In the past 6 years, multiple laboratories have reported the successful directed cardiomyocyte differentiation from human pluripotent stem cells (hPSCs; reviewed in Burridge et al). These stem cell–derived cardiomyocytes beat spontaneously, express the expected sarcomeric proteins and ion channels, and exhibit cardiac-type action potentials and calcium transients. They show similar functional properties to the cardiomyocytes in the developing heart with comparable excitation–contraction coupling (ECC) mechanism and neurohormonal signaling. After transplantation into infarcted hearts of mice, rats, and guinea pigs, hPSC cardiomyocytes (hPSC-CMs) engraft, form gap junctions with the host cardiomyocytes, reduce adverse remodeling, and enhance cardiac mechanical function. Despite these promising results, many lines of evidence indicate that under the conditions currently used, hPSC-CMs do not exhibit the morphological and functional characteristics of adult cardiomyocytes.

Engineering Adolescence
Maturation of Human Pluripotent Stem Cell–Derived Cardiomyocytes
Xiulan Yang, Lil Pabon, Charles E. Murry

Abstract: The discovery of human pluripotent stem cells (hPSCs), including both human embryonic stem cells and human-induced pluripotent stem cells, has opened up novel paths for a wide range of scientific studies. The capability to direct the differentiation of hPSCs into functional cardiomyocytes has provided a platform for regenerative medicine, development, tissue engineering, disease modeling, and drug toxicity testing. Despite exciting progress, achieving the optimal benefits has been hampered by the immature nature of these cardiomyocytes. Cardiac maturation has long been studied in vivo using animal models; however, finding ways to mature hPSC cardiomyocytes is only in its initial stages. In this review, we discuss progress in promoting the maturation of the hPSC cardiomyocytes, in the context of our current knowledge of developmental cardiac maturation and in relation to in vitro model systems such as rodent ventricular myocytes. Promising approaches that have begun to be examined in hPSC cardiomyocytes include long-term culturing, 3-dimensional tissue engineering, mechanical loading, electric stimulation, modulation of substrate stiffness, and treatment with neurohormonal factors. Future studies will benefit from the combinatorial use of different approaches that more closely mimic nature’s diverse cues, which may result in broader changes in structure, function, and therapeutic applicability.

Key Words: disease modeling ■ human pluripotent stem cell–derived cardiomyocytes ■ maturation ■ pharmacologic screening
One might reasonably ask, why do we need to mature these human cardiomyocytes? Indeed, researchers have used immature rodent cardiomyocytes as model systems for decades without ever maturing them to an adult phenotype. There are several answers to this question. First, adult rodent cardiomyocytes are readily available for study, whereas adult human cardiomyocytes are difficult to obtain. Second, learning how to mature human cardiomyocytes may teach us how our own hearts mature in postnatal life. Third, mature human cardiomyocytes may better reflect the physiology of the adult heart and therefore be more useful in disease modeling and drug screening. Finally, for myocardial repair, it is desirable to have hPSC-CMs whose electric and mechanical properties more closely resemble those of native myocardium. Such matured cells would be expected to pose less arrhythmic risk and have enhanced contractile performance.

In this review, we discuss the state of the current knowledge on the maturation, disease modeling, and drug screening prospects of hPSC-CMs. Because maturation of hPSC-CMs is a relatively new field, we discuss it in the context of what is known in other systems including in vivo animal models and isolated rodent cardiomyocytes. We address the factors that have been shown to influence cardiomyocyte maturation and also discuss issues that need to be addressed to generate bona fide mature human heart tissue, starting with a comparative discussion of immature and adult cardiomyocytes in relation to what we currently know about hPSC-CMs. Please note that all 3 types of cardiomyocytes (ventricular-, atrial-, and nodal-type) can be obtained from hPSCs. Because virtually all studies to date have not used purified subtypes, we refer here to mixed cardiomyocyte populations. Cardiomyocyte subtype differentiation and purification is under intensive investigation, and its advances will benefit this area.

Overview of Cardiomyocyte Maturation

Morphology

As heart development progresses, cardiac muscle cells undergo a complex series of structural changes that ultimately lead to their adult phenotype (Figure 1; Table 1). The growth of the embryonic/fetal heart is primarily achieved by cardiomyocyte proliferation. In the postnatal heart, hypertrophic growth becomes predominant, and the increase in cardiomyocyte size for both rodents and humans can be 30- to 40-fold. It is worth mentioning that increase in cell size may also result from pathological and physiological hypertrophic responses. In this review, we consider cardiomyocyte maturation to involve physiological hypertrophy because it is part of normal heart development. For a review discussing physiological versus pathological hypertrophy, we refer the readers to the work by Maillet et al. The human embryonic stem cell cardiomyocytes (hESC-CMs) have been reported to be ≈600 μm² on average, which is significantly smaller than an adult cardiomyocyte that has spread out with prolonged culture. Membrane capacitance is directly proportional to cell surface area. The capacitance of the hESC-CMs is 17.5±7.6 pF, in comparison with the ≈150 pF typically reported for adult human ventricular myocytes. Geometric considerations indicate that the cells show even greater differences in volume than cell area. In any case, cell size is an important parameter because it influences impulse propagation, maximal rate of action potential depolarization, and total contractile force.

In addition to size, the shape of the cardiomyocytes also has important functional implications including ECC. It is well known that adult cardiomyocytes have elongated anisotropic shapes (Figure 1B) with a length-to-width ratio of 7 to 9.5 and are aligned in the context of cardiac tissue. In vivo, immature cardiomyocytes are rod shaped, similar to the adult ones, but when cultured in vitro, the immature cardiomyocytes flatten and spread in all directions, whereas the adult ones maintain their cylindrical morphology in short-term culture. To date, under commonly used conditions, hPSC-CMs have irregular shapes (Figure 1A), and they do not typically show alignment in 2-dimensional (2D) cultures.

Contractile Apparatus

The sarcomere is the fundamental unit for cardiomyocyte contraction. Monitoring the expression levels of sarcomeric proteins, such as cardiac troponin T, cardiac troponin I,
α-actinin, and β-myosin heavy chain (β-MHC), provides a basic assessment of specialization and maturation of hPSC-CMs. Reverse transcriptase polymerase chain reaction and immunocytochemical analyses from various groups have confirmed the expression of sarcomeric genes in hPSC-CMs.18–20 Because relatively immature cardiomyocytes express these proteins, the detection of sarcomeric genes should not be used as the only assessment of maturation. Electron microscopy has shown that hPSC-CMs have immature ultrastructural characteristics with various degrees of sarcomeric organization (Figure 1C).21,22 During cardiomyocyte development, sarcomeric structure becomes more organized, and sarcomeric length increases to facilitate force generation. In a relaxed adult human cardiac muscle cell, sarcomere length is \( \approx 2.2 \) μm, whereas immature hPSC-CMs have an immature ultrastructural copy has shown that hPSC-CMs have immature ultrastructural copy has shown that hPSC-CMs have immature ultrastructural copy has shown that hPSC-CMs have immature ultrastructural copy has shown that hPSC-CMs have immature ultrastructural

### Myofibrillar Isoform Switch

During development, several myofibrillar protein isoforms undergo switching, which modulates the contractile function of cardiomyocytes. Titin, which is involved in the maintenance of sarcomere integrity and elasticity, has been shown to shift from relatively compliant isoforms (with a size range 3200–3700 kDa, designated N2BA) to a shorter and stiffer form (3000 kDa, designated N2B). This alteration regulates the passive tension of maturing cardiomyocytes.25–27 In human postnatal left ventricles, N2B is the dominant titin form.28 Titin, which is involved in the maintenance of sarcomere integrity and elasticity, has been shown to shift from relatively compliant isoforms (with a size range 3200–3700 kDa, designated N2BA) to a shorter and stiffer form (3000 kDa, designated N2B). This alteration regulates the passive tension of maturing cardiomyocytes.25–27 In human postnatal left ventricles, N2B is the dominant titin form.28

### Sarcomere Structure

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Immature Cardiomyocytes</th>
<th>Adult Cardiomyocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>Circular</td>
<td>Rod shaped</td>
</tr>
<tr>
<td>Membrane capacitance</td>
<td>17.5±7.6 pF</td>
<td>=150 pF</td>
</tr>
<tr>
<td>Structure</td>
<td>Disarrayed</td>
<td>Highly organized</td>
</tr>
<tr>
<td>Length</td>
<td>( \approx 1.6 ) μm</td>
<td>( \approx 2.2 ) μm</td>
</tr>
<tr>
<td>Titin</td>
<td>N2BA</td>
<td>N2B</td>
</tr>
<tr>
<td>ssTnI</td>
<td>cTnI</td>
<td></td>
</tr>
<tr>
<td>α-MHC</td>
<td>( \beta ) &gt; ( \alpha )</td>
<td>( \beta ) ( \gg ) ( \alpha )</td>
</tr>
<tr>
<td>Transverse tubules</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Irregular reticular network in cytoplasm; occupies a small fraction of cell volume</td>
<td>Regularly distributed; occupies ( \approx 20% ) to ( 40% ) of cell volume</td>
</tr>
<tr>
<td>Metabolic substrate</td>
<td>Glucose</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>Multinucleation</td>
<td>Mononucleated</td>
<td>( \approx 25% ) multinucleated</td>
</tr>
<tr>
<td>Electrophysiological properties</td>
<td>Upstroke velocity</td>
<td>( \approx 50 ) V/s</td>
</tr>
<tr>
<td></td>
<td>Resting membrane potential</td>
<td>( \approx -60 ) mV</td>
</tr>
<tr>
<td>E-C coupling</td>
<td>Partially developed (refer to Calcium Handling and ECC)</td>
<td>Mature</td>
</tr>
<tr>
<td>Contractile force</td>
<td>( \approx ) nN range/cell</td>
<td>( \approx ) ( \mu )N range/cell</td>
</tr>
<tr>
<td>Gap junction distribution</td>
<td>Circumferential</td>
<td>Polarized to intercalated disks</td>
</tr>
<tr>
<td>Conduction velocity</td>
<td>( \approx 0.1 ) m/s</td>
<td>0.3–1.0 m/s</td>
</tr>
<tr>
<td>Responses to β-adrenergic stimulation</td>
<td>Chronotropic response</td>
<td>Chronotropic response</td>
</tr>
<tr>
<td></td>
<td>Lack of inotropic reaction</td>
<td>Inotropic reaction</td>
</tr>
</tbody>
</table>

Data refer to human pluripotent stem cell derivatives when possible. cTnI indicates cardiac troponin I; MHC, myosin heavy chain; and ssTnI, slow skeletal troponin I.

Functional analyses indicate that the troponin complex containing cardiac troponin I has decreased Ca2+ sensitivity for tension production, compared with complexes containing slow skeletal troponin I.33–35 For MHC, it is well known that in rodents there is a switch from β-MHC before birth to α-MHC after birth,36 which correlates with a postnatal heart rate increase. In human hearts, there are more β-MHC than α-MHC at all stages, but there is more α-MHC in fetal than adult hearts,37 correlating with the postnatal heart rate decrease. To our knowledge, the issue of contractile protein isoform switching remains open in the context of the maturation of hPSC-CMs. Because of the clear impact of myofibrillar protein isoform switching in cardiac function, future studies will benefit from taking this parameter into consideration.

### Transverse Tubules

Transverse tubules (T-tubules) are the membrane invaginations along the Z-line regions, with regular spacing (\( \approx 2 \) μm) along the longitudinal axis of adult mammalian ventricular myocytes (Figure 1D). Because they represent a key component of ECC in adult-sized myocytes, T-tubule appearance is a hallmark of cardiomyocyte development.38 Fetal rat cardiomyocytes lack T-tubules; however, by postnatal days 6 to 9, there are some T-tubule-like subcellular structures in the subsarcolemmal region. The myocytes of 12- to 14-day neonatal rats contain T-tubules that extend to the cell interior although still irregular. At \( \approx 1 \) month after birth, the T-tubule system is fully developed and displays the pattern of an adult cardiomyocyte. T-tubules make it possible for an adult cardiomyocyte to have rapid electric excitation, initiation, and
synchronous triggering of sarcoplasmic reticulum (SR) calcium release, and therefore, coordinated contraction throughout the entire cytoplasm. Studies from various groups have shown that hESC-CMs have few to no T-tubules in 2D culture, and further experiments showed that these cardiomyocytes had unsynchronized Ca²⁺ transients, as reflected by nonuniform calcium dynamics across the cell and greater calcium peak amplitude in the cell periphery than at the central region.

Mitochondria and Metabolic Substrate
Mitochondrial structural and functional changes are critical components of maturation during heart development. In immature cardiomyocytes ex vivo, mitochondria distribute throughout the cytoplasm in a reticular network, and they only account for a small fraction of the cell volume. Ultrastructurally, the inner membrane does not exhibit well-formed cristae. Interestingly, the mitochondria permeability transition pore, whose opening leads to pathological condition that initiates cell death in adult hearts, is open in embryonic mouse hearts, and its closure results in mitochondrial structural and functional maturation, evidenced by increased mitochondria length and mitochondrial membrane potential, and decreased reactive oxygen species production. As development proceeds, mitochondria develop more mature lamellar cristae and, in the adult cardiomyocytes, they are regularly distributed. Mitochondria occupy ≈20% to 40% of the adult myocyte volume and are distributed throughout the cell in a crystal-like lattice pattern. Because they are restricted by tightly packed distribution along myofibrils or beneath the sarcolemma, mitochondrial motility is highly limited. Corresponding with the structural changes from an immature to a mature state, the oxidative capacity of the mitochondria increases, as reflected in a metabolic substrate switch. During early cardiac development, glycolysis is a major source (80%) of energy. As cardiomyocytes mature and become terminally differentiated, mitochondrial oxidative capacity increases, with fatty acid β-oxidation (80%) becoming a major source of energy. In hPSC-CMs, long and slender mitochondria are clustered next to nucleus or in the cell periphery. Accordingly, human-induced pluripotent stem cell cardiomyocytes (hiPSC-CMs) rely on glycolysis rather than fatty acid β-oxidation.

DNA Synthesis, Multinucleation, and Nuclear Ploidy
During early stages of development, cardiomyocytes exhibit a robust proliferative capacity. Studies in diverse model systems have shown that as the heart matures, this proliferative capacity diminishes and, before cell-cycle withdrawal, many cardiomyocytes exhibit binucleation and, in some instances, extensive polyplody. Work by the Clubb and Bishop, Soonpaa et al., Li et al., and others revealed that during murine development, DNA synthesis occurs in 2 distinct phases: the early fetal phase is associated with cell division, whereas the second phase leads to binucleation. At birth, few cardiomyocytes are binucleated; however, within 2 weeks after birth, binucleated cardiomyocytes reach the adult level (≈75% in rodents). The timing of binucleation varies among different species. In sheep, there is a sharp increase in binucleation before birth. In humans, cardiomyocytes proliferate for the first few months after birth and the estimate of binucleation percentage of 25%. In addition, mature human cardiomyocyte nuclei commonly exhibit polyplody, which is the result of DNA replication without nuclear division or cytokinesis. Ploidy in human cardiac nuclei can reach 8 N in healthy hearts (diploid=2 N), with levels as high as 64 N in overloaded hearts.

Electrophysiological Properties
To generate the adult cardiomyocyte action potential, it takes the orchestrated activity among several ionic channels. During cardiac development, expression and function of distinct channel types occur with time. Indeed, studies in animal models show that channels undergo fetal and postnatal developmental changes that lead to the acquisition and maintenance of a mature cardiac electrophysiological phenotype. In freshly isolated cells, transient outward current (Iₒ) density has been shown to double at day 15 compared with day 5 rat neonatal cardiomyocytes. Only Iₒ is functionally expressed in the young canine ventricles, whereas both Iᵌᵣ and Iₑᵣ are present in adult canine myocardium, suggesting a greater dependence on Iᵌᵣ for repolarization in young dogs. Because of the low level of Iᵌᵣ channel, the resting membrane potential is higher (≈−60 mV) in immature cardiomyocytes than their mature counterparts (≈−90 mV). In addition, lower levels of the sodium channel Nav1.5 and the L-type calcium channel in immature cardiomyocytes lead to slower upstroke velocity and to a lack or a shorter plateau phase, respectively.

Calcium Handling and ECC
One of the most widely characterized functional parameters in hPSC-CMs is calcium handling. Multiple reports have shown that hPSC-CMs express critical Ca²⁺ handling proteins and exhibit [Ca²⁺]i transients. However, the maturation status and the basic mechanism that regulate the ECC of these cells remains controversial. Early studies indicated that in hPSC-CMs, [Ca²⁺]i transients and contraction were insensitive to drugs that interfere with SR Ca²⁺ release or reuptake. Further analyses of the expression of proteins known to be involved in Ca²⁺ handling could not detect key SR regulatory proteins such as calsequestrin or phospholamban. This study suggested that in hPSC-CMs, the rise in [Ca²⁺]i transients is the result from trans-sarcolemmal entry via calcium channels and that internal Ca²⁺ stores do not contribute significantly to this process. A subsequent report identified 2 different subpopulations of hESC-CMs, one that was caffeine-sensitive and another insensitive. Follow-up studies by this group showed that compared with control cells, hESC-CMs transduced with the regulatory protein calsequestrin had increased transient amplitude, upstroke velocity and transient decay, suggesting that calsequestrin has a significant impact in the functional maturation of hESC-CMs. Extensive evidence from 2 other groups support the notion that hPSC-CMs have functional SR Ca²⁺ stores and that, unlike cardiomyocytes in model species such as rat and rabbits, rabbits and mice, the establishment of a tight and localized control of ECC is an early event in the maturation
process of hPSC-CMs. Two more recent reports characterizing hPSC-CMs have shown the expression of key regulatory proteins, such as calsequestrin, and have also provided evidence supporting the presence of functional SR-dependent Ca2+ handling. It is possible that differential Ca2+ handling properties may arise from variability in the maturation status of different hPSC-CMs analyzed.

**Contractile Force**

For both immature and mature cardiomyocytes, contractile force is one of the least studied parameters. At the tissue level, Hasenfuss et al. reported a peak twitch tension for strips of human myocardium of 44±11.7 mN/mm², and similarly 56.4±4.4 mN/mm² for rat myocardium. In studies from our own group, hPSC-CMs in collagen constructs generated ≈0.08 mN/mm² (at a passive tension of 0.4 mN/mm²), which is ≈550-fold less than the adult human myocardium. The collagen constructs made of neonatal rat ventricular cardiomyocytes (NRVM) generated 0.4 to 0.8 mN/mm² at a resting tension of 0.1 to 0.3 mN/mm. These studies may underestimate force generation, because the collagen gels in which the cells are embedded are compliant and may stretch rather than transmit all force, and the cell density of these constructs is lower than native tissue. Nevertheless, the force generation of engineered human or rat myocardium is clearly much less than adult myocardium.

At a single cell level, Korte et al. reported a force of ≈1μN range by skinned adult rat cardiomyocytes. For hESC-CMs, Kita-Matsuo et al. used dynamic traction force microscopy (which measures lateral force generation) to analyze individual cells that were plated onto polyacrylamide gels with an elastic modulus of 4 kPa and surfaces functionalized with chemically cross-linked gelatin. Selected cardiomyocytes contracted with an average total force of 144±33 nN. Surprisingly, the contractile force was not changed with culture time ≥90 days. Under the same conditions, NRVM contracted with a total force of 222±54 nN. Liu et al. measured the contractile force of hESC- and hPSC-CMs by using atomic force microscopy (which measures vertical force generation) and showed that hPSC- and hESC-CMs beat similarly, with no differences in contractile force generation. However, the total force output is probably much higher, because the method used could not measure the lateral modes of contraction. The fact that the force data produced were not correlated with the cell area makes it hard to compare cardiomyocyte force generation between studies.

**Gap Junction Distribution and Conduction Velocity**

The adult human left ventricle myocardium has a conduction velocity of 0.3 to 1.0 m/s, whereas the conduction velocity in immature human heart has not to our knowledge been determined. In neonatal canine ventricular muscle, the average propagation velocity is 0.33 m/s, whereas in the adult canine the number increases to 0.50 m/s. In addition to the contribution of cell geometry, the distribution of gap junctions is an important factor that regulates conduction velocity. The gap junction protein connexin 43 and the adherens junction protein N-cadherin are circumferentially distributed during fetal life, and, as the postnatal rodent heart matures, these proteins become progressively concentrated into intercalated disks at the ends of the cells. This subcellular redistribution results in a much accelerated conduction velocity. Thus far, connexin 43 and N-cadherin show circumferential distribution in hPSC-derived cardiomyocytes in 2D culture, supporting the notion of an immature phenotype.

**Cardiac Gene Expression**

When considering gene expression during maturation, it is worth mentioning that, when cells grow 30-fold, there is more of everything on a per-cell basis. Because most RNA and protein studies normalize expression internally, for example, to housekeeping genes, total RNA or protein, global increases in transcript and protein content per cardiomyocyte are missed. Despite this limitation, multiple studies have shown that when compared with adult heart tissue, hPSC-CMs have lower expression of sarcomeric genes as well as genes encoding proteins for ion transport, and calcium handling. Because of space limitations, Table 2 briefly summarizes these data.

**Responses to Calcium and β-Adrenergic Stimulation**

With the above-mentioned differences between immature and mature cardiomyocytes, it is not surprising that the hPSC-CMs could respond differently to pharmacological stimulations. One study investigated the response of hESC-CMs to extracellular calcium and β-adrenergic stimulation using beating clusters that were cocultured with noncontractile, avital slices of neonatal mouse ventricles. Removing calcium or administration of a Ca2+ channel blocker stopped the spontaneous beating. Increasing Ca2+ concentration results in accelerated beating rate and increased developed isometric force up to a [Ca]2+ of 2.5 mmol/L. If [Ca]2+ was increased further, spontaneous beating rate decreased, whereas the developed force continued to increase.

These same investigators found that the β-adrenergic agonist, isoproterenol, induced a dose-dependent increase of the beating. Increasing Ca2+ concentration results in increased developed isometric force up to a [Ca]2+ of 2.5 mmol/L. If [Ca]2+ was increased further, spontaneous beating rate decreased, whereas the developed force continued to increase.

**Table 2. Major Cardiac Genes Upregulated (by ≥2-fold) in Adult Hearts Compared with the Immature hPSC-CMs**

<table>
<thead>
<tr>
<th>Genes Upregulated in Adult Heart Compared with hPSC-CMs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcomere</td>
</tr>
<tr>
<td>Ion transporters and some of their regulatory proteins</td>
</tr>
<tr>
<td>Sarcoplasmic reticulum</td>
</tr>
</tbody>
</table>

hPSC-CM indicates human pluripotent stem cell cardiomyocyte.
did not significantly change the developed isometric force during spontaneous contractions or during electric stimulation at a constant rate. The lack of an inotropic reaction despite a pronounced chronotropic response after β-adrenergic stimulation most likely indicates immaturity of the SR. In contrast with this study, Liu et al. detected the inotropic effect of norepinephrine (mixed α- and β-agonist) in both single hiPSC- and hESC-CMs using atomic force microscopy. Yokoo et al. found similar increases in shortening with a video-edge detecting system. It is not clear what leads to the discrepancy, but further investigations are warranted to clarify this important issue.

Modulation of Cardiomyocyte Maturation

To maximize the therapeutic benefits of hPSC-CMs, a critical challenge is to enhance their maturation status. It takes human cardiomyocytes years to reach adult form in terms of size, shape, molecular composition, metabolism, and physiological function in vivo. In this section, we will discuss the variety of approaches used to improve the maturation status of immature cardiomyocytes, including hPSC-CMs and rodent cardiomyocytes. The overall goal will be to mimic nature’s work, although at this stage, studies have only used 1 or 2 biological cues, leading to an intermediate state. Because of space limitations, the signaling pathways involved in maturation will not be discussed here; for this purpose, readers may refer to the excellent review by Heineke and Molkentin.

Long-Term Culture

Human neonatal cardiomyocytes require 6 to 10 years in vivo to reach their adult phenotype. Because of obvious practical constraints, the current length of differentiation for hPSC-CMs is much shorter. Beating cardiomyocytes can be generated from hPSCs within 15 days of differentiation. Thus, a logical approach to obtain more mature cardiomyocytes is by long-term culture. Studies have shown that similar to mouse neonatal cardiomyocytes, hPSC-CMs display autonomous hypertrophy in serum-free medium. The cells undergo a process of cell-cycle withdrawal and ultrastructural maturation within 35 days of culture period. Electrophysiological studies showed that the I_{Ca,L} and I_{K1} transient outward rectifier potassium currents, as well as the calcium current I_{Ca,L} and the pacemaking current I_{Kr}, underwent developmental maturation during 3-month culture period (Figure 2G). Surprisingly, in these studies, the generation of contraction stress did not increase by long culture time up to 3 months or up to 2 months. Spontaneous beating rate after 2 months culture was reported to decrease in some culture conditions; whereas other studies reported increased beating rate. Conduction velocity was reported to be significantly upregulated after 2 months in culture. M-band appearance, a hallmark of sarcomeric structural maturation, was reported in hPSC-CMs after 1 year in culture.

Most recently, Lundy et al. performed long-term hPSC-CM culture studies ≤120 days and found dramatic changes in cell size, anisotropy, sarcomere length, and percentage of multinucleated cardiomyocytes (Figure 2). Functionally, the long-term cultured cells showed a doubling in shortening magnitude with slowed contraction kinetics. An increase in calcium release and reuptake rates was also reported. Electrophysiologically, the long-term cultured cells have hyperpolarized maximum diastolic potentials, increased action potential amplitudes, and faster upstroke velocities. These data demonstrate that hPSC-CMs mature with time both morphologically and functionally, implying either a timekeeping mechanism or a time-dependent signaling pathway. Although long-term culture enhances many aspects of hPSC-CM maturation, this approach offers limited throughput. For this reason, many laboratories are currently attempting to accelerate and improve this process using diverse manipulations (discussed below).

Substrate Stiffness

The environment of the cardiomyocyte changes dynamically during development. Collagen accumulation, for example, begins during embryonic development and continues until several weeks after birth. This, and other related changes, result in a 3-fold increase of elastic modulus (passive stiffness) from embryonic to neonatal stages in mice and 2-fold increase in rats.
from neonatal to adult stage.\textsuperscript{85} This process of myocardium stiffening coincides with postnatal elevations in blood pressure and the heart’s increased capability of pumping blood.

Several studies investigated the effect of substrate stiffness on maturation. In NRVM on collagen-coated polyacrylamide gels with varying elastic moduli (1–50 kPa),\textsuperscript{82} extracellular stiffness close to that of native myocardium (10 kPa) significantly enhances their maturation as reflected by aligned sarcomeres, greater mechanical force (examined using traction force microscopy) and the largest calcium transients and sarco/endoplasmic reticulum calcium-ATPase 2a expression. A similar effect of substrate stiffness on cell morphology was observed in hESC-CMs in the same culture context.\textsuperscript{83} Rodríguez et al\textsuperscript{84} developed a novel approach that combines high-speed line scanning with microfabricated arrays of flexible posts. This provides temporal resolution to measure the power of NRVM cultured on post arrays of different stiffness. The study found that NRVM had a 6-fold greater twitch forces but slower twitch velocity when cultured on substrates with higher stiffness. Cardiomyocytes on stiffer arrays had more mature myofibril structure as reflected by a greater sarcomere length and Z-band width. Intracellular calcium levels also increased with stiffness during a twitch. Similar results were found by Hazeltine et al.\textsuperscript{78} who tracked the motion of fluorescent beads embedded in polyacrylamide hydrogels (4.4–99.7 kPa), and showed that both NRVM and hPSC-CMs generated greater mechanical force on gels with higher stiffness. Cell area was greatest on a substrate stiffness of 49.4 kPa. In contrast to the other studies, this group observed well-defined sarcomeres on all substrate stiffness; the reason for this difference is not clear.

**Cell Patterning**

In vivo cardiomyocytes are exposed to physical stimuli including topographical cues to keep them elongated and rod-shaped. It has been elegantly demonstrated that cardiomyocyte shape regulates sarcomere alignment.\textsuperscript{85} With a native cell length-to-width ratio of 7:1, cardiomyocytes exhibit directional anisotropy. Thus, a potential way to mature the cardiomyocytes is to develop advanced cell culture systems that mimic the in vivo microenvironmental topographical cues. For instance, cultivation of the cells on patterned substrates provides topographical cues that significantly enhance cell alignment.\textsuperscript{86,87} McDevitt et al\textsuperscript{88} seeded NRVM onto microcontact-printed laminin layers 5 to 50 μm wide. The NRVMs assumed rod-shaped geometries with myofibrils aligned parallel to the laminin lanes, and strikingly, a bipolar localization of N-cadherin and connexin 43 resembling intercalated disks. Kim et al\textsuperscript{89} constructed an anisotropically nanofabricated substrate with polyethylene glycol patterned with ridges and grooves ranging from 150 to 800 nm, designed to closely reproduce a nanoscale structure of the myocardial extracellular matrix composed of aligned fibrils ≈100 nm in diameter. Although a single cardiomyocyte spans >10 nanoridges, the cells still aligned along the direction of the topographical cue. Considerable differences were noticed between the nonaligned and aligned cell cultures in terms of cell geometry, conduction velocity, and Cx43 expression, with aligned cultures exhibiting properties more similar to the native heart. Combining surface topography and substrate stiffness cues together, Wang et al\textsuperscript{86} found that morphology and orientation of NRVM were mainly influenced by nanogrooved structures, whereas the contractile function of the cells was regulated by both surface topography and substrate stiffness with better contraction on the soft substrate with deep grooves.

**Electric Stimulation**

In vivo cardiomyocytes are constantly subjected to electric signals that promote synchronous contractions. It has been hypothesized that electric stimulation of hPSC-CMs would enhance their maturation, but there has been surprisingly little work performed in this area. NRVM in collagen sponges,\textsuperscript{90} showed better cell alignment and coupling, and an increase in contraction amplitude after electrical stimulation. The stimulated cells also showed increased ultrastructural organization. Electric stimulation of NRVM monolayers\textsuperscript{91} resulted in an increase in sodium calcium exchanger, increased action potential duration, and enhanced conduction velocity,\textsuperscript{91} as well as increased mitochondrial content and activity.\textsuperscript{92} In human ESC-CMs, electrical stimulation leads to longer action potential duration and higher caffeine-induced calcium flux.\textsuperscript{93} Also, it has been reported that in hESC-CMs electrical stimulation leads to higher Kir2.1 expression.\textsuperscript{94}

**Biochemical Cues**

**Adrenergic Receptor Agonists**

Adrenergic agonists have been studied extensively in rodent cardiomyocytes and are well known to induce hypertrophy. Traditionally, these are considered to be models of pathological hypertrophy; however, it is interesting to consider their potential roles in physiological maturation. Norepinephrine is an α- and β-adrenergic receptor agonist that can significantly increase protein synthesis after treatment of fetal and neonatal mouse myocytes and NRVM.\textsuperscript{94} In hESC-CM, administration of the α-adrenergic receptor/Gq agonist phenylephrine\textsuperscript{97} resulted in a significant 1.8-fold increase in cell area, 3.8-fold increase in cell number with organized sarcomere structure, and a 2-fold increase in cell volume. Total cellular protein to DNA content was also higher in phenylephrine-treated cells.

**Triiodothyronine**

Thyroid hormone is essential for normal cardiac development.\textsuperscript{95} In the perinatal period, it regulates the isomorph switching of several myocardial proteins, including MHC and titin.\textsuperscript{96} Chattergoon et al\textsuperscript{97} reported that triiodothyronine is a major inducer of cardiomyocyte maturation in the sheep fetus by increasing cell width, increasing binucleated cell percentage, reducing cell proliferation, and increasing the sarco/endoplasmic reticulum calcium-ATPase 2a expression. Studies in murine ESC-CMs also showed that triiodothyronine increases Nkx2.5, myosin light chain-2v, α-MHC, sarco/endoplasmic reticulum calcium-ATPase 2a, and ryanodine receptor-2 expression.\textsuperscript{88} Electrophysiological studies showed that triiodothyronine-treated cardiomyocytes exhibited decreased resting membrane potential and more adult-like calcium homeostasis properties, including a significantly larger maximal upstroke velocity, a higher maximal decay velocity, and a larger peak amplitude of caffeine-induced calcium transients.
Insulin-like Growth Factor I

Insulin-like growth factor I (IGF-1) is essential for the regulation of cardiomyocyte proliferation, differentiation, postnatal growth, and maturation of the heart. IGF-1 treatment of NRVM leads to upregulation of myosin light chain-2 and troponin I, cell size doubling, with an increase in protein synthesis. IGF-1 has also been reported to influence the maturation of cardiomyocyte metabolism by enhancing the effect of the nuclear receptor/transcription factor peroxisome proliferator–activated receptor α and increasing the expression of the fatty acid oxidation enzymes medium chain acyl-CoA dehydrogenase and the muscle-type carnitine palmitoyltransferase I in NRVM. Although IGF-1 has been shown to be a mitogen for hESC-CMs, to our knowledge it has not been studied in regulating their maturity.

MicroRNA

Analysis of micro RNA (miR) profiles of human ESCs, hESC-CMs, fetal human, and adult human ventricular cardiomyocytes identified miR-1 as a potential modulator of cardiomyocyte maturation. Further studies showed that miR-1 overexpression decreased action potential duration and hyperpolarized both the resting membrane potential and the maximum diastolic potential in hESC-CMs because of increased ICa,L, ICa,C, and decreased ICa,T. In addition, miR-1 augmented the immature Ca2+ transient amplitude and kinetics. Because of its involvement in thyroid hormone responsiveness and the myosin isoform switch, miR208 is a potential candidate that also may promote maturation.

Tissue Engineering and Mechanical Loading

The myocardium consists of a 3D arrangement of rod-shaped cardiomyocytes, which form myofibers that are adjacent to interstitial fibroblasts, blood vessels, and extracellular matrix. Cardiomyocytes are also continuously subjected to cyclic mechanical strain induced by rhythmic heart beating. It is thus not surprising that even adult cardiomyocytes begin to dedifferentiate when they are placed in 2D culture without mechanical loading. An attractive hypothesis is that seeding immature cardiomyocytes in appropriate 3D matrix and subjecting them to mechanical stress will enhance their maturation. This hypothesis was first elegantly proven by the work of Zimmerman et al. NRVMs were cast in hydrogels of collagen I plus basement membrane proteins and subjected to phasic mechanical stretch to generate engineered heart tissue. After 14 days, the cardiac cells in engineered heart tissue exhibited interconnected, longitudinally oriented cardiac muscle bundles with morphological features resembling adult native tissue. It is worth noting that cardiomyocytes in the engineered heart tissue exhibited a well-developed T-tubular system and dyad formation with the SR. The engineered heart tissue displayed contractile characteristics of native myocardium with a high ratio of twitch to resting tension and a strong β-adrenergic inotropic response. Action potential recordings demonstrated lower stable resting membrane potential, faster upstroke kinetics, and a prominent plateau phase. To our knowledge, this study generated cardiomyocytes with the closest morphological and electrophysiological properties to adult cardiomyocytes from NRVM.

Liau et al introduced 3D cell alignment cues consisting of elliptical posts within a fibrin-based hydrogel matrix. Mouse ESC-CMs were seeded, and they assembled into a dense, structurally, and functionally aligned 3D syncytium with supporting nonmyocytes within a total culture time similar to period of mouse embryonic development. Those cells had a conduction velocity between 22 and 25 cm/s. It is worth mentioning these values are close to the conduction velocity of neonatal mouse hearts (≈29 cm/s), whereas adult mouse hearts has a conduction of 62 cm/s. The cardiomyocytes produced significant contractile forces ≤2 mN. Because the authors reported cross-sectional areas of 49 mm², this corresponds to force production of 0.04 mN/mm², achieving levels of functional differentiation similar to those of neonatal mouse hearts. To date, this tissue engineering approach has produced the fastest propagating electric signals. It would be interesting to see to what extent this system could mature the hPSC-CMs.

Our group was the first to adapt hydrogel-based tissue engineering techniques to generate human engineered cardiac tissue from hPSC-CMs. The cells were cast in 3D collagen and basement membrane protein matrix followed by uniaxial mechanical stress conditioning. Cyclic stress markedly increased cardiomyocyte alignment and hypertrophy, and proliferation rates were moderately upregulated. Addition of endothelial cells further enhanced cardiomyocyte proliferation, and addition ofstromal supporting cells augmented vessel-like structure formation. Importantly, the optimized human cardiac tissue constructs generated Starling curves (Figure 3), increasing their active force in response to increased resting length.

Most recently, Nunes et al developed a biowire system in which hPSC-CMs with supporting cells were seeded into a PDMS template around a surgical suture in collagen gels. The biowire platform leads to the generation of 3-dimensional, aligned cardiac tissues with frequent striations. By combining biowire cultivation system with electrical stimulation, the authors reported increased myofibrillar ultrastructural organization, higher conduction velocity, and enhanced electrophysiological and calcium handling properties.

Cell–Cell Interaction and Cardiomyocyte Maturation In Vivo

During development in vivo, cardiomyocytes interact closely with several other cell types, such as fibroblasts, endothelial, and smooth muscle cells. Therefore, either direct cell–cell contact or paracrine factors affect cardiomyocyte maturation. Indeed, using genetically purified early hESC-CMs, Kim et al showed that noncardiomyocytes are required for the development of some intracellular calcium handling proteins. Ion channel development (HCN4) and electrophysiological maturation were also enhanced by the presence of the noncardiomyocytes. It will be of considerable interest to identify the molecular signals that mediate this cross-talk.

Using ventricular slices of recipient hearts, Halbach et al performed time-course recordings on the electrophysiological maturation of fetal mouse cardiomyocytes after transplanting them into cryoinjured mouse hearts. Fetal cardiomyocytes that electrically integrated with the host hearts matured much faster than cells that were embedded in the cryoinjury, which showed...
no electric integration. Twelve days after transplantation, the action potential duration at 50% repolarization of the integrated cells matched the action potential duration at 50% repolarization of the host cardiomyocytes, whereas the cells embedded in cryoinjured tissue still displayed immature electrophysiological properties. Structurally, transplanted cells displayed an increased cell area and length/width ratio. Although fetal mouse cardiomyocytes were used in this study, it provides some possible clues that may be applied to promote hPSC-CM maturation.

Recent work in direct reprogramming further shows the importance of the in vivo environment. The combination of 3 transcription factors (Gata4, Mef2c, and Tbx5) in vitro reprograms fibroblasts into cardiomyocyte-like cells, some of which express cardiac-specific genes, exhibit spontaneous calcium flux and beat. These induced cardiomyocytes morphologically and functionally resemble immature hPSC-CMs rather than adult cardiomyocytes. When reprogramming was performed in vivo, however, the induced cardiomyocytes displayed morphological and functional properties of adult cardiomyocytes with elongated cell shape, intercalated disks with polarized connexin 43 expression, and electrophysiological parameters similar to the adult cardiomyocytes.

Again, this study highlights the importance of in vivo cues to the maturation of cardiomyocytes.

**hiPSCs and Cardiac Disease Modeling**

The contribution of animal models to our overall understanding of diseases has been enormous. Nonetheless, significant differences exist between humans and common experimental animals, for example, size, heart rate, ion channel contributions, distinct developmental processes, and many diseases cannot be modeled well in animals. With the advent of induced pluripotent stem cells, functional cardiomyocytes can be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.

**Pharmacological Studies**

One of the principal reasons that drugs are withdrawn from the market after initial approval is cardiovascular toxicity that went undetected despite extensive testing in animals. This points to the significant difference between human and animal physiology. Because of the lack of sufficient and healthy human cardiac tissue for experimental research, hPSC-CMs have been suggested as a new, attractive model. The effects of a variety of pharmacological agents have been assessed in hPSC-CMs. These drugs have effects on beating frequency and contractility that were compatible with empirical results in the clinic, suggesting that hPSC-CMs could become an attractive tool for investigating drug effectiveness and safety. Another group used hiPSC-CMs as a platform to characterize the proarrhythmic potential of 28 different compounds with established cardiac effects. The hiPSC-CMs responded to drug treatment in a manner similar to what has been observed clinically.

**Figure 3. Tissue engineered human pluripotent stem cell cardiomyocytes collagen constructs were subjected to a series of stretches to increase resting tension.** Note the increased twitch heights at higher preload (insets). A, Starling curves showing a linear increase in active force in response to increased diastolic length. Adapted with permission from McDevitt et al. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.

Kadota et al developed a reentrant arrhythmia model using hPSC-CM sheets. By optically imaging the calcium signal, the authors found that sodium channel blockers affected the velocity and propagation pattern of activating waves. High-frequency stimulation of the sheets generated reentrant spiral waves, which were terminated by current antiarrhythmic drugs. This model may be useful for future screening and testing drugs with antiarrhythmic potential.
One study,124 however, showed that caution should be used before extrapolating the results from the hESC-CMs to the adult cardiomyocytes because lack of functional $I_{Na}$ channels, enhanced $I_{K}$ density, and some other differences between them may limit the potential of the hPSC-CMs to respond to drugs, especially proarrhythmic triggers. This study suggested that in some cases, maturation of the electric phenotype is necessary for future implementation in drug safety testing. As mentioned earlier in Overview of Cardiomyocyte Maturation, hPSC-CMs may respond to some stimulation (eg, response to high concentrations of calcium and β-adrenergic agonists) differently from their adult counterparts,14 and most studies have currently screened one or at best only a handful of cell lines.

Conclusions and Future Directions

As illustrated in Figure 4, hPSCs have diverse potential applications in cardiovascular biology and medicine; however, to reach this potential we need to learn to promote their maturity. Cardiomyocyte maturation in vivo is regulated by diverse factors, including topographical, electric, adhesive, mechanical, biochemical, and cell–cell interaction cues. It is natural to want to break these components down, decipher the signaling pathways that control the phenotype, and try to harness them to accelerate the process. Maturation of cells, as in people, is a complex trait, and we suspect it is unlikely to be controlled by a single master pathway. Thus, regulating a single pathway may control only a subset of the overall network, and this could lead to a distorted or partially matured phenotype. To achieve a better maturation status of the hPSC-CMs, it might be necessary to expose the cells to multiple regulatory cues simultaneously. It is natural to think that the best outcome would be a fully adult phenotype. However, for cell transplantation, adult cardiomyocytes do not survive to form new myocardium, whereas fetal and neonatal cardiomyocytes do.125 Therefore, for cell transplantation purposes, the best option might be to obtain a cardiomyocyte phenotype with an yet-to-be-identified optimal maturation status that leads to the best engrafting and functional improvements.

As summarized in Figure 5, although progress toward maturation of hPSC-CMs has been made, significant challenges remain. An immediate future application for hPSC-CMs (either in 2D or 3D culture) is to perform high-throughput experiments to find the interventions that efficiently promote their maturation. For such screens to be successful, however, the end point indicating maturation needs to be carefully selected, as you get what you select for. Another exciting possibility of the use of hiPSC-CMs is the potential for personalized drug screening because different individuals can have distinct responses to a specific medicine. If the drug’s possible cardiotoxic effects could be identified in the individual’s hiPSC-CMs, this could greatly advance personalized medicine. In this case, the importance to enhance the cell maturation is self-evident if the drug is for adult-onset diseases.

For patients with genetic diseases, it is possible that mutations could be repaired and the hiPSC-CMs be used for autologous cell replacement therapy. With the current available techniques, investigation of the patient-derived iPSC-CMs in 3D cardiac tissue constructs may lead to novel findings about the targeted diseases. One can also envision using hPSC-CMs for an in vitro human myocardial infarction model in which the cells are exposed to ischemic conditions. Because immature and mature cardiomyocytes respond to ischemia differently, optimizing the maturation status again will be necessary.

In summary, substantial progress has been accomplished in just a few years since the development of hESCs and hiPSCs.

Figure 4. Potential application of human pluripotent stem cell cardiomyocytes (hPSC-CMs). hPSC-CMs can be used and already show promising results in regenerative medicine. These cells are also valuable for disease modeling and pharmacological studies, as discussed in the main text. Enhancing hPSC-CM maturation state may allow for improved therapeutic applications. As we develop methods to generate cardiomyocytes of different maturation states, we will be able to gain significant insights into human cardiac development.

Figure 5. Summary of current maturation approaches and phenotypes achieved to date. A and B, Representative immature human pluripotent stem cell cardiomyocytes (hPSC-CMs) and intermediate hPSC-CMs, respectively. The cells were stained for α-actinin (green) and filamentous actin (phalloidin, red). Nuclei (blue). Elongated cell shape, more organized α-actinin (green) and filamentous actin (phalloidin, red). Nuclei (blue). Elongated cell shape, more organized sarcomere structures, longer sarcomeres, and more abundant mitochondria were achieved with manipulations. Compared with immature hPSC-CMs, the intermediate cardiomyocytes display higher conduction velocity,26 contractile force, and increased calcium transient kinetics.27 Scale bars in A and B, 25 μm; C and E, 0.5 μm; D, 5 μm. B through E were adapted from Warren et al27 with permission from Dr Michael A. Laflamme. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.
Successful differentiation of cardiomyocytes from hPSCs shows great promises in several applications such as human development, cell transplantation, disease modeling, and drug screening. Promoting maturation of hPSC-derived cardiomyocytes will be an essential step toward achieving these goals.

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Disclosures
C.E. Murry is co-founder and equity holder in BEAT Biotherapeutics. The other authors report no conflicts.

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