Prospects for *In Vitro* Myofilament Maturation in Stem Cell-Derived Cardiac Myocytes

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**ABSTRACT:** Cardiomyocytes derived from human stem cells are quickly becoming mainstays of cardiac regenerative medicine, *in vitro* disease modeling, and drug screening. Their suitability for such roles may seem obvious, but assessments of their contractile behavior suggest that they have not achieved a completely mature cardiac muscle phenotype. This could be explained in part by an incomplete transition from fetal to adult myofilament protein isoform expression. In this commentary, we review evidence that supports this hypothesis and discuss prospects for ultimately generating engineered heart tissue specimens that behave similarly to adult human myocardium. We suggest approaches to better characterize myofilament maturation level in these *in vitro* systems, and illustrate how new computational models could be used to better understand complex relationships between muscle contraction, myofilament protein isoform expression, and maturation.

**KEYWORDS:** cardiomyopathy, disease modeling, engineered heart tissue human pluripotent stem cell-derived cardiomyocytes, maturation, protein isoforms

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**Introduction**

Cardiac muscle cells derived from human embryonic stem cells (hESC-CMs) and human-induced pluripotent stem cells (hiPSC-CMs) hold great potential in many different medical applications such as regenerative medicine,1 drug screening,2 and disease modeling.3,4 Since the advent of robust methods for inducing cardiac differentiation in stem cells,5 several groups have turned their efforts toward assembling this new cell source into functioning engineered heart tissue (EHT). EHT specimens have enabled the first experiments quantifying the physiological behavior of stem cell-derived myocardium. While initial results are encouraging, more work is required in order to establish the degree to which these initially fetal-like cells have been successfully transformed into mature cardiomyocytes.

During cardiogenesis and maturation, the heart is exposed to marked changes in hemodynamic loading, hormonal regulation, and oxygen levels. These perturbations coincide with adjustments to contractile performance,6 which can be observed through *in vitro* measurements of muscle mechanics. Developmental changes to contraction are achieved in part by shifts in protein isoforms found in the myofilament contractile apparatus. Given the extensive literature on myofilament mechanical function and protein isoform expression during development,6,7 these characteristics constitute a powerful set of markers for assessing the maturity of hESC/hiPSC-CMs. Although a few studies have measured mechanical function in various forms8–11 or the expression of certain myofilament genes in hESC/hiPSC cultures,10–16 the full potential of these markers has not been exploited.

In this commentary, we consider the use of stem cell-derived cardiomyocytes as a tool for disease modeling, with an eye toward how this application may be impacted by the degree of myofilament maturation. We examine the state of the art in achieving maturation by comparing the reported contractile performance of EHTs to adult human myocardial data from the literature. We discuss how remaining phenotypic gaps might relate to immature myofilament protein isoform expression, and endeavor to show that computational analyses, in conjunction with the extensive literature on cardiac myofilament maturation, will point the way toward new milestones on the path to mature *in vitro* models of cardiac muscle.

**Efforts to Model Genetic Heart Disorders with Stem Cell-Derived Cardiomyocytes**

With the emergence of induced pluripotent stem cell (iPSC) technology, it is possible to generate cardiomyocytes from patients with inherited heart disorders. Although these cells
are immature in many respects, they do exhibit some in vitro phenotypes that resemble clinical pathologies.\textsuperscript{2,17,38} The genetic specificity of abnormal phenotypes is enhanced by the possibility of creating control iPSCs from close family members that lack the mutation of interest.\textsuperscript{19} A further advantage is that genetic engineering has become much more accessible with the emergence of new genetic editing techniques such as CRISPR/Cas9.\textsuperscript{20} Patient-derived iPSCs can be genetically engineered to remove mutations, or, alternatively, disease-causing mutations can be introduced into control cells to test the true pathogenicity of a suspected mutation.\textsuperscript{19}

To date, iPSC lines have been created from patients with arrhythmogenic right ventricular cardiomyopathy,\textsuperscript{21–23} catecholaminergic polymorphic ventricular tachycardia,\textsuperscript{24–26} cardiac hypertrophy,\textsuperscript{27} duchenne muscular dystrophy,\textsuperscript{28} dilated cardiomyopathy (DCM),\textsuperscript{18,29} familial hypertrophic cardiomyopathy (HCM),\textsuperscript{17} Friedreich ataxia-associated HCM,\textsuperscript{30} hypoplastic left heart syndrome,\textsuperscript{31} Jervell and Lange-Nielsen syndrome,\textsuperscript{32} Leopard syndrome,\textsuperscript{33} and Long QT syndrome.\textsuperscript{34–37} Cardiomyocytes differentiated from these lines have been studied to search for phenotypes and disease mechanisms in an in vitro setting. These efforts are reviewed in greater detail elsewhere.\textsuperscript{3,4,38}

Although these initial reports demonstrate great potential for hiPSC-CMs in cardiac disease modeling, specific findings must be interpreted with caution. In their detailed review on current limitations of hiPSC, Eschenhagen et al.\textsuperscript{38} highlight that it remains subject to debate whether cultured hiPSC-CMs from patients with sarcomeric mutations recapitulate aspects of the disease pathology or only the influence of the sarcomeric mutation on induced differentiation and cell culture.

For instance, Lan et al.\textsuperscript{17} generated iPSC-CMs from a 10-member family cohort carrying a missense mutation in the myosin heavy chain gene (MYH7). These cell lines exhibited several characteristics of the HCM phenotype, such as cellular hypertrophy, calcineurin–NFAT (nuclear factor of activated T cells) activation, upregulation of hypertrophic transcription factors, as well as irregular calcium transients and contractile arrhythmias when compared to healthy control iPSCs. They observed that the Arg663His MYH7 mutation leads to arrhythmias and elevated intracellular diastolic Ca\textsuperscript{2+} at the single-cell level even before the onset of cellular hypertrophy.

The implication of these findings is that the primary mechanism by which myosin mutations trigger hypertrophy is through altered intracellular Ca\textsuperscript{2+} cycling. Such a mechanism is feasible: Increased myosin–actin affinity, which could be a consequence of this mutation, is known to increase the Ca\textsuperscript{2+} sensitivity and the amount of Ca\textsuperscript{2+} buffered by the myofilaments. Increased buffering capacity would likely have the effect of elevating the diastolic Ca\textsuperscript{2+} levels in cells, as observed. Persistently elevated Ca\textsuperscript{2+} levels would in turn chronically overactivate the hypertrophic calcineurin–NFAT signaling pathway. However, it is possible that such a mechanism is only observed in immature iPSC-CMs whose myofilaments may already have increased Ca\textsuperscript{2+} sensitivity and buffering capacity. Against a mature background that buffers less Ca\textsuperscript{2+},\textsuperscript{39} the effects of the myosin mutation may be not be sufficient to trigger a pathogenic hypertrophic response. Hence, while the study exposes an interesting potential disease mechanism, its application to adult physiology cannot be assumed without knowing more about the status of myofilament maturation in these particular cells. In other words, the relevance of in vitro model results to clinical disease depends critically upon adequate cellular maturation. In this regard, results obtained with hiPSC-CMs in vitro are still somewhat limited.\textsuperscript{38}

**Efforts to Characterize and Improve Maturation in Stem Cell-Derived Cardiomyocytes**

In general, stem cell-derived cardiomyocytes tend toward an immature fetal phenotype (reviewed elsewhere\textsuperscript{40,41}). hESC/hiPSC-CMs are less likely to be multinucleated than adult cardiac cells, and the overall expression levels of contractile and cytoskeletal genes are below those commonly seen in fetal or adult cardiomyocytes.\textsuperscript{42,43} Furthermore, in hESC/hiPSC-CMs the subcellular structures required for normal excitation contraction coupling and Ca\textsuperscript{2+} handling are missing or poorly developed.\textsuperscript{40} Transverse tubules in particular are conspicuously absent.\textsuperscript{40,44–47} As a consequence, in hESC/hiPSC-CMs most of the intracellular Ca\textsuperscript{2+} transient comes from influx through sarcolemmal channels rather than release from the sarcoplasmic reticulum. This process differs greatly from adult EC coupling, during which calcium-induced calcium release accounts for 70% released Ca\textsuperscript{2+}.\textsuperscript{48} This explains why hESC/hiPSC-CMs typically exhibit smaller and slower Ca\textsuperscript{2+} transients than their adult counterparts.\textsuperscript{49} Some experiments suggest that these deficits in Ca\textsuperscript{2+} handling maturation are more severe in hiPSC-CMs than cells derived from hESCs.\textsuperscript{50}

Other indications of cellular immaturity in hESC/hiPSC-CMs are a smaller overall size, lack of characteristic sarcomere formation, spontaneous beating, fetal-like action potentials, dependence on glycolosis rather than fatty acid oxidation for the production of ATP, and fewer numbers of mitochondria.\textsuperscript{40,51–53}

The degree of maturation observed in hESC/hiPSC-CMs cells is subject to a variety of factors such as the differentiation protocol, length of culture, presence of growth factors, co-culture with other cell types, and the spatial configuration of cells [two-dimensional (2D) vs three-dimensional (3D) culture].\textsuperscript{40,54} Efforts to promote maturation have ranged from altering culture conditions, increasing culture time, and applying physical cues (mechanical and electrical).\textsuperscript{41,54–57} Given that human neonatal cardiomyocytes require several years to achieve their adult phenotype, Lundy et al.\textsuperscript{56} investigated the influence of culture duration on maturation. In comparison to 20–40 days, a culture period of 80–120 days led to faster Ca\textsuperscript{2+} transient kinetics, increased contractile performance, better organized sarcomeres with Z-disks and organized...
A- and I-bands, 10-fold increase in the fraction of multinucleated CMs, and expression levels of MYH6 and MYH7 that almost match the expression profiles in the adult human heart. In a different study, Yang et al treated hiPSC-CMs with the growth hormone tri-iodo-L-thyronine (T3) for 1 week. 58 This led to significant increases in cell size and sarcomere length, increased mitochondrial function, higher peak force, improved contractile kinetics, and faster Ca2+ transients.

There is ample evidence to suggest that forming immature cardiomyocytes into structures resembling native cardiac tissue (as opposed to 2D culture) enhances maturation. Three-dimensional culture increases action potential propagation speed, force transduction, and contractile tension. 58 A study directly comparing hESC-CMs seeded in 3D hydrogels with 2D monolayer cultures also found higher conduction velocities, longer sarcomeres, and higher expression levels of key cardiac genes such as cardiac troponin T (TnT), alpha myosin heavy chain, and SERCA2.10

Another critical advantage gained by assembling hESC/iPSC-CMs into 3D tissue specimens is the ability to apply specific mechanical loading regimes to the cells in culture and measure their overall mechanical performance under near-physiological conditions. Currently, creating ribbon-like EHTs using molded hydrogels is the most common approach. 9,39-61 This technology was initially implemented with rat neonatal ventricular myocytes, but more recently EHTs seeded with human stem cell-derived cardiomyocytes have emerged.9-11,16,59,62

The advent of EHTs seeded with hESC/iPSC-CMs has coincided with an increased effort to design bioreactors that mimic the native cardiac environment by applying mechanical loads and electrical stimulation. Mechanical stretch16,62 and electrical pacing14 of EHTs have both been extensively used to improve function and maturation levels of engineered cardiac constructs seeded with hESC-CMs. Recently, efforts have also been made to simultaneously apply both stimuli in a realistic manner. Morgan et al.63 were able to develop a bioreactor system for rat EHTs that could apply a delayed electrical stimulation after applied cyclical mechanical stretch in an effort to mimic isovolumetric contraction, which ultimately led to higher protein expressions of SERCA2 and cardiac TnT when compared to less physiological culture conditions.

Although efforts to characterize and improve maturation of hESC/iPSC-CMs are well under way, we cannot confidently state how close the field is to recapitulating adult cardiac muscle behavior. One reason is that maturation is multifaceted, and not clearly defined by any widely accepted standard. Another is that tools for quantitative analysis, which would help identify and explain molecular aspects of maturation, have not yet been applied to the problem. One way forward could be to focus on isometric twitch kinetics as a simple but comprehensive readout of EHT maturity and an objective of quantitative analysis.

**Characterization of Twitch Dynamics as a Comprehensive and Integrative Measure of Myofilament Maturation**

**Introduction to myocardial twitch dynamics.** The time course of cardiac muscle twitch force (Fig. 1A) has been studied extensively under diverse circumstances. In a typical experiment, linear muscle specimens such as papillary muscles or trabeculae are dissected and mounted in a bath that maintains physiological conditions. Muscles are attached to a force transducer that quantifies twitch force as a function of time, and electrodes placed in the bath allow the contraction frequency of the muscle to be controlled. In the most common experimental procedure, muscle specimens are held at constant length during contraction (isometric conditions). The overall muscle length for a series of several contractions is often adjusted in order to examine the force–length relationship.

After twitches are measured, it is common practice to transform the force recordings into descriptive scalar properties. These include the peak twitch tension, minimum and maximum time derivatives of tension (dT/dt min and max), time from stimulus to peak tension (TTP), and time from peak tension to 50% relaxation (RT50), among others (Fig. 1B). Typical values for these scalar properties, as well as their dependence on muscle length, stimulation frequency, and adrenergic stimulation constitute the “physiological” behavior of intact cardiac muscle. These tests or some subset thereof are frequently invoked in studies of ESC- or iPSC-derived myocardial mimics. The work of Turnbull et al.31 is perhaps the most comprehensive example.

Isometric twitch responses are known to depend upon the expression of specific myofilament protein isoforms, many of which are developmentally regulated. Hence, the measurement of twitch dynamics can function as a means of assessing the maturation of cardiac myocytes, including maturation of myofilament protein expression. Indeed, some investigators are viewing maturation from a functional standpoint as achieving twitch force properties that mimic available data from adult myocardium.31 What is less commonly recognized is that the twitch force characterizations of in vitro cardiac muscle differentiation hold abundant information that could be related to the expression of specific myofilament protein isoforms.

**Comparison of twitch dynamics in adult and ESC/iPSC-derived myocardium.** A comparison of published twitch force records from human EHTs shows substantial diversity. This can be seen easily in the qualitative comparison presented in Figure 1C–E. We have digitized records from human EHTs seeded with hESC-CMs30,31 and hiPSC-CMs8 and overlaid them on normalized twitch traces from adult human myocardium.34,65 All of the records shown in Figure 1C–E were collected at physiological temperature and a pacing rate of 1 Hz. The twitch measurements in panels C and D resemble adult kinetics to some degree, while that of panel E is clearly slower to contract and relax when compared to the adult traces. Although its kinetics are substantially slower, it should be
noted that the twitches measured by Zhang et al.\textsuperscript{10} are the only ones to date that achieve peak tension values close to native adult myocardium, with all others being approximately 10-fold smaller.

Each of the human EHT studies cited here extracted scalar properties from twitch records in order to quantify contractile behavior. Unfortunately, the analysis details and selected properties are not consistent across these studies, meaning that quantitative comparisons between them are limited. They are also limited by the fact that twitch records were obtained under slightly different muscle length protocols in each study. However, some general comparisons are possible, and from them we can glean a general feeling for the state of the art with regard to twitch maturation.

For adult myocardial preparations, typical contractile values at 1 Hz pacing rate range around a peak tension of 20 mN/mm\textsuperscript{2}, a TTP of about 190 ms, and RT50 of 120 ms in left ventricular muscle strips.\textsuperscript{64} Healthy myocardium shows a bell-shaped force–frequency curve peaking at around 3 Hz. Increasing the stimulation frequency from 1 to 2.5 Hz causes the twitch tension to double.\textsuperscript{64} The increase in twitch tension with faster pacing is termed force–frequency response.

Human EHT preparations reach peak tension ranging from 0.57\textsuperscript{11} to 11.8 mN/mm\textsuperscript{2}.\textsuperscript{10} Properties of twitch kinetics can only be sparsely compared to those of the adult humans due to different metrics used in most cases, but Turnbull et al report a TTP of 90 ms, which is substantially faster than reported for adult myocardium.\textsuperscript{11} Relaxation kinetics were reported in the same study using an RT90 value that, on average, was 118 ms. We could not locate this exact metric for human adult myocardium, but Wiegerinck et al report a value of 400 ms for RT90 in human neonatal myocardial preparations.\textsuperscript{66} In terms of the force–frequency response, all of the stem cell–derived cardiac tissues show no change or even a reduction in peak twitch tension over frequency changes that elicit drastic increases in tension when applied to adult human myocardium.\textsuperscript{8,10,11} A flat or negative force–frequency response is associated with failing or immature/newborn human heart tissue in multiple studies.\textsuperscript{64,66,67}

One additional aspect of contractile function commonly tested is the length–tension relationship (Frank–Starling response). In these tests, a specimen is stretched to a particular length and its peak twitch tension recorded. Repeating this measurement at different muscle lengths in adult myocardium gives the classic Frank–Starling relationship, that is, the peak contractile tension increases with increasing muscle length until reaching a saturation point. We found that a positive length–tension relationship was the only physiological response unanimously reproduced by human EHTs.\textsuperscript{10,31,36}

Although the conditions for comparison are not ideal, we feel it is safe to conclude that none of the human EHTs characterized to date recapitulates all aspects of reported human

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\text{Tension} \quad \text{Time} \\
\text{Stimulus} \\
\text{Peak tension} \\
\text{Time from stimulus to peak tension} \\
\text{50% peak tension} \\
\text{Time from peak to 50% relaxation} \\
\text{10% Peak tension} \\
\text{Time from peak to 90% relaxation} \\
\text{Time derivative of tension} \\
\text{Maximum rate of tension change} \\
\text{Normalized Tension} \\
\text{Human RV trabecula [Nejad & Janssen\textsuperscript{65}] } \\
\text{Human LV Myocardial Strip [Mulieri et al.,\textsuperscript{64}]} \\
\text{Human ESC-CM Tissue [Turnbull et al.\textsuperscript{11}]} \\
\text{Human iPSC-CM Tissue [Stoehr et al.\textsuperscript{8}]} \\
\text{Human ESC-CM Tissue [Zhang et al.\textsuperscript{10}]} \\
\text{Minimum rate of tension change} \\
0 \quad \text{Time (ms)} \\
1000 \\
\text{Figure 1. An overview of myocardial twitch dynamics. (A) A schematic representation of a cardiac muscle twitch tension record. (B) Illustration of descriptive scalar properties commonly extracted from twitch records. (C–E) Overlay of native cardiac tissue twitch records and published engineered heart tissues.}}
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adult myocardial twitch behavior. While it is tempting to classify hESC/hiPSC-CMs as having immature or fetal behavior, the obvious diversity in twitch kinetics among published studies (Fig. 1C–E) prohibits a simplistic interpretation. Going forward, it seems that the field must give specific attention to the different facets of contractile maturation, both to measure them and to identify their molecular underpinnings. The latter requires a detailed understanding of myofilament protein isoform expression.

Protein Isoforms of the Contractile Apparatus

Activation of the contractile apparatus is the end point of a process known as excitation–contraction coupling (EC coupling). The initial event in EC coupling is depolarization of the cell membrane, which leads to an influx in Ca\(^{2+}\) ions through voltage-dependent Ca\(^{2+}\) channels. This inward Ca\(^{2+}\) current triggers the release of Ca\(^{2+}\) ions from the sarcoplasmic reticulum. For an in-depth discussion of EC coupling, see reviews by Bers\(^{48}\) or Satin et al. in this supplement. Once released, intracellular Ca\(^{2+}\) binds to troponin C of the myofilament, triggering contraction of the sarcomere.

Sarcomeres are the repeating structures that comprise the contractile apparatus within cardiomyocytes (Fig. 2). Sarcomeres start and end with dense protein aggregations known as Z-disk, which anchor each sarcomere's hexagonal lattice of actin thin filaments. The thick filaments of the sarcomere, composed primarily of the motor protein myosin, interdigitate with thin filaments to allow actin–myosin cross-bridge formation. Thin and thick filaments together contain the necessary molecular machinery to produce and regulate contractile force, and are often collectively referred to as the myofilament system.

Many of the myofilaments' constituent proteins exist as distinct isoforms, arising either from distinct genes or alternatively spliced transcripts. In the following subsections, we consider myofilament proteins that experience substantial shifts in isoform expression during development (Fig. 3). For each, we examine these shifts in the context of their role in modulating myofilament function and cardiac twitch dynamics.

**Myosin heavy chain.** Myosin, the protein that couples ATPase activity with mechanical work, is expressed in two myosin heavy chain (MHC) isoforms, \(\alpha\) and \(\beta\). These two isoforms have 93% of their amino acid sequence in common and are functionally similar in general terms.\(^{68}\) \(\alpha\)- and \(\beta\)-MHCs are encoded by two distinct genes \(MYH6\) and \(MYH7\), respectively.\(^{69}\)

The expression of cardiac MHC isoforms changes during development in a species-dependent manner. The ratio of \(\alpha\)- to \(\beta\)-MHC expression is also responsive to perturbations in the environment in the form of cardiovascular stress or certain hormones.\(^{70,71}\) In rodent hearts, both genes are co-expressed in the ventricle at birth, with the \(\beta\) expression being dominant.\(^{72}\) During fetal development, \(\beta\)-MHC transcription decreases and is replaced by \(\alpha\)-MHC,\(^{72,73}\) which ultimately leads to an adult ventricular myocardium that expresses mostly the \(\alpha\) isoform (\(\sim 90\%\)).\(^{74}\) Rodents experience a postnatal surge in thyroid hormone, which is known to induce a switch from \(\beta\)- to \(\alpha\)-MHC.\(^{72,75}\) Addition of T3 into culture media is now a tool used to promote the developmental switch in tissue-cultured cardiomyocytes.\(^{55,76,77}\)

Figure 2. Schematic of the sarcomere and its constituent proteins. Adapted from: Campbell SG, McCulloch AD. Multi-scale computational models of familial hypertrophic cardiomyopathy: genotype to phenotype. *J R Soc Interface*. 2011;8:1550–1561.

**Abbreviations:** cMyBP-C, cardiac myosin binding protein C; Tm, tropomyosin; TnT, troponin T; TnI, troponin I; TnC, troponin C; MHC, myosin heavy chain; MLC 1/2, myosin light chain 1/2.
The atria both rodents and humans mainly express the α isoform (90%–100% of α-MHC in human), which remains constant under normal conditions. In heart failure, the expression decreases to 50%–55%. Nonfailing adult human ventricular myocardium expresses mainly β-MHC with a low (0%–15%) but detectable α-MHC content. Reiser et al. found that fetal ventricular tissue at gestational week 12 already expressed the adult level of α-MHC. The amount of α-MHC in samples from patients with heart failure or hypertrophy was reduced further to 0%–4%. These small shifts can have a profound influence on contractile function, underscoring their role in cardiac development and the cardiac response to different hemodynamic perturbations.

The MHC isoforms are known to have different ATPase activities, actin sliding velocities, and power output. More specifically, α-MHC, the faster of the two, has several-fold higher myofibrillar actomyosin-activated ATPase activity. This means that this isoform sacrifices economy of tension for the ability to contract at greater speed. It was also found that myocytes expressing α-MHC are able to develop higher twitch tensions and a significantly higher power output. The relationship between power output and α-MHC content has been found to be linear in rat skinned myocytes. Finally, α-MHC exhibits a higher sarcomere length dependence of the absolute loaded shortening velocity and power output. On the other hand, the lower velocity of β-MHC allows it to be more energy efficient, potentially making it physiologically favorable in larger mammals such as humans.

MHC isoform expression is not only of interest due to functional differences but also because they interact differently with other sarcomeric proteins. In the case of cardiac myosin binding protein C (cMyBP-C), its PKA-mediated phosphorylation increases ATPase activity of α-MHC but not β-MHC systems. Furthermore, Mamidi et al. have shown that α- and β-MHC have different effects on how cardiac contractility dynamics get regulated by cTnT. It is therefore of importance to know the relative isoform expressions of MHC when modeling cardiac diseases using stem cell-derived cardiomyocytes. Especially in the case of HCM, a "disease of the sarcomere," this can be significant. The arginine to glutamine mutation at amino acid 403 (termed R403Q) is a missense mutation in MHC that is known to cause familial HCM.

| Protein name                        | Abbreviation | Human gene | Approximate expression level during maturation |
|-------------------------------------|--------------|------------|-----------------------------------------------|
| α Myosin heavy chain                | α-MHC        | MYH6       | Rodent → Neonatal → Adult                     |
| β Myosin heavy chain                | β-MHC        | MYH7       | Human                                         |
| Ventricular myosin regulatory light chain | MLC2v       | MYL2       |                                               |
| Atrial myosin regulatory light chain | MLC2a        | MYL7       |                                               |
| Cardiac troponin T (splice variant 1) | cTnT 1      | TNNT2      |                                               |
| Cardiac troponin T (splice variant 2) | cTnT 2      | TNNT2      |                                               |
| Cardiac troponin T (splice variant 3) | cTnT 3      | TNNT2      |                                               |
| Cardiac troponin T (splice variant 4) | cTnT 4      | TNNT2      |                                               |
| Cardiac troponin I                   | cTnI         | TNN13      |                                               |
| Slow skeletal troponin I             | sTnI         | TNN11      |                                               |
| Titin (large/elastic splice variant) | N2BA         | TTN        |                                               |
| Titin (small/elastic splice variant) | N2B          | TTN        |                                               |

*Figure 3.* Adapted diagram from Marston illustrating the changes in isoform composition of the thick and thin filament during development (fetal to adult) in rodent (red) and human (blue) left ventricular tissue. Relevant sources are cited in the text.
et al.\textsuperscript{2} applied the same mutation to an $\alpha$- as well as $\beta$-MHC backbone and found opposing functional consequences. The R403Q mutation in an $\alpha$-backbone showed faster actin filament velocity as well as increased actin-activated ATPase activity, whereas the same mutation in a $\beta$-backbone showed decreased actin filament velocity and actin-activated ATPase activity.

Mutations in cTnT at residue R92 had different effects depending on the presence of $\alpha$- and $\beta$-MHC. Only in the presence of $\beta$-MHC was there a significant decrease in the Frank–Starling response and increase in the responsiveness of cardiac myofilaments to Ca\textsuperscript{2+} ions at a longer sarcomere length.\textsuperscript{93} Furthermore, rates of crossbridge cycling were faster in a $\beta$-MHC background but unaffected on a background of $\alpha$-MHC. It is therefore of great importance to quantify the presence of isoforms to account for functional differences due to immature isoform expressions when investigating functional impacts of cardiac diseases.

Determining the ratio of MHC isoforms in cultured hESC/hiPSC-CMs and EHTs seeded with such cells has so far been limited to the probing of relative MYH6 and MYH7 transcript abundance.\textsuperscript{10,21,15,55,56,94} With their cardiac patch, Zhang et al.\textsuperscript{50} achieved a $\beta$-MHC/$\alpha$-MHC ratio of about 3:5 in comparison to an approximate ratio of 9:1 in adult human tissue.\textsuperscript{80–82} Their construct hence expresses predominantly the fast MHC isoform. Turnbull et al. express an even lower $\beta$-MHC/$\alpha$-MHC ratio, which might explain why their twitch kinetics are faster than observed in adult human tissue.\textsuperscript{11} Increasing culture period might be one possible way of encouraging a more complete switch from $\alpha$-MHC to $\beta$-MHC. Culturing iPSC- and hESC-derived cardiomyocytes for about 80–120 days led to expression levels of both MHC isoforms that almost matched adult left ventricular tissue, and coincided with a significant slowing of contraction kinetics.\textsuperscript{56}

However, results based on transcript abundance must be interpreted with caution, since MHC transcripts and expressed proteins do not always correlate. Human ventricles that have undergone pathological hypertrophy show an increase in gene expression of the fetal TnT isoform.\textsuperscript{92,94} Expression exerts meaningful physiological effects on contraction, and hence specific attention to its exact composition in iPSC-CMs seems warranted.

During development of the rodent heart, solo expression of either MLC2v or MLC2a delineates ventricular and atrial anatomy.\textsuperscript{101} Accordingly, MLC2v has been successfully targeted as a means for enriching populations of iPSC-CMs for ventricular phenotypes. Bizy et al. used viral transfection of iPSC-CMs with the green fluorescent protein (GFP) driven by MLC2v-specific promoter to isolate a subpopulation of cells, which ultimately exhibited longer action potential durations than cells sorted using an MLC2a promoter.\textsuperscript{102} Although this concept has never been directly tested, these studies suggest that the expression of ventricular light chain over the atrial isoform occurs early in development and is strongly associated with a ventricular action potential morphology. Hence, current techniques aimed at enhancing ventricular cardiomyocyte differentiation (most commonly characterized by action potential morphology) seem likely to simultaneously select cells that express MLC2v.

MLC2v contains phosphorylatable serine residues targeted by myosin light chain kinase (MLK). Phosphorylation of MLC2v increases the step size of myosin\textsuperscript{103} which ultimately increases the contraction force and duration of the cardiac twitch.\textsuperscript{104} Work conducted in rat cardiac muscle suggests that MLC2v phosphorylation is a key component of the positive force–frequency relationship.\textsuperscript{105} It is interesting to note that the force–frequency relation in EHTs has been reported as being flat or negative in published work to date.\textsuperscript{10,11} The pathway responsible for mediating the frequency-dependent phosphorylation of MLC2v may therefore be absent or otherwise deficient in neonatal cardiac cells and hiPSC-CMs. Verifying that cardiac MLK\textsuperscript{106,107} is expressed in hiPSC-CMs would be an important initial step in this process.

**Troponin T.** TnT binds to tropomyosin as well as the other troponin subunits, effectively anchoring the troponin complex to the thin filament and buttressing end-to-end overlap of adjacent tropomyosin molecules.\textsuperscript{108} Cardiac troponin T (cTnT) is expressed as several different isoforms that arise from alternative splicing of the TNNT2 gene. Rats show co-expression of cTnT2 and cTnT4 at birth, with cTnT2 getting entirely replaced by cTnT4 during development.\textsuperscript{109,110} In humans, the isoforms cTnT1 and cTnT3 are both expressed in the fetal heart, but the cTnT3 isoform predominates expression in the adult myocardium (Fig. 3).\textsuperscript{111} Evidence suggests that TnT isoforms are capable of modulating cardiac contraction to some degree. For instance, Nassar et al. examined the Ca\textsuperscript{2+} sensitivity of force in ventricular myocardium from neonatal rabbits and found that sensitivity increased with greater expression of the fetal TnT isoform.\textsuperscript{112} Although the precise impact of fetal TnT expression on cardiac twitch dynamics...
has not been specifically examined, enhanced Ca\textsuperscript{2+} sensitivity would tend to cause slower relaxation in the intact heart.

**Tropinin I.** Tropinin I (TnI) gets its name from its ability to inhibit myosin ATPase activity in vitro.\textsuperscript{113} TnI exerts its regulatory activity through interactions with actin and troponin C (TnC). The inhibitory region of the protein binds to actin under low-Ca\textsuperscript{2+} conditions, preventing movement of tropomyosin to expose myosin binding sites on actin. The N-terminal domain of TnC binds the switch region of TnI in the presence of Ca\textsuperscript{2+}, and this event facilitates dissociation of the inhibitory region from actin (reviewed in detail elsewhere).\textsuperscript{114,115}

Slow skeletal TnI (ssTnI, human gene TNNI3) is expressed in the fetal heart, but expression switches entirely to the cardiac isoform (cTnI, human gene TNNI2) in the months following birth (Fig. 3).\textsuperscript{110,116} This isoform switch is of great physiological significance, because of an N-terminal extension present in cTnI (N-cTnI). Under normal conditions, N-cTnI interacts with TnC to stabilize Ca\textsuperscript{2+} binding, but phosphorylation of N-cTnI at two serine residues by protein kinase A appears to reduce N-cTnI/TnC interactions and subsequently Ca\textsuperscript{2+} binding affinity of the myofilaments.\textsuperscript{117} Hence, cTnI allows the Ca\textsuperscript{2+} responsiveness of the myofilaments to be tuned through adrenergic signaling\textsuperscript{118} and indirectly through the heart rate.\textsuperscript{105} Transgenic expression of ssTnI in mouse hearts resulted in altered twitch characteristics, including slower relaxation and an absence of accelerated relaxation following treatment with isoprenaline.\textsuperscript{118} TnI has also been shown to contribute to the length dependence of myofilament contractile force in an isoform-dependent manner.\textsuperscript{119,120}

These studies of TnI isoform effects suggest that measurements of twitch dynamics in artificially differentiated iPSC-CMs under adrenergic stimulation and at various pacing frequencies are important metrics of functional maturation. Initial attempts to characterize the cTnI to ssTnI protein isoform ratio in iPSC-CM showed a fetal TNNI signature even under long-term culture with only limited amounts of cTnI protein even after 9.5 months of culture.\textsuperscript{121}

**Titin.** Titin (TTN) is a long, spring-like protein that runs from the z-disk to the m-line in sarcomeres. TTN plays essential roles in sarcomere formation, passive cardiomyocyte stiffness, mechanical signaling, and active muscle contraction.\textsuperscript{122} When expressed, the titin gene is alternatively spliced to form shorter (N2B) or longer (N2BA) isoforms, and these are developmentally regulated. The longer and more compliant N2BA isoform predominates fetal expression but quickly declines in favor of the stiffer N2B isoform immediately after birth (Fig. 3),\textsuperscript{123–125} leading to a passive resting tension increase.\textsuperscript{126} The ratio of N2BA to N2B protein expression in adult human left ventricular tissue is around 0.5.\textsuperscript{127}

TTN isoform switching has not been directly examined in human ESC- or iPSC-derived myocardium, but work with neonatal rat cardiomyocyte cultures suggests that the expression of N2B increases with time in culture.\textsuperscript{128} Furthermore, transition from fetal to mature TTN is promoted by thyroid hormone\textsuperscript{129} and insulin.\textsuperscript{129} While TTN is perhaps most often thought of in its role as a determinant of passive myocardial stiffness, evidence suggests that it is also a key component of length-dependent activation.\textsuperscript{130} Fukuda et al.\textsuperscript{130} reported that myocardium expressing the adult (N2B) TTN isoform was more sensitive to length than that expressing primarily N2BA TTN. Hence, incomplete transition from N2BA to N2B TTN isoform expression could manifest functionally as a blunted Frank–Starling response in stem cell-derived cardiomyocytes.

**Computational Modeling to Analyze Cardiomyocyte Maturation**

Both the approaches for gauging contractile maturation discussed here have important benefits and limitations. Twitch kinetics can describe the overall character of a complex gene expression milieu with just a few functional measurements. This approach also keeps the focus on reproducing physiological function, which, it could be argued, is a more practical objective than precise recapitulation of the entire adult myofilament expression profile. On the other hand, when twitches do not exhibit mature phenotypes (Fig. 1C–E), pinpointing the underlying molecular cause is difficult. The effects of a given protein isoform shift or post-translational modification are not necessarily confined to a single functional attribute such as relaxation rate. For instance, β-adrenergic responsiveness, the force–frequency relationship, twitch relaxation, and other contractile properties can all be affected by TnI isoform expression (see Solaro et al for review\textsuperscript{131}).

Assessing hESC/hiPSC-CM maturation using myofilament gene and protein expression data is perhaps underutilized and would certainly lend great insight into the remaining deficits in maturation. However, a comprehensive study of expression under each of the many differentiation and maturation protocols now in use is probably unrealistic given the scale of such an undertaking.

Another major complicating factor is that the intracellular Ca\textsuperscript{2+} transients driving muscle contraction also show maturation deficits in ESC/iPSC-derived cardiomyocytes (see the review by Satin et al in this supplement). Without some form of quantitative analysis, it is difficult to know whether immature twitch behavior is due to an abnormal input signal (Ca\textsuperscript{2+} transient), expression of fetal myofilament isoforms, or a combination of the two.

In our own work, we have developed computational tools to predict the isometric twitch time course in response to a Ca\textsuperscript{2+} transient input (Fig. 4B). Model parameters reflect the biophysical and biochemical properties of myofilament proteins, which can include the effects of different protein isoforms\textsuperscript{132} and post-translational modifications such as phos-
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In this way, we can account quantitatively for twitch modifications, separating Ca\textsuperscript{2+} transient effects from those originating in the myofilaments.

The utility of this approach may be seen in our recent study comparing adult cardiomyocytes isolated from wild-type and Pkd\textsuperscript{2+/-} mouse strains. Simultaneous recordings of Ca\textsuperscript{2+} transients and unloaded sarcomere shortening in these cells revealed a significant increase in the magnitude and duration of the intracellular Ca\textsuperscript{2+} transient in Pkd\textsuperscript{2+/-} cells compared with wild type, but no difference in their twitch dynamics. Although these data directly indicate the existence of some differences in the myofilament composition between wild-type and Pkd\textsuperscript{2+/-} cells, they do not reveal their identity. Using the model, we first determined a parameter set that would reproduce the wild-type twitch profile in response to an average wild-type Ca\textsuperscript{2+} transient (Fig. 4 A and B). Keeping this parameter set, we switch the input signal to that of an average Pkd\textsuperscript{2+/-} Ca\textsuperscript{2+} transient. As expected, this yielded a simulated twitch response that differed substantially from wild type. Given that no such twitch differences were observed experimentally, the conclusion that the myofilaments themselves must be altered was further reinforced. Numerical experiments allowed us to seek the identity of this myofilament alteration. We found that assuming a lower Ca\textsuperscript{2+} affinity of the troponin complex in Pkd\textsuperscript{2+/-} cardiomyocytes caused the twitch dynamics to be identical in the two strains (Fig. 4C). The well-known relationship between troponin Ca\textsuperscript{2+} affinity and cTnI phosphorylation at serine residues 22/23 led us to suspect elevated cTnI phosphorylation in Pkd\textsuperscript{2+/-} hearts. Western blots subsequently confirmed this hypothesis (Fig. 4D).

Given the complex interplay between myofilament protein isoforms, phosphorylation sites, and the Ca\textsuperscript{2+} transient in determining twitch dynamics, we consider the use of computational analysis to be an important emerging tool in evaluating in vitro maturation of cardiac cells and in determining specific gene-level deficiencies in competing approaches.

**Markers of maturation**

1. **Functional markers of maturation**
   - Maximum peak tension
   - Positive force-frequency behavior
   - Decreasing twitch duration and kinetics (RT 50 and TTP) with increasing pacing frequency
   - Frank-starling behavior

2. **Biomolecular markers of maturation**
   - β-MHC content 95% of total MHC
   - 100% expression of cardiac Tnl isoform
   - Dominant expression of cardiac TnT splice variant 3
   - Titin isoforms N2BA and N2B are expressed 1:1
Recommendations for Twitch Phenotyping to Assess Functional Myocardial Maturation

In our view, the challenge of measuring and enhancing myofilament maturation in hESC/hiPSC-CMs could be greatly improved by the adoption of some basic standards. In particular, twitch phenotyping should be done in a uniform and consistent manner (Summarized in Table 1). To facilitate comparison, experimental conditions and analysis metrics should be chosen to match existing human data. Muscles should be tested in 37 °C tyrode’s solution containing 1.8 mM CaCl₂. Pacing rates should be selected to include at a minimum the three rates used in the most comprehensive data sets, namely 0.5, 1, and 2.5 Hz.

When determining which scalar properties to use in describing twitch characteristics, it is certainly desirable to compute the peak twitch tension, but twitch kinetic parameters such as TTP and relaxation rates contain a wealth of important information. An important opportunity can also be missed when twitch kinetics are not analyzed as a function of pacing frequency. This is due to the fact that mature myocardium achieves steady-state force production upon changes in stimulation frequency in two stages: a rapid phase, characterized by immediate changes in twitch force and Ca²⁺ transient amplitude; and a delayed phase in which force changes independently of the Ca²⁺ signal. Interestingly, in the second/late phase, twitch timing kinetics (such as relaxation and TTP) achieve steady-state values before peak tension itself.

The subtlety inherent in these different phases is not currently detected in typical EHT test protocols. We therefore recommend that twitch relaxation rates as well as peak tension be examined in a time-resolved fashion when testing the force–frequency relationship in human EHT preparations. These same quantities ought to be examined during the response of EHTs to adrenergic stimulation, since decreased twitching patterns, and cell morphology sometimes used to assess maturation, owing to the transition from slow skeletal TnI to cardiac TnI. In order to detect such an effect, it would be necessary to compute the normalized Frank–Starling gain: the change in active tension between the two muscle lengths divided by the passive tension change. A negative Frank–Starling response would likely indicate immature cells, and is also observed in failing myocardium.

Finally, harnessing the power of computational modeling to perform mechanistic analyses of twitch dynamics requires the measurement of calcium transients. Stoehr et al have recently illustrated the ability of an automated system to measure calcium transients using the ratiometric fluorescent dye Fura-2.

Conclusions

Even though the field has made great progress in producing human stem cell–derived myocardium, the available data suggest that the goal of physiologically mature tissue has yet to be attained. Furthermore, the standards by which we assess maturity are still evolving and will require careful thought. The required markers needed to assess the maturation stage of iPSC-CMs are still a topic of debate in the stem cells field. This is highlighted by the fact that isolated adult cardiomyocytes start reexpressing fetal protein isoforms when cultured in vitro. Furthermore, external stresses can alter physiological markers such as calcium handling, beating patterns, and cell morphology sometimes used to assess maturity. Hence, a mix of quantitative molecular and functional markers next to the usually used structural markers is needed (See box on page 9). In light of these facts, we feel that caution is required when interpreting disease models that are based on hESC/hiPSC-CMs. Understanding the influence of individual sarcomeric protein isoforms on muscle function should figure prominently in such analyses, especially when the disease in question involves a sarcomeric mutation.

As of now, only a few protein isoform expression profiles have been assessed in hESC/hiPSC-CMs. Studies of cardiac development suggest that isoform profiles are powerful markers of myocardial maturation, and may represent an untapped resource. Given the work that remains before mature stem cell–derived myocardium can be produced, we strongly endorse routine measurement of these critical myocellular components. At the same time, twitch characteristics of engineered myocardial preparations can provide valuable insights into functional maturity.
especially when protocols and analysis methods are carefully selected. Finally, in cases where maturation is shown to be incomplete, either through functional or biochemical means, computational analysis can be used to elucidate potential molecular-level remedies.

Even though this commentary has focused on some of the shortcomings of human EHTs as a platform for disease modeling, we feel that these limitations can be satisfactorily addressed in the short term through properly selected controls and computational analysis. Furthermore, we are optimistic that in the long run, maturation in these systems will be improved such that the bulk of adult myocardial phenotypes are reproduced.

Author Contributions
JS and SGC wrote the manuscript. Both authors reviewed and approved of the final manuscript.

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