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**Title:** From fetus towards adult: maturation and functional analysis of pluripotent stem cell-derived cardiomyocytes  
**Issue Date:** 2016-10-13
Chapter 4

Contractile defect caused by mutation in MYBPC3 revealed under conditions optimized for human PSC-cardiomyocyte function

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Adapted from Cell reports 2015, 13: 1–13
Abstract

Maximizing baseline function of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) is essential for their effective application in models of cardiac toxicity and disease. Here, we aimed to identify factors that would promote an adequate level of function to permit robust single cell contractility measurements in an induced PSC (hiPSC) model of hypertrophic cardiomyopathy (HCM). A simple screen revealed the collaborative effects of thyroid hormone, IGF-1 and the glucocorticoid analog dexamethasone on the electrophysiology, bioenergetics and contractile force generation of hPSC-CMs. In this optimized condition, hiPSC-CMs with mutations in MYBPC3, a gene encoding myosin-binding protein C and causing HCM, showed significantly lower contractile force generation than controls. This was recapitulated by direct knockdown of MYBPC3 in control hPSC-CMs, supporting a mechanism of haploinsufficiency. Modelling this disease in vitro using human cells is an important step towards identifying therapeutic interventions for HCM.
Introduction

Human pluripotent stem cells (hPSCs) are becoming increasingly used in biomedical research. Their extensive growth and differentiation potential enables routine production of cell types otherwise difficult to obtain. Cardiomyocytes are an important example, since collecting biopsies from the heart is highly invasive yet the cells they contain are those most pertinent to heart disease and drug-associated toxicity. Although many reports have now demonstrated the utility of hPSC-derived cardiomyocytes (hPSC-CMs) for modelling heart disease [1–4], the baseline functional performance of these cells is still less than that in adult heart tissue. Low maximum diastolic potential (MDP) and slow action potential upstroke velocities are frequently observed across all cardiomyocyte subtypes [5–7], coupled with a low force of contraction [8,9].

The poor functional output of hPSC-CMs is in large part due to their developmental immaturity [6]. While efforts are ongoing to advance the developmental state beyond their fetal equivalents, another important and valid approach is to also consider ways to maximise cell function more immediately, which might not necessarily be coupled to or regulated in the same way as developmental maturation. This might be achieved, for example, by improving aspects of cell metabolism and bioenergetics. Cardiomyocytes maintained outside their native environment within the heart may be disconnected from many factors important for their basic physiology, including relevant growth factor and hormone signalling, extracellular matrix proteins, other cell populations in the heart and mechanical- and electrical-stimulation. As protocols for hPSC-CM generation have improved and the generation of pure populations of cardiomyocytes in serum-free media well established, this separation becomes even more notable. Many physiological factors that would normally be circulating in the heart are absent under most basal culture conditions and it is unclear which should be provided in a defined formulation to promote optimal cardiomyocyte function in bioassay development.

The MDP is a fundamental determinant of excitability, and in cardiomyocytes from adult “working myocardium” is around -85 mV [10]. The lack of I_{K1} current, due to low expression of the KCNJ2 gene, is one reason that ventricular- and atrial-like cardiomyocytes from hPSCs are insufficiently polarised [5,11,12]. However, many other factors affect the resting membrane potential. These include the activity of the energy demanding Na,K-ATPase, and potassium conductance as determined by other channels [13,14], which may also not be appropriately expressed or function optimally. The MDP is a strong predictor of contractile output in hPSC-CMs [9], and so improving MDP is an important prerequisite for faithfully modelling diseases affecting excitability or contractility.

One disease predicted to affect cardiomyocyte contractility is hypertrophic cardiomyopathy (HCM). HCM is highly prevalent, affecting 1:500 of the population and can be caused by mutations in various sarcomeric protein encoding genes [15]. Approximately 70% of cases are the result of mutations either in MYH7, encoding β-myosin heavy chain, or in MYBPC3, encoding cardiac myosin-binding protein C (cMyBP-C). As a disease with known genetic cause but ill-defined molecular pathogenesis, HCM is an excellent candidate for stem
cell modelling [16]. The absence of a cure or even an effective treatment strategy provides great urgency for in vitro human models to identify drugs able to restore contractile function and prevent the development of hypertrophy, cardiac remodelling and consequential arrhythmias. Induced pluripotent stem cells (hiPSCs) have already been generated from patients with mutations in MYH7 and MYBPC3, and cardiomyocytes derived from them [17–19], but their force generation, measured on physiologically-relevant substrate dimensions and stiffness, has not yet been described, and is currently hindered by poor baseline performance as noted above.

In this study, we began with a well characterised NKX2-5eGFP/w human embryonic stem cell (hESC) line, in which working cardiomyocytes are specifically marked by eGFP expression [20,21], to identify factors or combinations of factors which promoted an increase in their resting plasma membrane potential, or that had an anabolic effect, making the assumption that such factors could have functional benefit. We then tested these factors at the level of single cell traction force and action potential generation. Optimized conditions were then applied to an hiPSC model of MYBPC3 insufficiency and this revealed a contractile defect in cardiomyocytes derived from MYBPC3 mutation carriers.

Results

The identification of factors modulating hiPSC-derived cardiomyocyte plasma membrane potential and size

Populations of cardiomyocytes were generated by monolayer differentiation of NKX2-5eGFP/w hESCs and these were maintained until day 16. To screen for modulators of resting plasma membrane potential (ΔΨp), we assessed short-term accumulation of the cationic fluorescent probe tetramethylrhodamine methyl ester (TMRM) in cell suspensions. TMRM follows Nernstian behaviour across cell membranes, the early phase of TMRM accumulation into cells is dominated by the ΔΨp, while with longer loading times the mitochondrial membrane potential and matrix volume contribute increasingly to total accumulation [22]. Time-course measurement of TMRM accumulation was performed from control conditions (Figure 1a and b). Based on this, 8 mins loading time was chosen for relative assessments of ΔΨp, since this was in the linear phase of accumulation but gave signal substantially above background. Additionally, total cellular eGFP fluorescence was used as an estimate of cell size/volume, on the assumption that eGFP levels should correlate with total cell protein. There are several caveats to this reasoning, but modulators can be subsequently validated with more direct assays. In support of a general correlation, fetal calf serum exposure, known to increase the size of these cells [23], increased eGFP levels after 5 days of exposure (Fig. S1).

The following factors, each with putative roles in cardiomyocyte function, were tested: insulin-like growth factor 1 (IGF-1), the hedgehog signalling agonist SAG, the synthetic glucocorticoid dexamethasone (Dex), triiodothyronine hormone (T3), the α-adrenergic agonist phenylephrine (PE) and the β-adrenergic agonist isoproterenol (ISO) [24–29]. Testing was performed in a serum-free, low-insulin medium that has become a widely used standard for
Figure 1- Using TMRM to identify modifiers of ΔΨp in hESC-derived NKX2-5+ cardiomyocytes. A. Time-course measurement of TMRM accumulation after cardiac differentiation of NKX2-5eGFP/w hESCs. FACS plots show eGFP and TMRM fluorescence after 0, 4, 8, 13 and 22 mins of loading with TMRM. A minor cross-bleed correction has been applied to all. B. Median TMRM fluorescence intensity in eGFP+ cells plotted against loading time. C. eGFP and TMRM fluorescence values in eGFP+ cardiomyocytes relative to a vehicle-only control after 5 days of incubation with the factors shown (n=6-34). D. eGFP and TMRM fluorescence values in eGFP+ cardiomyocytes relative to a T3-treated control after 5 days of incubation with the factors shown (n=10-22). E. Upper panel: example FACS
maintenance of these cells [30]. Contracting monolayers of differentiated cells (day 16) were maintained for 5 days in each test condition before measurement (Figure 1c). IGF-1 was the only factor to significantly increase eGFP fluorescence intensity, and TMRM accumulation increased only proportionally in this condition. IGF-1 may therefore mildly increase cell volume without affecting ΔΨp. T3 was the only factor that significantly increased TMRM accumulation, while eGFP fluorescence remained unaffected. This indicated an increase in ΔΨp by T3, but not in cell volume. While ISO mildly increased TMRM accumulation, neither ISO nor PE induced an increase in eGFP fluorescence; inconsistent with their established role as hypertrophic agonists in other cardiomyocyte systems but consistent with a reported variable response in some hPSC-CM lines [31–33]. Dex had little effect on either eGFP or TMRM, consistent with unpublished work from our group showing a lack of effect by Dex-alone on cardiomyocyte electrophysiology in hPSC-derived cardiomyocytes (Kosmidis et al. unpublished results), but inconsistent with its previously described role in cardiomyocyte maturation [28,34], suggesting that competence factors may be lacking.

These results support a role for T3 in determining ΔΨp in hPSC-derived cardiomyocytes, but not for maturation-related growth. To explore the possibility of enhancing this condition further, we tested the effect of additional IGF-1 or Dex compared to T3-alone (assigned as the new reference control and normalized to 1) (Figure 1d). The addition of IGF-1 again mildly increased eGFP levels over T3-alone, while T3+Dex mildly, but significantly increased TMRM accumulation. However, the combination of T3+IGF-1+Dex (TID) increased both eGFP and TMRM significantly, suggesting an enhancement in both size and possibly ΔΨp (Figure 1e), changes which could be associated with further improved cardiomyocyte function. The increased eGFP fluorescence with T3+IGF-1 and TID was not associated with, and therefore explained by, a relative increase in eGFP mRNA expression in these cells (Fig. S2). Upregulation (~4-fold) of the glucocorticoid-response gene FKBP5 confirmed increased glucocorticoid signalling by Dex (Fig. S2). The action of TID on eGFP levels and TMRM accumulation was confirmed in cardiomyocytes from an independent NKX2-5eGF-P/w knock-in reporter M1 hESC line as well as an NKX2-5eGF-P/w knock-in reporter hiPSC line (Fig. S1).

As insulin was present in the basal medium at 1 µg/ml for all these assays and at these concentrations can cross-react with the IGF-1 receptor, we separately tested the impact of insulin and IGF-1 in this T3+Dex condition. Addition of 1 µg/ml insulin-alone to the medium containing T3+Dex significantly increased eGFP fluorescence, but addition of 100 ng/ml IGF-1 increased eGFP fluorescence further and also increased TMRM accumulation (Fig. S1). This suggests that activation of the IGF receptor is the primary mediator of this response.
Importantly, in the presence of TID, cardiomyocytes responded appropriately to stimulation by the adrenergic agonists: norepinephrine (NE), PE or ISO, with an increase in eGFP fluorescence, suggesting that a baseline level of bioenergetic- or anabolic-activity may be important for this hypertrophic response (Fig. 1f). Relative eGFP mRNA expression was not significantly affected by these factors (Fig. S2). The action of adrenergic agonists did not, however, increase TMRM accumulation beyond the increase in eGFP intensity, suggesting increased TMRM was probably due to increased cell volume and not due to a further improvement in ΔΨp by these factors.

Bioenergetic response in cardiomyocytes by the concerted action of T3, IGF-1 and dexamethasone

The response to IGF-1 and Dex in terms of cardiomyocyte growth and ΔΨp suggests a possible synergistic role in metabolic stimulation. To test this, bioenergetic profiling of contracting cardiomyocyte monolayers was performed using the Seahorse XF24 Analyzer on cells treated during the same experimental time-course. Contraction rates, measured prior to analysis, were not significantly different between groups (Figure 2a). Under standard conditions (15 mM glucose, 0.5 mM sodium pyruvate) normalized to total cell protein, T3 mildly increased the oligomycin-sensitive respiration rate as previously reported (Yang et al., 2014), indicating increased mitochondrial ATP turnover (Figure 2b and d). Neither IGF-1 nor Dex stimulated this further, but the combination of TID did have a further stimulatory effect (TID: 213±4 vs Vehicle: 124±6 pmoles O2/min/10 µg cell protein; p<0.05). The anaerobic glycolytic rate (calculated as previously described by Mookerjee et al., 2015) was also significantly increased by TID (TID: 68±4 vs Vehicle: 34±4 pmoles H+/min/10 µg cell protein; p<0.05) (Figure 2c). A conversion of these values to ATP production rates (see Experimental procedures) showed a large increase (1.7-fold) in combined ATP turnover with TID (Figure 2d). The oligomycin-inhibited rates, the maximum uncoupled respiration rates induced by FCCP, and the non-mitochondrial rates, were not significantly different between conditions (Figure 2b). These results support a synergistic effect for the actions of IGF-1 and Dex on basal cell activity. Inhibition of both excitation and contraction by co-injection of nifedipine and blebbistatin decreased the mitochondrial respiration rate by 39.3±4.2% in the vehicle-only condition and 43.9±9.0% in TID (Figure 1e) after subtraction of a minor buffer-only injection effect. As the starting respiration rate was higher with TID this showed that the activity of these processes had increased at least proportionally by TID.

Sensitivity to substrate supply was assessed by examining the change in basal respiration rate and respiratory capacity on inhibition of either long-chain fatty acid uptake into mitochondria by the carnitine palmitoyl transferase inhibitor etomoxir (40 µM) or inhibition of mitochondrial pyruvate uptake by inhibition of the pyruvate transporter with UK5099 (5 µM). These experiments were performed in a more physiological medium containing 5.5 mM glucose, 0.15 mM sodium pyruvate and 100 µM palmitate. Figure 2f and 2g show typical respiratory responses to etomoxir and UK5099 of cells from vehicle-only medium and TID-medium respectively. Figure 2h shows quantification of the sensitivity of basal and FCCP-stimulated mitochondrial respiration rates to these inhibitors. Basal respiration in cells from vehicle-only medium and TID-medium was inhibited 21.0±4.3% and 24.4±4.9%
respectively by etomoxir, and 27.7±4.2% and 31.9±3.3% respectively by UK5099. The basal respiration rate was again higher in TID-treated cells. FCCP-stimulated respiration in cells from vehicle-only medium and TID-medium was inhibited 18.5±2.1% and 20.2±2.2% respectively by etomoxir, and 27.7±4.2% and 31.9±3.3% respectively by UK5099. The basal respiration rate was again higher in TID-treated cells. FCCP-stimulated respiration in cells from vehicle-only medium and TID-medium was inhibited 18.5±2.1% and 20.2±2.2% respectively by etomoxir, and 27.7±4.2% and 31.9±3.3% respectively by UK5099. The basal respiration rate was again higher in TID-treated cells.
respectively by etomoxir, and 53.8±5.5% and 47.0±2.9% respectively by UK5099. Overall, these data suggest that TID increases the basal utilization of both glucose and fatty acids in cardiomyocytes in concert with increased ATP demand for excitation/contraction as well as other processes. In line with these changes, TID increased expression of PGC-1α and PGC-1β (Fig. S2), important regulators of myocardial FAO and mitochondrial function (Birket et al., 2013; Finck and Kelly, 2007). We additionally found that increased bioenergetic activity by TID exposure was associated with a decreased rate of dihydroethidium (DHE) oxidation, suggesting a decreased level of reactive oxygen species (Fig. S3), which may also support improved cardiomyocyte function.

**Electrophysiological and contractile improvements by the concerted action of TID**

To test whether the bioenergetic changes and improved resting ΔΨp resulted in improvements in electrophysiological function, action potentials were measured in spontaneously active cells. To focus on a possible effect of glucocorticoid signalling we measured cells maintained in three conditions: vehicle-only, T3+IGF-1 and TID. The results are shown in Figure 3 and summarized in Table 1. The MDP was increased by both T3+IGF-1 and TID (Figure 3b), aligning well with our estimates by TMRM uptake. The amplitude of the AP was also increased progressively by both treatments (Figure 3c). Interestingly, while the upstroke velocity was not significantly increased by T3+IGF-1 it was markedly increased by TID, from 16±5 V/s to 59±6 V/s (Figure 3d; p<0.05). This specific effect was significantly blocked by 6 µM GSK650394, an inhibitor of the serum- and glucocorticoid inducible kinase SGK1 [35] which was previously shown to block cardiac sodium channel degradation, and which is activated by IGF-1 [36]. We confirmed SGK1 upregulation in response to Dex (Figure 3g). SCN5A expression was not increased by dexamethasone (Fig. S2), and the inhibitor did not significantly affect any other aspect of the AP, together supporting this specific mechanistic explanation. Despite the increased MDP, AP frequency was increased by TID in this single cell format (Figure 3f), differing from measurements in the monolayer format in Figure 2a. In summary, the AP data also supports the conclusion that cardiomyocyte function was improved by the action of TID.

**Table 1- Action potential parameters of single spontaneously active cardiomyocytes.**

|                  | Vehicle (n=12) | T3+IGF-1 (n=15) | T3+IGF-1+Dex (n=33) | TID+SGK (n=16) |
|------------------|---------------|-----------------|--------------------|---------------|
| MDP (mV)         | −66.9±1.2     | −71.5±0.9*      | −74.2±0.8*         | −74.2±1.1*    |
| dV/dtmax (V/s)   | 15.8±2.2      | 21.5±4.9        | 58.9±4.6*          | 36.5±5.6*    |
| APA (mV)         | 97±2          | 104±2*          | 112±2*             | 114±2*       |
| APD90 (ms)       | 110±11        | 98±12           | 104±11             | 97±10        |
| APD90 (ms)       | 146±14        | 125±13          | 134±11             | 124±10       |
| Frequency (Hz)   | 1.6±0.2       | 2.1±0.2         | 2.5±0.2*           | 2.2±0.2*     |
| Capacitance (Cm) | 20±3          | 17±1            | 25±2               | 24±3         |

Data are mean±SEM; TID+SGK=T3+IGF-1+Dex+GSK650394, n=number of cells, MDP=maximal diastolic potential, dV/dtmax=maximal upstroke velocity, APA=action potential amplitude, APD50, and APD90=action potential duration at 50, and 90% repolarization, respectively. * p<0.05 compared to vehicle. One-Way ANOVA with a Tukey post-test.
We next examined single cell contractility under the same maintenance conditions with the exception that cardiomyocytes were plated on soft micropatterned polyacrylamide gels (20 µm-wide gelatin lines) containing fluorescent micro-beads. Bead displacement was imaged during spontaneous contraction of individual cells and converted to a value of trace.
tion stress (Figure 4a), as previously described [9]. In vehicle-only medium, mean traction stress was 0.15±0.02 mN/mm²; in T3+IGF-1 this was increased by 1.8-fold to 0.27±0.02 mN/mm² (p<0.05), and in TID medium it was increased by 2.9-fold to 0.44±0.04 mN/mm² (p<0.05) (Figure 4b). This corresponded to traction forces of 0.15±0.02 µN, 0.36±0.04 µN and 0.76±0.08 µN for vehicle-only, T3+IGF-1, and TID medium, respectively. The potentiated increase in traction stress by the addition of Dex, over the T3+IGF-1-condition, was significant (p<0.05). Cardiomyocyte area was increased 1.4-fold by T3+IGF-1 (p<0.05) and 1.8-fold by TID (p<0.05) (Figure 4c), although cells remained small compared to adult human equivalents (Ancy et al., 2003). The potentiated increase in cell area by Dex was significant (p<0.05), aligning with our estimates by eGFP intensity. Contraction frequency was increased by TID, however as the force-frequency relationship was negative in all conditions this did not explain the difference in traction stress between the conditions (Figure 4d). A co-staining of Troponin I and α-actinin showed that the structural organization of sarcomeres was improved by TID and sarcomeres were more uniform across the entire area of each cardiomyocyte (Figure 4e and Fig. S4). Expression of the contractility-related protein encoding genes: MYH6, ACTN2, MLC2V and SERCA2 were upregulated by TID, although they were not significantly different between T3+IGF-1 and TID (Fig. S2). T3 repressed MYH7 expression as previously reported (Iwaki et al., 2014). An increase in traction stress by TID was also recorded in cardiomyocytes from the M1 hESC line (Fig. S4).

**Figure 4** - Single cardiomyocyte traction force measurement. A. Typical examples of single aligned (spontaneously contracting) cardiomyocytes from vehicle-only and T3+IGF-1+Dex containing medium, showing a brightfield image of the relaxed form and a heat map of traction stress applied to the substrate calculated from the mean of the traction stress vectors (corresponding to Supplementary video 1). B. Traction stress, C. cell area and D. Traction stress-frequency relationship, of single spontaneously contracting cardiomyocytes maintained in vehicle (n=44), T3+IGF-1 (n=44) and T3+IGF-1+Dex (n=45). E. Immunostaining of typical aligned cardiomyocytes from vehicle- and TID-containing medium. Box and whisker plots show the median, interquartile range and 10-90 percentile range. The n signifies the number of individual cells measured, acquired over 3 independent experiments. Statistical significance was calculated using a one-way ANOVA with Tukey’s multiple comparison test * P<0.05. Scale bar = 10 µm. See also Figure S4.
Contractile dysfunction in a hiPSC model of hypertrophic cardiomyopathy

We have previously observed that single hiPSC-derived cardiomyocytes also generate very low contractile forces when maintained under standard basal conditions (~0.2 mN/mm²) (Ribeiro et al., 2015 and unpublished observations). This creates a very low level of sensitivity for detecting defects in function, as even the control cells may be functioning far from their full dynamic range. Therefore we took the opportunity to apply the TID-containing medium to an hiPSC disease model in which the derivative cardiomyocytes carry a c.2373dupG mutation in MYBPC3 [17]. This mutation causes HCM in patients [37]. The mutant MYBPC3 hiPSC-CMs were compared to cardiomyocytes derived from two hiPSC lines generated from healthy controls. Cardiac functional data of the HCM patients is provided in Table S1. A significant decrease in MYBPC3 protein relative to α-actinin was observed in the cardiomyocytes from all three HCM lines compared to both controls when measured at day 25 of differentiation (Figure 1a and b).

Attempts to measure these hiPSC-CMs in medium without TID proved unsuccessful as even the control cells failed to reliably generate robust bead displacement. However, in medium containing TID, traction force on the polyacrylamide substrate could be measured in all cell populations. Under these conditions, traction stress was significantly decreased in all three mutant lines: HCM1 0.31±0.02 mN/mm², HCM2 0.30±0.03 mN/mm², HCM3 0.29±0.03 mN/mm², compared to both controls: Con1 0.57±0.04 mN/mm², Con2 0.51±0.03 mN/mm²; p<0.05 (Figure 5c and d). This corresponded to traction forces of 0.43±0.04 µN, 0.46±0.06 µN, 0.44±0.05 µN and 0.81±0.16 µN, 0.86±0.10 µN for HCM1, HCM2, HCM3 and Con1, Con2, respectively. A difference in cardiomyocyte size as measured by cell area, was not evident between the control and the mutant cells, suggesting an overt hypertrophic response had not occurred by this stage of development under these conditions (Figure 5e). Contraction frequencies were not different (Fig. S4).

Discussion

Using a simple flow cytometry based assay we have identified a combination of defined factors, namely thyroid hormone (T3), IGF-1 and the glucocorticoid dexamethasone, which together enhanced the functional properties of hPSC-derived cardiomyocytes. We observed that T3 principally increased the resting membrane potential (ΔΨp), a critical factor in determining excitability and contractility, while IGF-1 and dexamethasone acted synergistically to stimulate cell energetics and traction force generation. In this optimized condition we assessed the impact of an HCM-causing mutation in MYBPC3, or gene knockdown. Decreased force generation was observed in both cases, recapitulating measurements in mutant mouse models and patient-derived cells, opening up the possibility of using this in vitro-based human model as a tool for future mechanistic studies and drug screening.

The positive influence of T3 on cardiomyocyte function is well supported in the literature. Levels increase markedly at birth in humans and have an important role in heart development and maturation [27,38]. T3 was previously shown to increase the activity of the Na,K-ATPase in cardiomyocytes [39], as well as increasing theIk1 current [40], both of
which could explain the increase in $\Delta \Psi_p$ observed here. T3 also affects sarcomeric gene expression, stimulating expression of $\alpha$-MHC, the fast ATPase activity MHC isoform, and repressing the expression of $\beta$-MHC, the slow ATPase activity isoform [41,42]. This effect was also shown in hESC-CMs by Yang et al. (2014), and confirmed here. These changes in MHC isoform expression may contribute to the increased contractile force generation observed with T3, as also previously reported by Yang et al. (2014). However, from this base condition we were able to extend these findings with the identification of additional physiologically relevant factors important for hPSC-CM function. IGF-1 also circulates in the developing heart and has an important role in myocardial cell growth and metabolism by signalling through the IGF-1 tyrosine kinase receptor [29]. We found that IGF-1 mildly increased the size of hPSC-CMs, but more importantly proved an essential factor in revealing a positive functional role for the glucocorticoid dexamethasone in this system. Synergistic effects for
IGF-1 and dexamethasone have been reported in skeletal muscle and heart, with pro-differentiation and anti-atrophic effects observed [43–45]. Glucocorticoids also promote the structural and functional maturation of fetal mouse cardiomyocytes [28,34], but have little effect on hPSC-CMs when added alone although do affect calcium handling (Kosmidis et al. unpublished results). Interestingly, the positive effect reported by Rog-Zielinska et al. in cultured cells was blocked by PGC-1α knockdown, a protein known to regulate mitochondrial respiration in cardiomyocytes [23]. Our observation here, that in combination with IGF-1 dexamethasone exerted a clear bioenergetic response, also supports the conclusion that the functional improvements in force generation and electrophysiology may at least partly be the result of enhanced energy production pathways. Basal respiration coupled to ATP synthesis was increased by TID, and as this rate remained proportionally sensitive to inhibitors of excitation and contraction these processes must be more active in these cells. Additionally, we showed that basal respiration was sensitive to inhibitors of both mitochondrial pyruvate uptake as well as fatty acid uptake, implying that substrate supply also exerts significant respiratory control in both basal and TID conditions. The higher respiratory rate in TID suggests that both pyruvate and fatty acid usage was increased. TID also stimulated anaerobic glycolysis which will provide additional pyruvate for mitochondria but can also drive the synthesis of biomass through precursor synthesis [46], and increase NADPH production important for ROS detoxification[47]. Reduced DHE oxidation in cells treated with TID indeed indicated lower ROS levels. Mitochondrial ROS production may also be lower as a result of the increased ATP turnover[48]. While ROS have been shown to have an important role in cardiomyocyte development[49], elevated levels can be inhibitory to cell function so a balance may be important[23,50–52].

At the electrophysiological level, in the presence of T3, the addition of IGF-1 and dexamethasone caused a large increase in hESC-CM AP upstroke velocity not explained solely by the MDP. This was not associated with increased SCN5A expression and was therefore predicted to involve altered cardiac sodium channel regulation. The functional improvement was highly sensitive to small molecule inhibition of the glucocorticoid-inducible kinase SGK1 which is known to regulate degradation of the channel through inactivation of the ubiquitin ligase NEDD4-2 [36,53]. This result supports a role for SGK1 in hPSC-CM function and will be an important avenue for future investigation. A further improvement was seen in the AP amplitude by TID, a parameter known to correlate with contractile force generation at a single cell level [9]. Indeed, TID substantially increased the traction force of hPSC-CMs as well as their sarcomeric structural organization, showing that all core aspects of cell function were enhanced.

Having identified conditions for maximising traction force, we were able to apply the system to a cardiac disease model. HCM is caused by mutations in sarcomeric protein-encoding genes important for contractile function, of which mutation in MYBPC3 accounts for approximately 35% of cases. While animal models of MYBPC3-deficiency and patient-derived cells have been well studied [54–60], a widely accessible human in vitro model has been lacking. Here, using two independent approaches, we assessed the impact of MYBPC3-deficiency on hPSC-CM contractile force generation. Cardiomyocytes from patient-derived
hiPSCs carrying a non-sense mutation in MYBPC3 had <50% of normal MYBPC3 levels and were found to exert significantly less force at the single cell level. Our results are supported by measurements in adult patient-derived cells assessed in vitro where a 30-40% decrease in maximum Ca2+-activated force development has been observed in cells with the c.2373insG mutation, and a mechanism of haploinsufficiency suggested [59–61]. Importantly, the force generation defects we observed here were in non-hypertrophic cells, suggesting a primary event not consequential to hypertrophy and related maladaptive processes. This observation is consistent with the diastolic dysfunction reported in non-hypertrophic cardiomyocytes of mice heterozygous for a point mutation in mybpc3 [62]. Together these results support the possibility that the contractile dysfunction is an early initiating event in HCM disease pathogenesis. The specific initiating cause of the force generation defect in the hPSC-CMs studied here is unknown, as in all other models of MYBPC3 mutation studied so far. Potential causes could include a defect in sarcomerogenesis leading to a reduced myofibril density, perturbed cross bridge cycling or increased sarcomere calcium sensitivity leading to diastolic dysfunction, or more general metabolic disturbance. Having a human model where this phenotype can be genetically induced and studied in a developmental context will present new opportunities to address this question. This could in turn lead to new therapeutic strategies for HCM, which is currently without cure.

In summary, providing functionally important physiological factors to hPSC-CMs may be critical for achieving robust baseline function and maximising their use in applications of disease modelling, drug discovery and development, and toxicity screening. These are major goals for the stem cell field. Here we found that three defined factors were sufficient to markedly improve the function of hPSC-CMs and facilitated their use in a disease model of HCM. The advance in culture conditions towards a fully defined formulation also revealed the potential of the single cell traction force measurement technology for studying diseases of contractility in a highly controllable system.

**Experimental Procedures**

**hPSC culture and differentiation**

H3 NKX2-5eGFP/w hESCs or M1 NKX2-5eGFP/w hESCs as previously generated [21], were maintained on mouse embryonic fibroblasts and passaged using TrypLE select (Life Technologies). The generation of transgene-free hiPSCs from skin fibroblasts of one healthy male donor (LUMC0004iCtrl [Con1]) and three patients each with a c.2373dupG mutation in MYBPC3 (LUMC0033iMyBPC [HCM1], LUMC0034iMyBPC [HCM2] and LUMC0035iMyBPC [HCM3]) was previously reported [17]. A second transgene-free control hiPSC line (LUMC0047iCtrl [Con2]) generated from another healthy male donor was included in this study. hiPSCs were maintained on Matrigel (growth factor reduced; Corning 354230) in mTeSR1 medium (Stem Cell Technologies) and passaged with 1 mg/ml Dispase (Life Technologies). NKX2-5eGFP/w hiPSCs (RPD, CLM unpublished) were maintained in Essential 8 medium (Life Technologies) and differentiated as previously described (van den Berg 2015).

Cardiac differentiation was induced from monolayer cultures on Matrigel in a serum-free
medium (BSA, polyvinyl alcohol, essential lipids [BPEL]) as described in Supplemental information. Contracting cultures were dissociated on day 13 and replated on Matrigel-coated 24-well plates. The following experimental factors were added on day 16, refreshed on day 20 and measured on day 21: 100 ng/ml Long R3 IGF-1 (in the main text: IGF-1), 1 µM SAG (Millipore), 1 µM dexamethasone, 100 nM triiodothyronine hormone, 10 µM phenylephrine, 1 µM isoproterenol, 1-1000 nM norepinephrine. Unless otherwise stated all factors were obtained from Sigma.

**Flow cytometry measurements for plasma membrane potential and reactive oxygen species**

For relative plasma membrane potential measurement, differentiated cultures were dissociated on day 21 (after treatment as above from day 16) using 5x TrypLE Select and resuspended in 2.5 nM TMRM (Life Technologies) in warm assay medium. Cells were incubated for 8 minutes at 37°C before being measured by flow cytometry. For superoxide detection, dissociated cells were labelled with 5 µM DHE for 30 mins at 37°C and then measured by flow cytometry. Appropriate compensation to correct from cross-bleed was performed for each.

**Respiration and acidification rates measured with the Seahorse XF-24 Analyzer**

Respiration and acidification rates were measured on adherent cells using a Seahorse XF-24 or an XF-96 Analyzer (Seahorse Bioscience). Cells were seeded on Matrigel-coated assay plates in BPEL medium 7 days before measurement. The assay was performed in bicarbonate-free DMEM as described in Supplemental information. The standard glucose concentration was 15 mM glucose and was supplemented with 0.5 mM sodium pyruvate. Cells were washed twice and pre-incubated in the assay medium for 1 h before measurement. For the standard profiling oligomycin was used at 0.5 µg/ml, FCCP titrated in 2 injections to 3 µM and rotenone and antimycin A were added at 1 µM and 2 µM respectively. A standard protein assay was used to normalize values to whole cell protein. Glycolytic rate calculations and ATP production rates are described in Supplemental information. To assess ATP demand for excitation and contractility nifedipine (10 µM) and blebbistatin (5 µg/ml) were co-injected and the respiration rate immediately recorded, followed by measurements after oligomycin and then rotenone and antimycin A injection. A vehicle-only injection was performed in parallel and the effect subtracted. Experiments analysing the effect of etomoxir and UK5099 were performed on the XF-96 format using an assay medium with the following modifications: the glucose concentration was 5.5 mM, sodium pyruvate 0.15 mM and palmitic acid was included at 100 µM conjugated to fatty acid-free BovoStar BSA as described in Supplemental information. 10 mM HEPES was also included in this assay medium to provide extra buffering (glycolytic rates were not calculated). Cells were washed twice and pre-incubated in this assay medium for 4 h before measurement. To assess the response to substrate uptake inhibitors, 40 µM etomoxir or 5 µM UK5099 were injected and respiration rates recorded after 45 mins, followed by injections of oligomycin, FCCP (3 µM) and then rotenone and antimycin A. A vehicle-only injection was performed in parallel and the effect subtracted.
**Electrophysiological characterization**

Action potential (AP) recordings were performed on single cardiomyocytes, 6-10 days after cell dissociation with the amphotericin perforated patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA). Signals were filtered and digitized at 5 and 40 kHz, respectively. Data acquisition and analysis were accomplished using pClamp10.1 (Axon Instruments) and custom software. Potentials were corrected for the liquid junction potential. Cells were continuously perfused in a perfusion chamber at 37°C (Cell MicroControls Norfolk VA, U.S.A.) using Tyrode’s solution containing (mM): NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 1.0, glucose 5.5, HEPES 5; pH 7.4 (NaOH). Pipettes (borosilicate glass; resistance ~2.5 MΩ) were filled with solution containing (mM): K-gluconate 125, KCl 20, NaCl 5, amphotericin-B 0.22, HEPES 10; pH 7.2 (KOH).

APs were recorded at spontaneous beating frequencies and characterized by duration at 50 and 90% repolarization (APD50, and APD90, respectively), maximal diastolic potential (MDP), AP amplitude, maximal upstroke velocity and frequency. AP parameter values obtained from 8-9 consecutive APs were averaged and data were collected from at least 2 independent differentiations per condition.

The SGK1-inhibitor GSK650394 [35] or vehicle-only control was applied at 6 µM 5 days before AP measurement. AP measurements were blinded in acquisition and analysis.

**Traction force measurements**

The traction force measurements were performed as previously described [8]. Cells were seeded onto the gelatin patterned acrylamide gels 4 days (see Supplemental information) before measurement in their normal culture medium in an environment maintained at 37°C with 5% CO2. Briefly, using a Leica AF-6000LX microscope, an image-series of aligned single spontaneously contracting cardiomyocytes was taken at 40x magnification at 20 frames per second, recording brightfield and fluorescent beads. Single frames from maximal relaxation and contraction of the brightfield and fluorescent beads image-series were analyzed by the LIBTRC software package (kindly provided by Dr. Micah Dembo), creating a mask of the cell outline from the brightfield image and a vector map from the difference between the relaxed and contracted fluorescent beads images. The vector map and the cell mask were used to calculate the total force that the cell applies on the substrate at its maximum peak of contraction. The traction stress generated by the cardiomyocyte during contraction was calculated by dividing the total force by the cell surface area. Measurements were blinded in acquisition and analysis.

**Quantitative real-time PCR**

RNA was isolated using a Minelute RNA extraction kit (Qiagen) and cDNA synthesized using an iScript cDNA synthesis kit (BioRad). Real-time PCR was performed on a BioRad CFX384 machine using IQ SYBR Green (BioRad). Gene expression values were normalized to the mean expression of the housekeeping genes human ribosomal protein (RPLP0), glucuronidase (GUSB), and RNF7. Primer sequences can be found in Supplemental information.
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Supplementary information

**Figure S1** - GFP and TMRM measurements in hPSC-derived cardiomyocytes maintained in different conditions. Related to Figure 1. FACS measurement after cardiac differentiation of NKX2-5eGFP/w hESCs and maintenance for 5 days under control conditions or in the presence of 5% FCS. (a) Scatter plots and P1 gating of control and (b) 5% FCS treated cells, (c) eGFP fluorescence of the P1 population of control and (d) FCS treated cells, (e) eGFP fluorescence histogram showing an overlay of the two P1 populations. The median eGFP fluorescence value within the P2 gate is shown above the histogram. (f) Relative TMRM and eGFP fluorescence values of M1 NKX2-5eGFP/w hESC-derived cardiomyocytes and (g) NKX2-5eGFP/w hiPSC-derived cardiomyocytes after 5 days of treatment with vehicle-only or TID medium. (h) Relative TMRM and eGFP fluorescence of cardiomyocytes after 5 days of treatment with T3+Dex (TD) plus insulin or IGF-1. Data are mean±SEM, n=6 (f), n=8 (g), n=9 (h). The n signifies the number of wells (cell populations) measured. Statistical significance was calculated using a one-way ANOVA with Dunnett’s correction, # P<0.05 for eGFP fluorescence, * P<0.05 for TMRM fluorescence.
Figure S2 - Gene expression profiles in hESC-derived cardiomyocytes maintained in different conditions. Related to Figure 1. (a) hESC-derived NKX2-5-eGFP+ cardiomyocytes were isolated by FACS after 7 days of treatment with the conditions shown. Gene expression is normalized to the housekeeping gene RPLP0 and shown relative to the vehicle-only treated control. (b) Gene expression in cardiomyocytes treated for 5 days with T3+IGF-1+Dex (TID) plus vehicle, 1 µM norepinephrine (NE), 10 µM phenylephrine (PE) or 1 µM isoproterenol (ISO). Gene expression is normalized to the housekeeping gene RPLP0 and shown relative to the TID+vehicle treated control. Data are mean±SEM from three independent experiments in a, and mean±SD for 3 replicate wells in b.

Figure S3 - Modulation of reactive oxygen species levels in hESC-derived cardiomyocytes. Related to Figure 2. (a) Dihydroethidium (DHE) fluorescence measurements reporting superoxide radical levels in eGFP+ cardiomyocytes shown relative to a vehicle-only control after 5 days of maintenance with the factors shown. (b) Histograms of eGFP and DHE fluorescence intensity in eGFP+ cells maintained with vehicle-only or T3+IGF-1+Dex (TID). Note that eGFP is slightly increased while DHE is decreased by TID. Data are mean±SEM from three independent experiments. Statistical significance was calculated using a one-way ANOVA with Dunnett’s correction, # P<0.05 for eGFP fluorescence, * P<0.05 for DHE fluorescence.
Figure S4—Structural imaging of hESC-derived cardiomyocytes and traction force analysis in an additional cell line. Related to Figure 4. (a) Immunostaining of Troponin I and α-actinin in cardiomyocytes maintained in (a) vehicle-only and (b) TID-medium, aligned on micropatterned PDMS gels. (c) Traction stress and (d) cell area of M1 hESC-derived cardiomyocytes maintained in vehicle or TID medium (n=15 individual cell measurements for each condition). Box and whisker plots show the median, interquartile range and 10-90 percentile range. Statistical significance was calculated with an unpaired t-test, * P<0.05.
Figure S5- HCM cardiomyocyte frequency analysis and testing TID in a defined, albumin-free, basal medium. Related to Figure 5. (a) Contraction frequency of single spontaneously contracting iPSC-derived cardiomyocytes from two control (Con1 [n=47] and Con2 [n=36]) and three MYBPC3 mutation lines (HCM1 [n=44], HCM2 [n=54] and HCM3 [n=43]). Box and whisker plots show the median, interquartile range and 10-90 percentile range. (b) eGFP and TMRM fluorescence values in eGFP+ cardiomyocytes relative to a vehicle-only control after 5 days of incubation with the concentrations of T3 shown (n=6). (c) eGFP and TMRM fluorescence values in eGFP+ cardiomyocytes maintained in T3 (100 nM) +IGF-1 supplemented with a range of dexamethasone concentrations, measured relative to the T3+IGF-1+vehicle control (T3+IGF-1). (d) Traction stress and (e) cell size of single spontaneously contracting cardiomyocytes maintained in T3+IGF-1+Dex (n=45) and T3+IGF-1+Dex in defined medium (n=43). The n signifies independent experimental replicates in b and c and the number of individual cells in a, d and e acquired over three independent experiments. Statistical significance was calculated with a one-way ANOVA with Tukey’s multiple comparison test in a, a one-way ANOVA with Dunnett’s correction in b and c, and an unpaired t-test in d and e. n.s. = not significant, * P<0.05.
Table S1. Clinical Data of HCM patients with 2373insG mutation in MYBPC3. Related to Figure 5.

| Patient | Age | Sex | Interventricular Septum in Diastole, mm | LVEDD, mm | LVESD, mm | % FS | LVEF% |
|---------|-----|-----|----------------------------------------|-----------|-----------|------|-------|
| HCM1    | 44  | m   | 13                                     | 54        | 32.9      | 39   | 56    |
| HCM2    | 14  | m   | 35                                     | 41        | 24        | 41   | 58    |
| HCM3    | 42  | m   | 24                                     | 55        | 42        | 24   | 48    |
| Healthy |    |     | <13                                    | 36-56     | 20-40     | 25-43| 55-70 |

LVEDD = left ventricular end diastolic diameter; LVESD = left ventricular end systolic diameter; % FS = fraction shortening (LVEDD-LVESD)/LVEDD*100%; LVEF% = left ventricular ejection fraction

Supplemental Experimental Procedures

hPSC culture and differentiation

H3 NKX2-5eGFP/w hESCs or M1 NKX2-5eGFP/w hESCs as previously generated (Elliott et al., 2011), were maintained on mouse embryonic fibroblasts and passaged using TrypLE select (Life Technologies). The generation of transgene-free hiPSCs from skin fibroblasts of one healthy male donor (LUMC0004iCtrl [Con1]) and three patients each with a c.2373dupG mutation in MYBPC3 (LUMC0033iMyBPC [HCM1], LUMC0034iMyBPC [HCM2] and LUMC0035iMyBPC [HCM3]) was previously reported (Dambrot et al., 2014). A second transgene-free control hiPSC line (LUMC0047iCtrl [Con2]) generated from another healthy male donor was included in this study. hiPSCs were maintained on Matrigel (growth factor reduced; Corning 354230) in mTeSR1 medium (Stem Cell Technologies) and passaged with 1 mg/ml Dispase (Life Technologies).

Cardiac differentiation was induced from monolayer cultures on Matrigel in serum-free medium (BSA, polyvinyl alcohol, essential lipids [BPEL]) as previously described (Ng et al., 2008). The formulation was as follows: 44% IMDM, 44% Ham’s F12, 5% protein-free hybridoma medium (PFHM-II), 0.25% Bovostar BSA (Bovogen, Australia), 0.125% polyvinyl alcohol, 1x chemically defined lipids, 400 µM α-thioglycerol (Sigma-Aldrich), 50 µg/ml L-ascorbic acid 2-phosphate (AA-2P), 2mM Glutamax, 0.1x Insulin-transferrin-selenium-ethanolamine (ITS-X) and 0.5% Pen/Strep. All BPEL components were obtained from Life Technologies unless otherwise stated. The following factors were present for the first 3 days 20 ng/ml BMP4 (R&D), 20 ng/ml Activin A (Miltenyi Biotech) and 1.5 µM CHIR 99021 (Axon Medchem). 5 µM XAV 939 (Tocris Bioscience) was present on days 3-6. NKX2-5eGFP/w hiPSCs (RD, CLM unpublished) were maintained in Essential 8 medium (Life Technologies) and differentiated as previously described (van den Berg 2015).

Contracting cultures were dissociated on day 13 and replated on Matrigel-coated 24-well plates. The following experimental factors were added on day 16, refreshed on day 20 and measured on day 21: 100 ng/ml Long R3 IGF-1 (in the main text: IGF-1), 1 µM SAG (Millipore), 1 µM dexamethasone, 100 nM triiodothyronine hormone, 10 µM phenylephrine, 1 µM isoproterenol, 1-1000 nM norepinephrine. The above factors were obtained from Sigma Aldrich unless otherwise stated.

Patterned Polyacrylamide gel fabrication

Patterned polyacrylamide gels were prepared as previously described (Rape et al., 2011). Briefly, a 1% gelatin solution was activated with 3.5 mg/mL Sodium Periodate (both Sigma-Aldrich). A polydimethylsiloxane (PDMS) stamp was casted from a SU8 master produced by standard soft lithography techniques and incubated with the activated 1% gelatin solution for 45 mins. The excess of gelatin was removed with a nitrogen gun and the stamp was used to contact-print a pattern of 20 µm thick with 20 µm spacing gelatin lines onto 10mm (electrophysiology) or 15 mm (contraction assay) coverslips. The polyacrylamide solution was prepared with a final concentration of 0.1% bis-acrylamide (Bio-Rad), 5% acrylamide (Bio-Rad) and 10 mM HEPE5 pH 8.5 in distilled water, followed by centrifugation for 1 min at 10,000 RPM for degassing. 0.086% (m/v) of ammonium persulfate (Sigma-Aldrich) and a 1:1000 dilution of 0.2-µm fluorescent beads (Ex/Em: 660/680nm - Molecular Probes) were added to the solution (for the contraction assay) and
briefly vortexed. The gel polymerization was initiated with TEMED (Bio-Rad) and 4.08 µl/9.2 µl of the final solution was added to a 15 mm/25 mm coverslip treated with plus Bind-Silane solution (GE Healthcare). The µcontact-printed coverslip was applied on top of the drop with the gelatin lines facing the gel. After 20 mins of polymerization the 10 mm/15 mm coverslip was removed. Each 25mm coverslip was mounted onto a well of a glassbottom 6-well plate (Mattek), replacing the initial glass. The 15mm coverslips were used directly. Plates were UV-sterilized and re-hydrated with culture medium for 30 mins before use. The polymerized gel has a Young’s modulus of 5.8 kPa (Frey et al., 2007).

Flow cytometry measurements for plasma membrane potential and reactive oxygen species

For relative plasma membrane potential measurement, differentiated cultures were dissociated on day 21 (after treatment as above from day 16) using 5x TrypLE Select and resuspended in 2.5 nM TMRM (Life Technologies) in warm assay medium. Cells were incubated for 8 minutes at 37°C before being measured by flow cytometry. eGFP and TMRM fluorescence intensities were recorded. For superoxide detection, dissociated cells were labelled with 5 µM DHE for 30 mins at 37°C and then measured by flow cytometry. Samples were measured with a MACSQuant VYB (Miltenyi Biotech) equipped with a blue (488 nm) and a yellow (561 nm) laser. eGFP and DHE fluorescence intensities were recorded. Appropriate compensation to correct from cross-breed was performed for each.

Respiration and acidification rates measured with the Seahorse XF-24 Analyzer

Respiration and acidification rates were measured on adherent cells using a Seahorse XF-24 or an XF-96 Analyzer (Seahorse Bioscience). The assay plates were coated with Matrigel (1:100). Cells were seeded in BPEL medium at a density of 6.5x10⁴ (XF-24) or 2.5x10⁴ (XF-96) cells/well 7 days before measurement. The assay was performed in bicarbonate-free DMEM (D5030; Sigma Aldrich), containing 0.25% fatty acid-free BovoStar (Bovogen, Australia), 1 mM sodium lactate, 0.15 mM sodium hydroxybutyrate, 2 mM GlutaMAX (Life Technologies) and 0.5 mM L-carnitine. The standard glucose concentration was 15 mM glucose and was supplemented with 0.5 mM sodium pyruvate. Cells were washed twice and pre-incubated in the assay medium for 1 h before measurement. For the standard profiling oligomycin was used at 0.5 µg/ml, FCCP titrated in 2 injections to 3 µM and rotenone and antimycin A were added at 1 µM and 2 µM respectively. A standard protein assay was used to normalize values to whole cell protein. Acid titration experiments were performed to calculate the buffering capacity of the Seahorse assay media and gave a value of 0.1 pmoles H+/mM in the 7 µl measuring volume. ECAR values were converted to anaerobic glycolysis/lactate production rates as previously described (Mookerjee et al., 2015), using a max H+/O2 value of 1.0 and converted 1:1 to an ATP production rate. The oligomycin-sensitive oxygen consumption was converted to an ATP production rate using a P/O ratio of 2.3 (Brand, 2005). To assess ATP demand for excitation and contractility nifedipine (10 µM) and blebbistatin (5 µg/ml) were co-injected and the respiration rate immediately recorded, followed by measurements after oligomycin and then rotenone and antimycin A injection. A vehicle-only injection was performed in parallel and the effect subtracted. Experiments analysing the effect of etomoxir and UK5099 were performed on the XF-96 format using an assay medium with the following modifications: the glucose concentration was 5.5 mM, sodium pyruvate 0.15 mM and palmitic acid was included at 100 µM conjugated to fatty acid-free BovoStar BSA in a 2:8:1 molar ratio. To prepare this a 4 mM palmitic acid solution was made in assay medium by heating at 65°C for 30 mins, then combined 1:1 with a 10% Bovostar BSA solution and heated at 37°C for 30 mins with mixing. 10 mM HEPES was also included in this assay medium to provide extra buffering (glycolytic rates were not calculated). Cells were washed twice and pre-incubated in this assay medium for 1 h before measurement. To assess the response to substrate uptake inhibitors, 40 µM etomoxir or 5 µM UK3099 were injected and respiration rates recorded after 45 mins, followed by injections of oligomycin, FCCP (3 µM) and then rotenone and antimycin A. A vehicle-only injection was performed in parallel and the effect subtracted.

Electrophysiological characterization

Action potential (AP) recordings were performed on single cardiomyocytes, 14-16 days after cell dissociation with the amphotericin perforated patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA). Signals were filtered and digitized at 5 and 40 kHz, respectively. Data acquisition and analysis were accomplished using pClamp10.1 (Axon Instruments) and custom software. Potentials were corrected for the liquid junction potential. Cells were continuously perfused in a perfusion chamber at 37°C (Cell MicroControls, Norfolk VA, U.S.A.) using Tyrode’s solution containing (mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1.0, glucose 5.5, HEPES 5; pH 7.4 (NaOH). Pipettes (borosilicate glass; resistance ~2.5 MΩ) were filled with solution containing (mM): K-glutamate 125, KCl 20, NaCl 5, amphotericin-B 0.22, HEPES 10; pH 7.2 (KOH).

APs were recorded at spontaneous frequencies and characterized by duration at 50 and 90% repolarization (APD50, and APD90, respectively), maximal diastolic potential (MDP), AP amplitude, maximal upstroke velocity and frequency. AP parameter values obtained from 8-9 consecutive APs were averaged and data were collected from at least 2 independent differentiations per condition.
**Traction force measurements**

The traction force measurements were performed as previously described (Hazeltine et al., 2012). Cells were seeded on gelatine patterned acrylamide gels 4 days before and measured in their normal culture medium in an environment maintained at 37°C with 5% CO2. Briefly, using a Leica AF-6000LX microscope, an image-series of aligned single spontaneously contracting cardiomyocytes was taken at 40x magnification at 20 frames per second, recording bright-field and fluorescent beads. Single frames from maximal relaxation and contraction of the brightfield and fluorescent beads image-series were analyzed by the LIBTRC software package (kindly provided by Dr. Micah Dembo), creating a mask of the cell outline from the brightfield image and a vector map from the difference between the relaxed and contracted fluorescent beads images. The vector map and the cell mask were used to calculate the maximum total force that the cell applies on the substrate at its peak of contraction. The traction stress generated by the cardiomyocyte during contraction was calculated by dividing the total force by the cell surface area. Measurements were blinded in acquisition and analysis.

**Quantitative real-time PCR**

RNA was isolated using a Minelute RNA extraction kit (Qiagen) and cDNA synthesized using an iScript cDNA synthesis kit (BioRad). Real-time PCR was performed on a BioRad CFX384 machine using IQ SYBR Green (BioRad). Gene expression values were normalized to the mean expression of the housekeeping genes human ribosomal protein (RPLP0), glucuronidase (GUSB), and RNF7. Primer sequences were as follows:

| Gene     | Forward primer                               | Reverse primer                           |
|----------|----------------------------------------------|------------------------------------------|
| RPLP0    | CACCATTGAAATCCTGTAGTGATGT                  | TGACCAGCCAAAGGAGAG                      |
| GUSB     | CCACCTAGAATCTCGTGCTAC                      | GTGCCCTGTAGTGTACTCAA                     |
| RNF7     | ACGCACCAGATAGTACAGG                       | CCAAGTGCAAGGGGAGA                       |
| eGFP     | GTGAGCAGAGGCGAGGAG                        | CCAGTAGTCAGGGTGGTC                      |
| FKBP5    | TTCCTTGTCTGCTCTGGT                       | ACCCTGGCGACTCACAATTCTGT                 |
| SCN5A    | GAGCTCTGTCAGGATTGAG                      | GAAGATGAGCACGGACGAGAG                   |
| SCN1B    | GTGGTGTAGTGACATGTTAGGT                    | GAAGGGCACTGAGGAGGTGTT                   |
| KCNJ2    | ACCGCTACAGCAGTCTCTCT                      | TCCACACACGTGTAGGAAG                     |
| PGC-1α    | AACTCATCAAAGCCAAACCA                      | GAGTTCAATAGTCCTGGTC                     |
| PGC-1β    | TCTTCAACTACCTCCTGGAC                     | CTCACAGTCAATCCGGAGAG                    |
| MYH6     | CCTCCTCACCCTAGCCCTGG                      | GGTGCCCTCCACTACAGA                      |
| MYH7     | CGAACCAGGGTCTTCTGCTCGA                    | GAGGAAGCTGCAACACACCT                   |
| ACNT2    | CTGCTCTTGGGTTGACAG                       | TACTAGAGCTAAGAGA                        |
| MYL2/MLC2V | TACGGTCCGGGAAAATTGCTGAC             | TACTAGAGCTAAGAGA                        |
| MYBP3    | GGATGCTCAAAGGCGCTCTCA                     | TCTCAGGAGCTAAGAGA                       |
| SERCA2   | ACCACATCTCAGGATGGAGA                      | CCAACGAGGCTACGATTG                      |

**Western blotting**

The samples were lysed with ice cold ELB (50mM HEPES pH 7.0; 250mM NaCl; 5mM EDTA; 0.1% NP-40) with 1:100 Protease Inhibitor Cocktail (Sigma-Aldrich) for 30 mins on ice. The samples were centrifuged at 7,000g for 10 mins and the supernatant was quantified for protein content using the Bio-Rad Protein Assay (based on the Bradford dye-binding method). 100µg of the hiPSC-CM lysates were run in a 6% polyacrylamide gel and 30µg of the hESC-CM with the shRNA lysates were run in a 10% polyacrylamide gel together with the protein ladder (Precision Plus Protein dual colour – Bio-Rad). The protein was transferred to an Amersham Hybond membrane (GE Heathcare- Life Sciences) overnight. The membrane was blocked with blocking buffer (2% milk in PBS) and each protein was detected with the specific antibody: Rabbit IgG anti- cMyBP-C (1:2000) kindly provided by Dr. Sakthivel Sadayappan from the Department of Cell and Molecular Physiology, Health Sciences Division, Loyola University Chicago, followed by Horse anti-Rabbit IgG – HRP (1:2000) (Cell Signaling). Mouse IgG anti-Actin (1:1000) (MAB1501 – Millipore) and Mouse IgG anti-α-actinin (A7811-Sigma-Aldrich), followed by Goat anti-mouse IgG-HRP (1:2000) (Cell Signaling).
Supplemental references

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