here does not take into account heterogeneity among the hPSC-CMs resulting for the differentiation process. Whilst electrophysiology measurements are often on single cells with specific AP morphology, such as nodal-, atrial-, or ventricular-like; gene expression is often determined on whole populations that include a mixture of subtypes of cardiomyocytes. It could thus be difficult to draw conclusions from gene expression profiles of each approach towards cardiomyocyte maturation. For example, $HCN4$ expression may be downregulated$^{10}$ or upregulated$^{11}$ in long-term cultures of hPSC-CMs corresponding to a population shift towards pacemaker cells. The myosin heavy chain transition ($\beta$-MHC/$\alpha$-MHC) might also reflect changes in the composition of cardiac population rather than maturation per se ($\alpha$-MHC is more abundant in atrial than in ventricular cells). The same applies for T-type calcium currents, which are expressed by more cardiomyocytes over time in culture$^{12}$ perhaps reflecting a population shift towards nodal-like cells within the culture. Therefore discrepancies in the expression of particular genes or functional properties among studies need to be evaluated carefully since they may be attributed to differences in cardiomyocyte subpopulation ratios rather than the maturation state per se. Methods to distinguish different hPSC-CM subtypes and attribute maturation to these specifically would greatly benefit research$^{156,157}$.

Important for determining whether hPSC-CM maturation has actually been achieved is that morphological, functional and electrophysiological characteristics are all assessed. Cell metabolism for instance has been measured in hPSC-CMs$^{100,158,159}$, but is rarely included in maturation studies. There remain other unresolved issues: the stiffness of the substrate upon which cardiomyocytes are cultured seems to be important but it is not yet clear whether rigid substrates (such as glass) promote maturity$^{28,146}$ compared to softer substrates or vice versa; the best time for electrical stimulation, frequency, pulse duration and the like also need optimization; much remains to be learnt about the effects of chemical compounds, miRNAs and growth hormones (including insulin-like growth factor 1 (IGF-1)$^{160}$. Ultimately, determining the exact molecular cues for hPSC-CM maturation would greatly benefit the attempts towards inducing an adult cardiomyocyte phenotype, however differences in materials and methods used, even as trivial as the presence or absence of media containing serum, confound clear conclusions at present. Moreover, hPSC-CMs are renowned for the line to line variability they manifest regarding their electrophysiological/functional output$^{161}$. This intrinsic feature that is attributed to various factors such as the reprogramming conditions, is not only a bottleneck for their
use in modeling disease, but should also be carefully taken into consideration when evaluating the functional outcome of maturation studies. Confirming the results of an approach towards cardiomyocyte maturity using more than one hiPSC or hESC line per study is advisable, but increases workload considerably.

To date, hPSCs have not yet yielded cardiomyocytes with a functional phenotype fully resembling that of an adult cardiomyocyte. Whether this goal will ever be reached, is questionable. After all, in vivo human cardiomyocytes undergo a developmental change even postnatally\textsuperscript{162} (where their proliferation halts and hypertrophic growth commences), making it doubtful whether such a mature state can be created in a dish in a considerable and practical time frame. However, one might wonder whether such a fully mature state is absolutely necessary for hPSC-CMs to function as a valid model. Obviously, this depends on the research question. With all the rapidly developing maturation strategies, it may be expected that hPSC-CMs will soon resemble adult cardiomyocytes at least more closely and that as a result hPSC-CMs will become a widely accepted “established” model, instead of just “promising” for the future. Until then, researchers using hPSC-CMs as a model should bear the shortcomings of this system in mind.

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Immaturity of stem-cell derived cardiomyocytes


