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# Concise Review: Maturation Phases of Human Pluripotent Stem Cell-Derived Cardiomyocytes

CLAIRE ROBERTSON,[a,b] DAVID D. TRAN,[b,c] STEVEN C. GEORGE[a,b,c,d]

[a]Department of Biomedical Engineering; [b]Edwards Lifesciences Center for Advanced Cardiovascular Technology; [c]Department of Chemical Engineering and Materials Science; and [d]Department of Medicine, University of California, Irvine, Irvine, California, USA

**Key Words.** Cardiomyocyte • Pluripotent stem cells • Phenotype • In vitro cell culture • Maturity

ABSTRACT

**Human pluripotent stem cell-derived cardiomyocytes (hPS-CM) may offer a number of advantages over previous cardiac models, however, questions of their immaturity complicate their adoption as a new in vitro model. hPS-CM differ from adult cardiomyocytes with respect to structure, proliferation, metabolism and electrophysiology, better approximating fetal cardiomyocytes. Time in culture appears to significantly impact phenotype, leading to what can be referred to as early and late hPS-CM. This work surveys the phenotype of hPS-CM, including structure, bioenergetics, sensitivity to damage, gene expression, and electrophysiology, including action potential, ion channels, and intracellular calcium stores, while contrasting fetal and adult CM with hPS-CM at early and late time points after onset of differentiation.** STEM CELLS *2013;31:829–837*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

There is an urgent need for novel cardiomyocyte models: ischemic heart disease remains the #1 killer in the western world [1], congenital cardiomyopathies affect 1%-2% of live births [2, 3], and drug-induced cardiotoxicity is a leading cause of market withdrawal [4]. Human pluripotent stem cell-derived cardiomyocytes (hPS-CM) may offer significant advances in the study of cardiac disease and treatments [5, 6]. Similar to currently available cardiomyocyte models, hPS-CM contract rhythmically [7] and respond appropriately to numerous cardioactive drugs [5, 8]. In addition, hPS-CM can also be manipulated genetically [9], maintained in in vitro culture long-term (1+ years) [10], and be created from adult patients with genetic conditions (in the case of cardiomyocytes sourced from induced pluripotent stem cells, hiPS) [11–14] and may engraft into damaged hearts in vivo [15–17].

Given the potential of these cells and the excitement surrounding them (>2,000 publications since the first report a decade ago [18]), it is timely to address the similarity of these cells to adult human cardiomyocytes, and how they might be used as models of such. Open questions surrounding these cells include: How do we best assess cardiomyocyte maturity? How well do hPS-CM model embryonic or adult CM in vitro? How does maturity change during in vitro culture? When can hPS-CM be used as models for adult CM?

It is frequently noted that hPS-CM resemble human fetal cardiomyocytes [7]; however, no previous review has systematically quantified the similarities. This is complicated by high variation in phenotype between hPS-CM studies, partially explained by differences in cell line of origin and culture conditions. Furthermore, evidence suggests that hPS-CM develop a more mature, adult-like phenotype with time in culture, yet differences between early and late phase hPS-CM have not yet been described. Therefore, this review will define "early" and "late" phase hPS-CM phenotype, and describe how hPS-CM resemble embryonic and adult cardiomyocytes with respect to key markers of maturity, including ultrastructure, metabolism, gene expression, and electrophysiology.

## hPS-CM STRUCTURE AND FUNCTION RESEMBLE EMBRYONIC CARDIOMYOCYTES

### Definition of Early and Late Phase hPS-CM

In this work, hPS-CM will be defined as spontaneously contractile cells derived from a human pluripotent cell line, to the exclusion of contractile cells derived from adult mesenchymal stem cells [19–22] or from mouse pluripotent stem cells, which have been described elsewhere [23–25]. Recent reviews have covered methods to create [17, 26–29] and purify hPS-CM [30], as well as their electrophysiology [31],

Author contributions: Claire Robertson, Conception and Design, Manuscript Writing; David Tran, Manuscript Writing, Graphic Design; Steven George, Financial Support, Final Approval of Manuscript.

Correspondence: Steven C. George, M.D., Ph.D., Edwards Lifesciences Professor, Director, Edwards Lifesciences Center for Advanced Cardiovascular Technology, Department of Biomedical Engineering, 2420 Engineering Hall, University of California, Irvine, Irvine, CA 92697-2730, USA. Telephone: (949) 824-3941; Fax: (949) 824-9968; e-mail: scgeorge@uci.edu Received October 29, 2012; accepted for publication December 21, 2012; first published online in STEM CELLS *EXPRESS* January 25, 2013.  1066-5099/2013/$30.00/0 doi: 10.1002/stem.1331

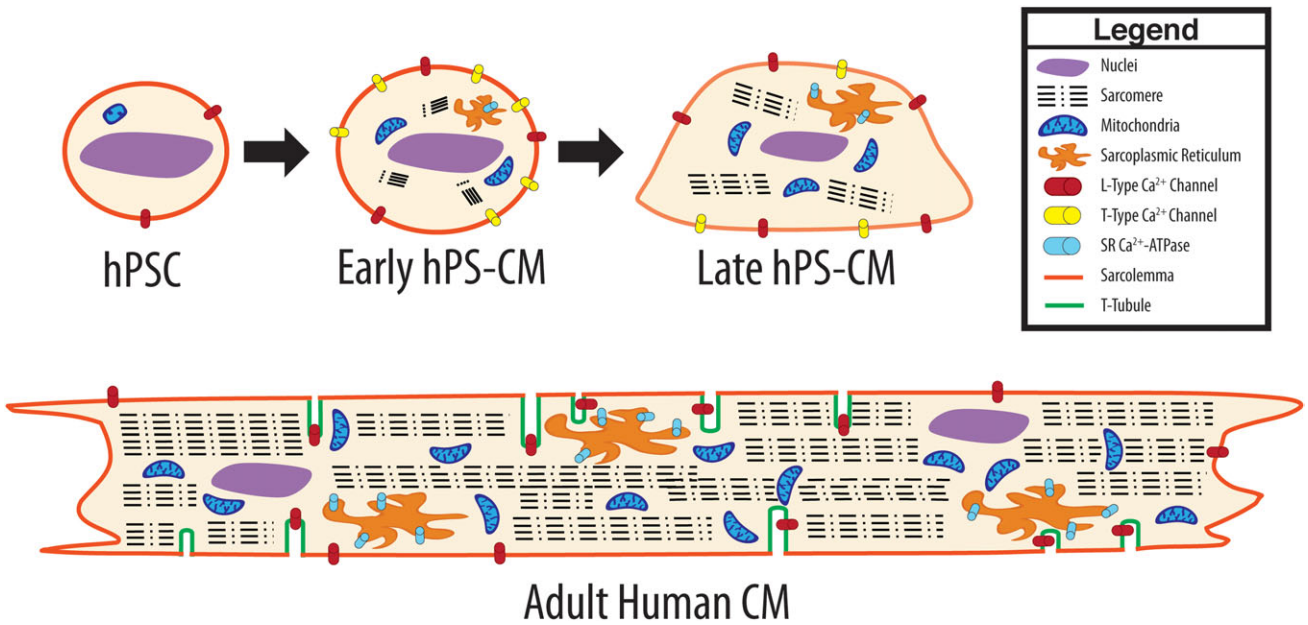


**Figure 1.** A visual comparison of early hPS-CM, late hPS-CM and adult CM morphology. Characteristics of hPS-CM depend strongly on time in culture since the initiation of contraction (early-proliferative, late-nonproliferative). Note that late hPS-CM differ from early hPS-CM with respect to shape, sarcomeric area and receptor expression. Adult CM are far larger, with multiple nuclei, large sarcomeric area, and large numbers of mitochondria. Abbreviations: hPSC, human pluripotent stem cell; hPS-CM, human pluripotent stem cell-derived cardiomyocytes.

drug response [5, 8], and function after transplant in vivo [16, 32]. CM derived from hiPS (hiPS-CM) and human embryonic stem (hES-CM) cells appear to be relatively similar but will be compared when data describing differences are available.

hPS-CM vary in maturity, thus, we will define hPS-CM as either early phase, defined as contractile cells, with some proliferative capacity and with embryonic like electrophysiology (i.e., small negative membrane potential and small action potential amplitude), or late phase, defined by loss of proliferative capacity and more adult-like electrophysiology. hPS-CM show early phase characteristics for generally the first month after initiation of contraction, with development of late phase characteristics arising afterward. Different elements of maturity appear to be affected by line [33–35], time in culture [35, 36], cocultured cells [37], and culture conditions [38, 39]; however, the factors affecting maturity remain largely unknown. This suggests that after initiation of contraction, genetic and environmental factors interact leading to a more mature phenotype; however, the process is incompletely understood.

## Morphology

It has been widely reported that hPS-CM structurally resemble embryonic or fetal cardiomyocytes [40, 41]. However, potentially important differences are seen when these cells are compared to embryonic or adult CM. Adult CM are large and cylindrical (approximately 150 $\times$ 10 $\mu$m for ventricular cells) [42], while embryonic and fetal CM are smaller [43]. Similarly, early hPS-CM (initiation of contraction, 21 days) are small and round to slightly oblong, approximately 5-10 $\mu$m in diameter [7, 33, 44] (Fig. 1). Late hPS-CM (>35 days) develop a more oblong morphology (30 $\mu$m $\times$ 10 $\mu$m), similar to the dimensions of human embryonic CM but remain small compared to adult [7]. In addition, most adult CM are bi- or multinucleated, whereas hPS-CM are mono-nuclear, similar to early embryonic cardiomyocytes [43].

The extensive t-tubule network present in adult ventricular CM is absent in both hPS-CM and embryonic CM [42]. As a result, excitation-contraction coupling is slower, and calcium primarily enters the cell through the sarcolemma instead of releasing from the sarcoplasmic reticulum (SR) [45–48]. Thus, early hPS-CM structurally resemble embryonic CM. With increasing time in culture, late hPS-CM develop a more adult-like morphology but do not appear to develop t-tubules or multinucleation (Fig. 1).

## Function: Proliferation

Early hPS-CM proliferate [7, 39, 49], similar to embryonic or fetal mammalian cardiomyocytes [50–52]. In contrast, adult CM are among the most slowly dividing cell types [53]. Over time in culture, proliferative capacity of hPS-CM decreases from that of stem cells (24-48-hour doubling time [54]) to low levels: at 4 weeks, only 10% cells were $BrdU^+$ after a 24-hour incorporation assay [39] and no $Ki\text{-}67^+$ cells were observed [7], similar to changes seen in fetal cardiac development [50] (Fig. 2). Atrial natriuretic factor (involved in cardiomyocyte proliferation [55, 56]) is expressed in hPS-CM [57, 58]. In summary, early hPS-CM proliferate at a lower rate than their pluripotent progenitors whereas late hPS-CM can be considered nonproliferating cells.

## Function: Gene Expression

The transcriptional profile of hPS-CM is starkly different from their originating pluripotent stem cells. Important differences include loss of pluripotency transcription factors and upregulation of mesodermal and cardiac markers [59–62]. Once differentiated, hPS-CM display a relatively homogeneous, cardiac-like gene expression program. Interestingly, gene expression of hiPS-CM and hES-CM is surprisingly similar, with only 1.9% of genes differentially expressed in these two cell types, despite dramatic differences between expression profiles in the undifferentiated hiPS and hES sources [63].

hPS-CM expression of contractile genes was not discernibly different from fetal heart tissue (20-week gestation) in one study of enriched early hPS-CM (age unknown) [59].

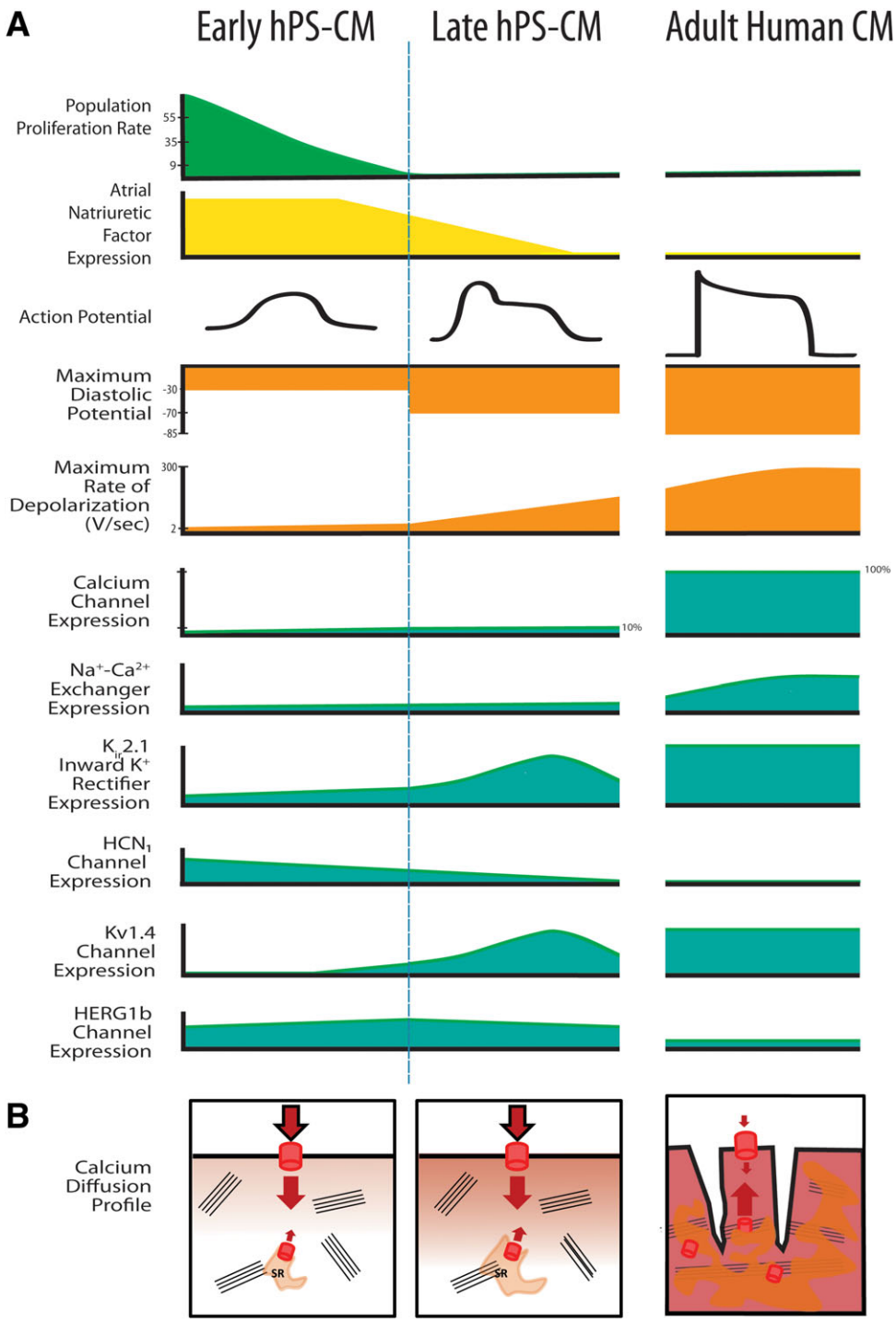


**Figure 2.** Visual comparison of early hPS-CM, late hPS-CM and adult CM phenotype. (**A**): An overview of major changes seen with increasing time in culture. Large changes in action potential characteristics (orange) are seen with time in culture, as well as expression of key ion channels (teal). (**B**): Calcium influx profiles for early and late hPS-CM compared with adult CM. Note that in early hPS-CM, almost no calcium is released from the sarcoplasmic reticulum, leading to slow, diffusion-limited calcium influx. Late hPS-CM perform better, but still show slow influx compared to adult. Abbreviation: hPS-CM, human pluripotent stem cell-derived cardiomyocytes.

Global gene expression profile of purified early hPS-CM is more similar to fetal cardiac tissue (age unspecified) than to adult cardiac tissue; however, hPS-CM gene expression clustered more closely with either fetal or adult cardiac tissue than with pluripotent stem cells [64]. Bigger differences are seen when comparing hPS-CM and adult heart tissue, with important differences seen in a number of cardiac ion channel and calcium handling genes, once again highlighting the immature phenotype of hPS-CM [61, 65].

## Function: Metabolism and Bioenergetics

Contractile machinery and mitochondria fill two-thirds of the cytoplasmic volume in adult CM (myofibril cell area, 40%

[66] to 52% [67] and mitochondria, 15% [66] to 25% [67, 68]). In contrast, both hPS-CM [30] and embryonic CM [69] show smaller sarcomeric regions [58, 70] and have more moderate numbers of mitochondria [68] (Fig. 1). Similarly, expression of contractile and cytoskeletal genes is much lower in hPS-CM (unknown age) compared to fetal (20 weeks) or adult cardiomyocytes [59, 64].

Adult CM are highly metabolically active and depend on oxidative metabolism for synthesis of ATP (fatty acid oxidation accounts for 90% of acetyl-CoA production [71, 72]). In comparison, embryonic and fetal cardiomyocytes rely on glycolysis for production of ATP [69, 73] (fatty acid oxidation $<$15% of acetyl-CoA production [74]) resulting in a relatively hypoxia resistant phenotype and providing substrates for protein production [69]. hPS-CM showed primarily glycolytic metabolism in one study (in late, nonproliferative hPS-CM) which evaluated oxygen consumption rates [75] and mixed glycolytic and oxidative metabolism in another which assessed incorporation of radiolabeled carbon into metabolites (age unknown) [68]. hPS-CM are also able to metabolize lactate, unlike hPS [68]. Higher expression of oxidative phosphorylation genes and proteins are seen in hPS-CM compared to pluripotent stem cells, suggesting that these cells have the potential to use this metabolic pathway [68, 76, 77], though the expression level lags behind fetal tissue [59]. It remains unclear whether time in culture can alter hPS-CM preferred energy substrate.

## Sensitivity to Damage and Apoptosis

It is unclear to what extent in vitro adult CM mimic the in vivo response to noxious stimuli. In vivo, adult CM may survive an entire lifetime ($>$80 years), while in vitro adult CM rarely survive more than a few days [78]. In stark contrast, hPS-CM are already culture-adapted, with reports of cells maintaining viability and contractility for a year [10, 79]. These observations clearly complicate comparisons of the sensitivity to damaging insults of hPS-CM with both in vivo and in vitro adult CM, and additional work is required to fully understand the differences in apoptotic cascades between these conditions.

Despite these limitations, some evidence suggests that hiPS-CM respond similarly to stimuli that cause damage to adult CM. For example, cardiotoxic tyrosine kinase inhibitors such as sunitinib and sorafenib demonstrate arrythmogenicity and increased apoptosis in hiPS-CM at clinically cardiotoxic doses of the drug [75, 80]. Likewise, doxorubicin, a cardiotoxic chemotherapeutic which is believed to act through oxidative stress [81], can induce apoptosis in hiPS-CM [82], as well as microtubule derangement [83]. Similarly, direct application of oxidizers such as hydrogen peroxide, induce apoptotic responses in hPS-CM [84, 85]. This process is mediated by opening of the mitochondrial permeability transition pore and could be prevented with anesthetic mediated preconditioning [84, 85], thus recapitulating the behavior seen in adult CM [86].

Embryonic human cardiomyocytes are resistant to hypoxia [87], whereas adult CM are highly dependent on an adequate oxygen supply [88]. As both hPS-CM and embryonic CM are predominantly glycolytic [75], it may be inferred that hPS-CM would likewise be resistant to hypoxia. However, the sensitivity of hPS-CM to ischemic stimuli has not been fully established. In summary, despite differences in metabolism, hPS-CM are sensitive to oxidative stress and cardiotoxic agents at levels expected from clinical use; however, their sensitivity to ischemia has not been characterized.

## Cardic-Specific Inotropic and Chronotropic Receptors

Several key chronotropic responses are observed in hPS-CM and may be affected by time in culture. $\alpha$, $\beta$1, and $\beta$2 adrenoceptor response have all been demonstrated in hPS-CM [89, 90]. A positive response to isoprenaline ($\beta$ receptor agonist) challenge is almost universally performed in studies of hPS-CM [18, 34, 35, 58, 89-98], suggesting that all hPS-CM have some $\beta$-receptor expression, regardless of cell line of origin, differentiation method. As in vivo, isoprenaline increases contraction rate (positive chonotropy), increases the amplitude of the calcium transient, and decreases the relaxation time [90]. However, unlike adult CM, isoprenaline does not increase contraction force [98], once again demonstrating the immaturity of this cell type. $\beta$2 response accounts for 17%-37% [90] of the total response to isoprenaline, akin to fetal CM. With increased time in culture, hPS-CM demonstrated increased chronotropic $\beta$ agonist response [90, 97]. In summary, $\beta$ adrenoceptor response is present in hPS-CM and shares characteristics with fetal CM and may be amplified with time in in vitro culture.

Several studies have demonstrated a chronotropic response to carbacholine [89, 91, 92], thus showing muscarinic receptor activity. Finally, increased intraceullular cAMP increases contraction rate in hPS-CM via the phosphodiesterase inhibitor IBMX [18, 58] and the adenylyl cyclase activator forskolin [58, 89]. It is unclear whether in vitro maturation time affects the magnitude of these responses or whether these responses affect force of hPS-CM contraction.

## Electrophysiology: Spontaneous Beating Rate

Spontaneous and synchronous contraction is seen as early as 5 days after the initiation of differentiation [99] and can be maintained for more than 1 year in culture [10] (in stark contrast to adult CM [78]). Different basal rhythms have been reported, ranging from 21 [93] to 52 beats per minute (BPM) [90], with most reporting $\sim$40 BPM [35, 92, 100]. The rate of contraction may be affected by cell line, cultures conditions, time since differentiation, and time since the onset of contraction. hiPS-CM from iPS from patients with long QT syndrome show slower repolarization, thus recapitulating the in vivo phenotype [11, 12, 101-103].

Time in culture affects beating rate, though magnitude and direction of this change appears to vary with study. Several studies have reported moderate increases in contraction rate (30-75 BPM at 70 days [90] and 40-85 BPM at 60 days [35]) though a decrease has also been reported (45-5 BPM over the course of 63 days [92]). hES-CM show faster and stronger rhythms than hiPS-CM [35], which may be due to earlier initiation of contraction or the differences between hiPS and hES cells [63, 104]. In summary, spontaneous beating is the principal hallmark of differentiated hPS-CM, and beating rate is affected by line of origin and by time in culture.

## Electrical Properties: Action Potential

hPS-CM contract spontaneously and synchronously, as noted previously, and are thus electrically active. Cells displaying atrial-, nodal-, and ventricular-like APs have been reported [105–107]. In addition, hPS-CM action potential characteristics vary between studies and within studies with different cell lines [35], differentiation methods [108], and time in culture [37]. Variation in a single population of hPS-CM has also been demonstrated, suggesting that even using the same cells, methods, and at the same time point, the

electrophysiological characteristics of hiPS-CM are more heterogeneous than those in an adult heart [108]. hPS-CM from the same embryoid body (EB) (a more homogeneous environment) showed greater homogeneity in action potential duration than hPS-CM from same population but different EBs [35], highlighting the potential important role of a common extracellular environment in hPS-CM maturation.

Most reported action potential characteristics are less mature than adult CM: maximum diastolic potential (MDP) for adult ventricular myocytes is −85 mV [109], whereas early hPS-CM MDP is approximately −30 mV [37] which improves to −60 to −75 mV in late hPS-CM [35, 102, 110-113]. The maximum rate of depolarization ($dv/dt_{max}$ or $V_{max}$) in adult CM is extremely fast, ranging from 300 V/second in healthy hearts [109] to about 100 V/second in heart failure [114]. In contrast, early hPS-CM show extremely slow depolarization depolarization speeds. Early hPS-CM depolarize at 2 V/second [37], improving in late hPS-CM to 10-40 V/second [35, 102, 112, 113] (with two studies reporting 130-150 V/second [108, 115] -Fig. 2). Similar parameters for embryonic or fetal cardiomyocytes are not available.

## Electrical Properties: Ion Channels

The major ionic currents normally present in adult CM are expressed in hPS-CM, though frequently at abnormal levels (Fig. 2). The calcium channels are necessary for contractility, as is NCX [116, 117] and HCN [110]. In early hPS-CM, sodium channel inhibition does not prevent spontaneous contraction, but in late hPS-CM the same inhibition blocked spontaneous contraction [37].

The potassium currents considered to be responsible for arrhythmias are expressed in hPS-CM [12, 102, 118] (Fig. 2). As a result, considerable interest in using hPS-CM for antiarrhythmic drug screening exists and has been reviewed [8, 34, 101, 57]. Some arrhythmias in hPS-CM are affected by time in culture, and thus may be a measure of in vitro maturity [108, 119].

## Electrical Properties: Intracellular Calcium

The extent of the SR and its necessity for automaticity in hPS-CM is a matter of debate. In adult CM, calcium induced calcium release (CICR) from the SR contributes almost 70% of the total calcium release [120]. In contrast, hPS-CM, which have very little SR function in the early phase [46, 121–125], demonstrate calcium transients that are smaller and slower [126], with most cation influx is through the cell membrane [124, 127]. This results in abnormal diffusion of calcium into the cell [122] and reduces the synchrony in contraction necessary for large force generation [98] (Fig. 2B).

Reports vary as to the presence and function of the SR, possibly due to changes with maturity [45-47, 121, 124]. However, there is consensus that intracellular calcium stores are smaller than in adult CM [121, 128]. Calcium handling and response to compounds that modify calcium handling (e.g., nifedipine and ryanodine) appear to vary significantly between lines [122, 129], and between embryonic and induced pluripotent derived CM [122, 130], including larger intracellular calcium stores, though line to line differences dominate differences between hES and hiPS class [35]. Over time in culture, increased sarcoplasmic reticulum function is seen as assessed by caffeine-induced calcium release [121].

When paced, adult CM show a positive force-frequency relationship; that is, at faster pacing rates, greater calcium transients and force of contraction are seen [120]. This relationship requires both significant intracellular calcium stores and electrical coordination across the cell (the t-tubule network again ensures that the entire cell depolarizes rapidly and homogeneously [120]). In contrast, hPS-CM have consistently shown negative force-frequency relationships [45, 97, 128]. In these cells, calcium primarily enters the cell across the cell membrane and diffuses though the cytoplasm, a slower process [42]. Similarly, postrest potentiation (i.e., an increased uptake in calcium in resting cardiomyocytes after rapid pacing) is not seen [45] or seen only to a low extent [97] in hPS-CM. It has not been studied whether these properties improve with time in culture, but the increased sarcoplasmic reticulum function seen in late hPS-CM suggests they may be more adult-like.

Some evidence suggests that non-SR calcium stores play a key role in excitation-contraction coupling in hPS-CM [46]. IP3 receptor (IP3R) is expressed and colocalizes with sarcomeres and the cell nucleus [121, 124], suggesting it may play a role in release of non-SR calcium stores. In adult CM, IP3R appears to regulate noncontractile calcium signaling only [131–133], although abnormal IP3R expression can cause arrhythmia [131]. In hPS-CM, IP3R may be involved in contractility as contraction rate is sensitive to IP3 and IP3R antagonists [121, 124]; however, this observation may depend on inhibition of ryanodine receptors (RYRs) [37].

## Structural and Functional Sarcoplasmic Reticulum Proteins

The structural and functional proteins in the SR show low and varied expression as would be expected from the evidence provided earlier on the underdeveloped SR in hPS-CM. Expression of the RYR is noted in a number of studies [37, 134], though at only a small fraction (0.1%) of the adult level [121]. Most reports state that application of ryanodine slows spontaneous contraction rate [14, 42, 97, 121, 124, 127, 135, 136], though two studies saw no such change [122, 125]. Similarly, one study reports close physical association between RYRs and L-type calcium channels [127], which would allow for efficient CICR [127], though other studies reported no such association [42, 134]. It should be noted that the colocalization of these two proteins in adult CM is debated [137]. SERCA, the sarcoplasmic reticulum $Ca^{2+}$ ATPase pump, is also expressed in hPS-CM [122, 138] at levels similar to fetal cardiomyocytes [134], but a variable response to its inhibitor thaspargin has been reported [45, 124].

Not surprisingly, proteins known to regulate SR function are also abnormally expressed in hPS-CM. Calsequestrin, which binds calcium and allows for dense packing of the ion in the SR, is absent in a number of studies [45, 46, 123, 134] though present in one [97]. Interestingly, transgenic calsequestrin overexpression was enough to improve calcium handling and SR maturity in hiPS-CM [123]. Phospholamban, an endogenous inhibitor of SERCA, is absent in some studies [45, 46], though present in others [138], and its presence is inferred from a positive drug response [90]. Some of this variability may be due to variable (widely unreported) hPS-CM age or manual selection of spontaneously beating cells, as more rapidly beating cells may have less phospholamban expression (in vivo phospholamban is known to repress cardiac contractility) [139, 140]. Junctin and triadin, which potentiate RYR [141], were expressed at low levels in one study [122] and absent in another [134].

## Conclusions

hPS-CM are a heterogeneous population of cells that recapitulate some features of embryonic and adult CM. hPS-CM contract spontaneously and synchronously, express numerous cardiac specific genes and proteins, and recapitulate several important electrophysiological features of adult CM. Recapitulation of fetal or adult CM phenotype may require novel culture methods better recapitulating the in vivo niche. Furthermore, time in culture, specifically time since the onset of differentiation or time since spontaneous contraction, is a major factor affecting proliferation, structure, intracellular calcium stores, and ion channel expression. It is unclear why time in culture should have such profound effects on hPS-CM phenotype, though several studies have emphasized the importance of paracrine signaling and cellular milieu in maturation, suggesting better recapitulation of the cardiac cellular niche will improve maturity. Nonetheless, it is convenient to define early and late phase hPS-CM based on phenotypic markers that include sarcomeric organization, sarcoplasmic reticulum, and membrane ion channels that impact such integrated behaviors as cell proliferation and the action potential. Despite limitations, hPS-CM demonstrate significant potential as a tool to enhance basic biological understanding, improve in vitro drug screening, and thus create new therapeutic options. Remaining challenges include improving the magnitude and consistency of intracellular calcium stores, improving sarcomeric volume and organization, creating consistent reproducible cell populations, and determining the mechanisms of increased maturity with time in culture.

## Acknowledgments

This work was supported in part by NIH UH2 TR000481 (SCG), NSF Graduate Research Fellowship Program (CR), and the ARCS Foundation Scholarship (CR).

## Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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