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Title: Coming of age of human stem cell derived cardiomyocytes: towards functional maturation of human pluripotent stem cell derived cardiomyocytes and their use in understanding inherited arrhythmia syndromes
Date: 2017-10-11
PGC-1α and reactive oxygen species regulate hESC-derived cardiomyocyte function

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Abstract

Diminished mitochondrial function is causally related to some heart diseases. Here we developed a human disease model based on cardiomyocytes from human embryonic stem cells (hESCs), in which an important pathway of mitochondrial gene expression was inactivated. Repression of PGC-1α, which is normally induced during development of cardiomyocytes, decreased mitochondrial content and activity and decreased the capacity for coping with energetic stress. Yet concurrently, reactive oxygen species (ROS) levels were lowered, and the amplitude of the action potential and the maximum amplitude of the calcium transient were in fact increased. Importantly, in control cardiomyocytes, lowering ROS levels emulated this beneficial effect of PGC-1α-knockdown and similarly increased the calcium transient amplitude. Our results suggest that controlling ROS levels may be of key physiological importance for recapitulating mature cardiomyocyte phenotypes and the combination of bioassays used in this study may have broad application in the analysis of cardiac physiology pertaining to disease.
Introduction

Pluripotent stem cells (PSCs) have the remarkable capacity to generate all cell types of the body [1]. Potential biomedical applications for the derivatives of PSCs are vast and diverse, including disease modeling, drug testing, tissue engineering and cell therapies. However, to fully realize the potential in any of these applications, it is essential to understand more about their functional properties and to identify the factors that control their stability and maturation since all differentiated derivatives of PSCs in vitro are immature, with fetal rather than adult characteristics [2].

Here, interest was in the properties of cardiomyocytes derived in vitro from human embryonic stem cells (hESCs). Electrically- and contraction-competent cardiomyocytes can now be generated efficiently under defined conditions from hESCs and human induced pluripotent stem cells (hiPSCs) [3]. These cardiomyocytes have potential to be used for all of the applications relevant to heart physiology and disease mentioned above. Now that the efficiency of differentiation is not rate limiting, a deeper study of the cardiomyocyte function is feasible and warranted. Of particular relevance to the heart as a pump is the ability of the cardiomyocytes to supply themselves with the necessary energy for their work. During development in vivo cardiomyocytes acquire a high density of mitochondria, which ultimately occupy 20-30% of the cell volume in the adult [4]. This gives these cells the huge capacity for ATP synthesis necessary to fund the high energy demands of ion pumping and contractility during strenuous activity. The importance of mitochondria for heart function is highlighted by the fact that functionally-important mutations affecting mitochondria frequently cause cardiomyopathy [5,6] and diminished mitochondrial function is an almost universal feature of cardiac disease [7].

Heart disease remains a major cause of morbidity and mortality in the Western world and there is an urgent need for better models and treatment strategies. Surprisingly though, investigation of mitochondrial involvement in heart disease has largely been limited to mice, which have markedly different cardiac physiology from humans [8] and have not proven to be a highly predictable model for mitochondrial disease. The advent of human PSC research has created opportunities to probe the functional relationship between mitochondria and heart failure and to study the specific cardiac pathogenic mechanisms of mitochondrial diseases using induced PSCs generated from patients. However, little is known about how mitochondrial function and bioenergetics change from a PSC to a cardiomyocyte or how important these functions are. Analysis of these fundamental character-
Statistics is thus first warranted. This has practical implications for investigating the response to an energetic stress such as a hypertrophic or a chronotropic stimulus and for studying disease phenotypes such as cardiomyopathy or cardiac hypertrophy where mitochondria are implicated.

Another important consideration is that if cardiomyocytes acquire a high density of highly polarized mitochondria then reactive oxygen species (ROS) production would be also expected to be high. It is unknown what impact this would have on cardiomyocyte function, stability or maturation in this in vitro context and therefore whether ROS levels should be controlled. ROS have been shown to affect a variety of important ion channels and pumps so the benefit of having a large energy reserve could be offset by a greater burden on the cell as a consequence of oxidative modifications and damage [9-11].

From a developmental perspective, if hPSC-derived cardiomyocytes do show developmentally related changes, then this system offers a robust model to learn about the regulation of these changes during formation of the human heart. For example, fundamental details, such as whether the increase in cardiomyocyte mitochondria is driven primarily by energy demand or as a genetic program, remain unknown. It is also not known which genes control mitochondrial biogenesis in human heart cells and whether these same genes are involved in heart disease. In the mouse, genes with known roles in mitochondrial biogenesis seem to have deterministic roles in heart failure [12] and some of these factors have also been additionally implicated in the perinatal maturation of the mouse heart [13].

In this study we have addressed fundamental aspects of hESC-derived cardiomyocyte bioenergetics, and identified PGC-1α as a major regulator of mitochondria and wider functionality in these cells.

Results

Differentiation of hESCs to cardiomyocytes involves a large increase in mitochondrial energy generating capacity despite little change in cell energetic demand

We utilized the targeted NKX2-5GFP/hESC reporter line in which eGFP (hereafter referred to as GFP) is expressed in cardiac progenitors and cardiomyocytes [14,15] to analyze changes in the cellular bioenergetic status during differentiation toward fully committed (i.e. minimally proliferative) cardiomyocytes. We used this cell line in combination with the Seahorse Bioanalyzer to assess respiration
and anaerobic glycolytic rates (proton production rates) in monolayer cultures (Figure 1). FACS-sorted embryoid body- (EB) derived cells (at day 12+7 post differentiation) contained 95.5±3% cardiomyocytes in the GFPpos fraction and 11.5±3% cardiomyocytes in the GFPneg fraction as assessed by troponin I immunofluorescence staining (Figure 1A). This is consistent with a sub-population of NKX2-5neg cardiomyocytes in the heart [16]. The GFPneg fraction is further composed of a heterogeneous population of mesodermal cell types (Figure S1A and B). The GFPpos cell population (hereafter referred to as cardiomyocytes), in this monolayer format, spontaneously contracted at a mean frequency of 0.50±0.04 Hz.

hESCs actively respire generating more than half of their ATP by oxidative phosphorylation [17]. The hESC line used here also showed this bias with an estimated 68±4% of ATP generated by oxidative phosphorylation (Figure 1B-D). Cardiomyocyte cultures (D12+7 GFPpos) showed a significant increase in coupled respiration, whereas the anaerobic glycolytic rate was significantly decreased resulting in little difference in total ATP output. By contrast, the GF-Pneg population showed no change in coupled respiration but the significantly decreased anaerobic glycolytic rate caused a reduction in total basal ATP output. However, mitochondrial inhibition by oligomycin increased anaerobic glycolysis in cardiomyocytes to a level as high as that observed in hESCs (Figure 1B). This shift in energy supply after oligomycin addition was permissive for contractility demonstrating that anaerobic glycolysis is robust enough to support beating. In fact, contracting cardiomyocytes were produced even when mitochondrial ATP production was inhibited from day 3 of EB differentiation. Thus, this ATP source is not essential for cardiomyocyte differentiation either (Figure S1C).

To determine the component contributors to energy demand we inhibited contraction and/or the action potential and calcium transient. Contraction was inhibited using the Myosin II ATPase inhibitor blebbistatin [18], which does not affect electrical excitability, and a combination of blebbistatin and the L-type calcium channel inhibitor nifedipine was used to additionally block the action potential (AP) and the calcium transient. The difference between the effect of the blebbistatin alone and the combination of the two inhibitors should give the energy demand of only the action potential and the calcium transient. These calculations showed that sarcomeric contraction alone accounted for 21.9±1.9% of coupled respiration, and the AP and the calcium transient combined accounted for 9±2.9% (Figure 1E and F).
Figure 1. Vastly increased respiratory capacity and mitochondrial volume in hESC-derived cardiomyocytes, with little change in total energy demand.

(A) Sorting of embryoid body-derived cells in an NKX2-5 reporter line into GFPpos and GFPneg fractions. Troponin I staining of representative sorted populations. (B) Oxygen consumption rates in monolayers of hESCs, day 12+7 GFPpos and day 12+7 GFPneg sorted cells, normalized to cell protein levels. Basal, endogenous rate; Oligomycin, ATP-synthase inhibited rate; FCCP, maximum stimulated rate; and Rot + Ant A, rotenone- and antimycin A-inhibited rate.
An obvious bioenergetic difference between these three cell populations was the vastly increased maximal respiratory capacity, and therefore theoretical ATP production capacity, of cardiomyocytes (Figure 1B and C). This suggests the possibility of increased mitochondrial respiratory chain content in these cells. Additionally, or alternatively, this could be due to changes in tricarboxylic acid (TCA) cycle enzymes, their state of activation, or differences in matrix [Ca\textsuperscript{2+}] between beating and non-beating cells. To assess mitochondrial content changes, possibly relating to cell maturation, the mitochondria-to-cell volume ratio was calculated. From a typical dissociation at day 12 of differentiation there was clear evidence of a gradual time-dependent increase in mitochondria in cardiomyocytes (Figure 1G and H). By contrast, the GFPneg fraction showed very little evidence of increased mitochondrial biogenesis. As there was little overall increase in energy demand between the hESC state and the cardiomyocyte culture at day 12+7, we reasoned that this process is unlikely to be driven in response to the basic energy demands of the cardiomyocyte. Supporting this prediction, the addition of nifedipine to reduce energy demand did not prevent the increase in mitochondria (Figure 1I).

This suggests that cardiomyocyte mitochondrial biogenesis might be regulated by a set developmental genetic program rather than occurring simply as a stochastic process in response to current energy requirements.

*C*P<0.05. See also Figure S1.
The progressive increase in mitochondrial volume in developing cardiomyocytes is driven by a developmental genetic program orchestrated by PGC-1α

Mitochondrial biogenesis is coordinated by the regulation of transcriptional co-regulators which act on DNA-binding transcription factors to modulate the expression of nuclear-encoded mitochondrial genes [19,20]. We examined the expression profile of three co-regulators, two activators: PGC-1α and PGC-1β and one co-repressor: RIP140. An obvious and universal feature was the large upregulation of PGC-1α specific to the GFPpos fractions with either “spin EB” differentiation or co-culture differentiation using END2 cells (Figure 2A). Of the three genes, this was the only strong candidate because PGC-1β was not upregulated in END2 co-culture-derived cardiomyocytes and RIP140 expression was elevated universally during differentiation. The spin EB cardiomyocyte gene expression changes were mirrored during human heart development (Figure S2A). Furthermore, GFP expression was detectable by day 7 (Figure 2B), corresponding exactly with the onset of PGC-1α expression (Figure 2C). In addition, PGC-1α expression was largely independent of energy demand related to cell contraction as the induction was only marginally affected by the presence of nifedipine, consistent with the mitochondrial biogenesis phenotype (Figure 2D). Recently four different isoforms of PGC-1α have been described and although the primers used here were able to detect both PGC-1α1 and PGC-1α4, isoform-specific PCR showed that PGC-1α4 was not detectable in our cells (Figure S2B) and the upregulation was of PGC-1α1, which is the isoform most important for mitochondrial biogenesis [21]. Although many transcription factor-binding elements have been studied within the PGC-1α1 proximal promoter and shown to coordinate its regulation, neither a 2.2kb nor 0.6kb promoter was sufficient to drive elevated luciferase expression at day 7 (Figure 2C), suggesting more complex regulation. Stronger activity from the 0.6kb promoter is consistent with the possible presence of repressive elements upstream of position -823 [22].

To test the involvement of PGC-1α in the cardiomyocyte-specific mitochondrial changes, short hairpin RNAs (shRNAs) targeting PGC-1α or a scrambled control were stably expressed from day 12 of differentiation and cardiomyocyte mitochondrial volume assessed after 7 days of gene knockdown. The efficacy of the shRNAs was confirmed (Figure S2C). Clone “shRNA #2” significantly inhibited mitochondrial biogenesis in cardiomyocytes and “shRNA #1” also marginally diminished its rate (Figure 2E and F). Constitutive over-expression of PGC-1α or PGC-1β resulted in a dramatic increase in mitochondrial volume. Together this data shows that PGC-1α plays an important role in the normal induction of mitochondrial biogenesis in these cardiomyocytes. Further, PGC-1β may be able to functionally substitute for PGC1α, despite not acting during normal cardiogenesis in this system.
Figure 2. Mitochondrial biogenesis is driven by PGC-1α during cardiac differentiation independent of energy demand for excitation/contraction.

(A) Cell sorting following cardiac differentiation via either “spin embryoid body” or “co-culture” protocols to separate GFPpos and GFPneg populations for RNA isolation. Normalized gene expression shown relative to hESCs for PGC-1α, PGC-1β and RIP140 from these sorted GFPpos and GFPneg populations. (B) GFPpos cell induction during early embryoid body cardiac differentiation. (C) The induction of PGC-1α mRNA in whole embryoid bodies during cardiac differentiation and the parallel activity of three PGC-1α-luciferase reporter constructs of 0 kb-, 0.6 kb- and 2.2 kb-promoter lengths. (D) PGC-1α mRNA in sorted populations at day 12 from embryoid bodies differentiated in the presence or absence of nifedipine (Nif). (E) Images of cardiomyocytes showing GFP, MitoTracker Deep Red (MTDR), and processed MTDR in cells transduced 7 days prior with: a scrambled (Scr) control shRNA, a PGC-1α-specific shRNA (#2) and a lentivirus for overexpression of PGC-1α. The scale bar is 10 μm. In panels A-D, data are represented as mean ± range (n=2 independent experiments).
PGC-1α plays a key role in regulating hESC-derived cardiomyocyte mitochondrial respiration, contractile automaticity and superoxide production

To assess how depletion of PGC-1α affects mitochondrial function, a bioenergetic analysis was performed 7 days after gene knockdown. Both PGC-1α shRNA sequences repressed mitochondrial respiration, with a very dramatic decrease in maximal respiration for hairpin #2, confirming that this gene alone plays a major role in increasing energy generating capacity during cardiac differentiation (Figure 3A). Surprisingly, the basal ATP turnover in these cells was also diminished by PGC-1α knockdown despite considerable reserves in respiratory capacity, which means that these cardiomyocytes are doing less work (Figure 3B). Consistent with the diminished ATP turnover, the beating frequency was reduced by around 50% (Figure 3C). The 25% reduction in ATP turnover following PGC-1α knockdown may, therefore, be largely explained by the reduced frequency in excitation/contraction. Thus, PGC-1α seems to be an important regulator of cardiomyocyte contractile automaticity. The slower beating rate will result in decreased mitochondrial [Ca^{2+}], potentially decreasing TCA cycle activity. Lower ATP demand and lower mitochondrial [Ca^{2+}] could both be contributing to the decreased respiration rate.

Mitochondria are important producers of reactive oxygen species (ROS), which can be highly damaging and inhibitory to cardiomyocyte function. PGC-1α will increase the number of potential ROS-producing sites and may impact the rate of ROS production at these sites via changes in mitochondrial proton motive force. Figure 3D shows that in line with differential accumulation of the cationic probe TMRM between GFPpos and GFPneg cells during normal EB differentiation, probably reflecting the change in mitochondria-to-cell volume ratio (Figure 1H), the levels of superoxide measured by dihydroethidium (DHE) were also different, increasing in cardiomyocytes and decreasing slightly in the GFPneg fraction. Knockdown of PGC-1α was sufficient to repress superoxide production in cardiomyocytes, effectively eliminating the difference between the populations (Figure 3E).
Additionally, oxidative stress in fact proved to be an important second regulator of mitochondrial biogenesis in hESC-derived cardiomyocytes. Decreasing ROS levels by culturing the cells under 3% O₂ slowed the rate of mitochondrial biogenesis whereas increasing ROS with low concentrations of rotenone, antimycin A or H₂O₂ stimulated it (Figure S3A and B). PGC-1α mRNA is already elevated at day 7 (Figure 2C) whereas superoxide increases only after this time (Figure 3D), excluding the possibility that ROS are primarily responsible for at least the initial elevation in PGC-1α mRNA and consequential PGC-1α-dependent mitochondrial biogenesis, consistent with an equal upregulation of PGC-1α with differentiation at 3% O₂ (Figure S3C). However, the ability of PGC-1α to stimulate ROS production may act to potentiate mitochondrial biogenesis thereby reinforcing its action.

Given the striking effect of PGC-1α knockdown on the bioenergetics and ROS levels of cardiomyocytes, investigation of the action potential (AP) and the calcium transient was warranted.

**PGC-1α knockdown alters action potential characteristics and increases the amplitude of the calcium transient via a reduction in oxidative stress**

Figure 4, A and B, shows typical APs of clusters and single cardiomyocytes with control and PGC-1α knockdown; average AP parameters are summarized in Figure 4C. Clusters of cardiomyocytes as well as single cells were measured to avoid limiting the analysis to a subset of cells. Clusters show more robust activity during patch clamp analysis, which could make them less prone to potential sampling bias.

In spontaneously beating cell clusters the reduction in frequency remained evident upon PGC-1α knockdown (1.11±0.42 versus 1.47±0.30 Hz) (Figure 4A and C). The faster rates compared with those measured in the Seahorse plates (Figure 3C) may reflect the different experimental conditions (see Experimental procedures).

Maximum diastolic potential (MDP) and AP upstroke velocity did not differ significantly. However, AP amplitude was significantly increased on PGC-1α knockdown in both single cells (109±2 versus 101±2 mV; p=0.013) and clusters (116±2 versus 107±2 mV; p=0.009). Furthermore, AP duration was prolonged in both groups, although only statistically significant in clusters (APD₅₀ = 153±15 versus 102±17 ms, p=0.034; APD₉₀ = 182±16 versus 120±17 ms, p=0.015) (Figure 4C).
Figure 3. PGC-1α is responsible for increased respiratory capacity in cardiomyocytes but also regulates basal ATP turnover and ROS production.

(A) Oxygen consumption rates in monolayers of cardiomyocytes transduced with shRNA-expressing lentivirus on day 12 and measured 7 days later (Scr shRNA = scrambled control), values normalized to cell protein levels. Basal, endogenous rate; Oligomycin, ATP-synthase inhibited rate; FCCP, maximum stimulated rate; and Rot + Ant A, rotenone- and antimycin A-inhibited rate. (B) ATP production rates from oxidative phosphorylation or anaerobic glycolysis under basal conditions or at maximum stimulated rate. (C) Average beating rates of cardiomyocyte monolayers used for bioenergetic analysis. (D) TMRM and DHE FACS quantifications in GFPpos and GFPneg cells during embryoid body differentiation. The dot plot and corresponding histogram show DHE labelled cells gated based on GFP expression. (E) TMRM and DHE FACS quantifications in cells transduced with shRNA-expressing lentivirus. Values are relative to the GFPneg cells in the Scr shRNA control. All data are represented as mean ± SEM (n=4 independent experiments in A, B, C, E and 3 independent experiments in D). Statistical significance was calculated using a one-way ANOVA with Dunnett’s correction. *p<0.05.

See also Figure S3.
Figure 4. Action potential characteristics with PGC-1α knockdown or mitochondrial inhibition.

(A) Typical spontaneous action potentials in clusters of cells previously transduced with a scrambled (Scr) control shRNA or the PGC-1α-specific shRNA. (B) Typical action potentials elicited at 1 Hz in single cardiomyocytes. (C) Average AP characteristics. (D) AP parameters in spontaneously beating clusters of control cardiomyocytes at baseline or following perfusion with oligomycin. (AP = action potential, MPD = maximum diastolic potential, APD_{50} & APD_{90} = action potential duration at 50 and 90% repolarization respectively). Statistical significance was calculated using an unpaired or paired t-test in C and D respectively. *p<0.05. See also Figure S4.
To assess whether the changes in the AP following PGC-1α knockdown could be mimicked by direct mitochondrial inhibition, the mitochondrial ATP synthase inhibitor oligomycin was added to the extracellular solution during measurement of clusters of cardiomyocytes. Neither frequency nor any other parameter of the AP was significantly affected by application of oligomycin for 4 minutes (Figure 4D). Contraction frequency was also unchanged when measured by imaging after exposure to oligomycin for 15 minutes in normal culture conditions (Figure S4). This suggests that ATP generated by anaerobic glycolysis alone may be sufficient to fund the AP requirements of these cells, at least in the short term, and that the effect of PGC-1α knockdown on the AP may not necessarily be related to the energetic defect in these cells.

Figure 5A shows typical [Ca\textsuperscript{2+}]i measurements in a control and a PGC-1α knockdown cluster; average [Ca\textsuperscript{2+}]i values are summarized in Figure 5, B-D. The diastolic [Ca\textsuperscript{2+}]i was similar between control and PGC-1α knockdown clusters (Figure 5B). However, systolic [Ca\textsuperscript{2+}]i was increased (Figure 5C) and consequently the Ca\textsuperscript{2+} transient (Figure 5D) was larger in PGC-1α knockdown clusters. The positive response to rapid cooling to release SR calcium suggests that the SR is a major store of Ca\textsuperscript{2+} in both conditions (Figure 5A).

The increase in systolic calcium is unlikely to be due to a lower level of calcium buffering by mitochondria, as mitochondria are reported to remove only 1% of systolic calcium even in adult myocytes during a transient [23,24]. We tested whether the effect on [Ca\textsuperscript{2+}]i could be recreated by lowering ROS levels. Incubation at 3% O\textsubscript{2} in the presence of 6 mM N-acetyl cysteine (NAC) suppressed superoxide levels in cardiomyocytes to a value equivalent to PGC-1α knockdown cardiomyocytes (53% versus 56% decrease respectively) (Figure 5E). In cells pre-cultured in this low ROS condition the Ca\textsuperscript{2+} transient was similarly increased (Figure 5B-D). The same changes were evident when the clusters were paced at 1 Hz (Figure S5), although the decreased transient in all conditions at 1 Hz already reveals a negative force-frequency relationship above 0.5 Hz.
Figure 5. Calcium transient characteristics with PGC-1α knockdown and the effect of directly lowering ROS levels.

A) Indo-1 ratio values showing transients and the response to rapid cooling. B, C, and D) average diastolic (B), systolic (C) and transient (D) concentrations. Statistical significance was calculated using an unpaired t-test (data are from 3 independent experiments). (E) Superoxide levels measured by DHE following 5 days under test condition of control (21% O₂), low oxygen (3% O₂) or low oxygen plus the antioxidant N-acetyl cysteine (NAC). Data are represented as mean ± SEM (n=3 independent experiments). Statistical significance was calculated using a one-way ANOVA with Dunnet’s correction using the 21% O₂ GFPpos values as the control. *p<0.05. See also Figure S5.
PGC-1α is important for the maintenance of cardiomyocyte sarcomeric organization during fetal calf serum-induced hypertrophy and response to beta-adrenergic stimulation

Under baseline conditions, where these cardiomyocytes are using only about one third of their theoretical ATP production capacity (Figure 1C), mitochondrial function may be partially redundant. However, under more energy demanding conditions one would expect that an energetic compromise might be exposed. To investigate this possibility, a model of cardiomyocyte hypertrophy was established using fetal calf serum (FCS) as the inducing agent. 5% FCS was found to cause a hypertrophic phenotype resulting in a volume difference of 3.8 fold when measured after 18 days (Figure 6A and B), from a median of 2324 μm³ in BPEL to 8886 μm³ in 5% FCS (n=85 and 78 cells respectively; p<0.001). The question we wanted to address was whether during this volume increase PGC-1α-depleted cells would still be able to maintain their structural organization by correctly synthesizing, trafficking and recycling their specialized sarcomeric proteins. Following NKX2-5pos cell identification in live cells, α-actinin staining was used to categorize single cardiomyocytes into three structural classes (Figure 6C): class I indicates a cell with α-actinin anti-parallel bands present across more than half of the cell’s area; class II where many bands are also present but across less than half of the cell’s area; and class III where labeling is apparent but a banding pattern is almost completely absent. In cardiomyocytes transduced with the scrambled (Scr) control shRNA, 17.6% were class III type after chronic FCS exposure, whereas in PGC-1α-depleted cardiomyocytes a significantly higher proportion, 53.5% (chi-square test, p<0.001), displayed class III characteristics (Figure 6D). Chronic incubation with the mitochondrial uncoupler 2,4-dinitrophenol (DNP) caused a similar disturbance to structural integrity suggesting that energetic compromise may be the cause of this phenomenon (Figure 6E) (chi-square test, p<0.001).

In a second assay, the chronotropic response to beta-adrenergic stimulation was evaluated. 100nM isoproterenol raised the spontaneous beating frequency of Scr shRNA control cardiomyocyte clusters in 30 of 33 areas by a mean increase in rate of 79% (Figure 6F). In PGC-1α knockdown clusters, isoproterenol caused beating to cease completely in 29 of 48 areas, while 17 areas increased their rate by a mean of 74% (Figure 6G). Consistent with the imaging data, isoproterenol increased the ATP production rate of the Scr shRNA control cells by 43.5% with both increased oxidative phosphorylation and anaerobic glycolysis, while in
Figure 6. PGC-1α is important for maintaining cardiomyocyte structural identity during fetal calf serum-induced hypertrophy and for the chronotropic response to beta-adrenergic stimulation.

(A) Calcein-AM-labelled cells used for cell volume calculations, previously maintained for 18 days in serum-free “BPEL” media (i), or 5% FCS-containing media (ii). GFPpos cardiomyocytes were pre-identified before loading and are shown as “regions of interest”. (B) Histogram quantification of cell volumes for the two culture conditions. BPEL median = 2324 μm³ (n=85), 5% FCS median = 78 μm³, (n=78); p<0.001. (C) α-actinin-labelled NKX2-5pos cells (cultured in 5% FCS) classified by sarcomeric structure into 3 groups. (D) Frequency of each cardiomyocyte structural class in Scr shRNA and PGC-1α-specific shRNA conditions (n=160 cells counted from 3 independent experiments). (E) Frequency of each cardiomyocyte structural class in vehicle control cells compared to 200 μM DNP treated cells (n=80 cells counted from 2 independent experiments). (F) Contraction frequency of individual Scr shRNA-transduced clusters at baseline and after exposure to 100 nM isoproterenol.
Figure 6. PGC-1α is important for maintaining cardiomyocyte structural identity during fetal calf serum-induced hypertrophy and for the chronotropic response to beta-adrenergic stimulation.

(G) Contraction frequency of individual PGC-1α shRNA-transduced clusters at baseline and after exposure to 100 nM isoproterenol. (H) ATP production rates from oxidative phosphorylation or anaerobic glycolysis under basal conditions or after isoproterenol exposure (data represent the mean from 4 technical replicates). Statistical significance was calculated using an unpaired t-test in B and a chi-squared test in D and E.

PGC-1α shRNA cells ATP production rate was decreased overall by 19% (Figure 6H). The increased glycolytic rate in these PGC-1α shRNA cells by isoproterenol may reflect a much faster rate in the minority of cells able to increase their beating frequency under this condition.

Discussion

In this study, we have described the bioenergetics of hESC-derived cardiomyocytes and the impact of manipulating this on cardiomyocyte function. Our principal finding is that on induction of cardiac differentiation in hESCs, a PGC-1α-dependent developmental program was engaged, which strongly stimulated mitochondrial biogenesis, consistent with cardiomyocyte maturation. Inactivating this pathway blocked mitochondrial biogenesis but also lowered levels of ROS; this led to an increased action potential and calcium transient amplitude, while at the same time made these cells vulnerable to metabolic stress.

Cardiomyocytes are rather unique in that during embryonic and fetal development they undergo dramatic maturation-related changes, progressing from small rounded cells with immature sarcomeric organization and limited energy generating capacity, to large rectangular cells with dense striated myofibrils organized in parallel to densely packed elongated mitochondria [25,26]. Cardiomyocyte mitochondria are essential in funding the ATP requirements of excitation/contraction during intense heart beating, and if respiratory function is compromised as a result of mutation, damage, or dysregulated in other ways, there may be pathological consequences [5,27,28].

Much of what we know about the genetics of mitochondrial regulation in the heart still comes from the mouse. Widely regarded as mitochondrial gene “master regulators”, PGC-1α and PGC-1β are known to be highly expressed in the heart and overexpression of PGC-1α has previously been shown to induce mitochondrial
proliferation in both the mouse heart and in cultured rat cardiomyocytes [29,30]. Single \textit{PGC-1\alpha} or \textit{PGC-1\beta} gene deletions have only a mild effect on the increase in mitochondrial mass that normally occurs during heart development and the cardiac phenotypes are subtle, mostly only apparent under metabolic challenge. By contrast, hearts of the \textit{PGC-1\alpha}/\textit{PGC-1\beta} double knockout mice are severely affected; the mice die soon postnatally and have markedly diminished mitochondrial mass and density even though up to mid-gestation mitochondria are apparently normal [13,27,31-33]. This suggests that other, \textit{PGC-1}-independent mitochondrial biogenesis-promoting genes may be involved in early mouse heart development.

Our system models human cardiac mitochondrial biogenesis using non-transformed cells and allows this to be studied in parallel with function. We found that \textit{PGC-1\alpha} alone was strongly upregulated at the very initiation of cardiac differentiation specifically in NKX2-5pos cells, regardless of the differentiation method used. The cardiomyocytes showed a regular and progressive increase in mitochondrial mass over many weeks of culture that depended on \textit{PGC-1\alpha}. Mitochondrial expansion still occurred in the complete absence of excitation/contraction, demonstrating that cardiomyocyte identity is linked to the mitochondrial biogenesis phenotype irrespective of increased energy demand. Significantly raising energy demand may stimulate it further, as we observed with DNP (Figure S3), but was not essential for the basal rate of mitochondrial biogenesis.

Since mitochondria are a principal source of ROS production, we also investigated whether \textit{PGC-1\alpha}-dependent mitochondrial changes resulted in altered ROS levels in cardiomyocytes. Although \textit{PGC-1\alpha} can induce the expression of antioxidant enzymes [34], in this context the impact of having a greater number of mitochondrial ROS-producing sites, must outweigh the induction of detoxification pathways because ROS levels were increased.

Remarkably, on \textit{PGC-1\alpha} knockdown, the amplitude and duration of the AP and the maximum amplitude of the calcium transient were increased to values closer to those in adult cardiomyocytes, despite a marked energetic defect. Energetic compromise in cardiomyocytes typically shortens the action potential as a result of increased ATP-regulated potassium current, and it decreases calcium transient amplitude [35-37]. Yet counter to this, there is evidence for an inhibitory effect of oxidative stress on the calcium current and AP duration [9,38]. Given our evidence for the oligomycin-insensitivity (at least on short term exposure) of the AP in these early cardiomyocytes, the benefit of lowered oxidative stress may be dominant in determining the overall outcome. So remarkably, repression of the
mitochondrial biogenesis program in these cells resulted in an improvement in the calcium transient. Fortunately however, the ROS levels could be lowered directly in control cardiomyocytes with a combination of culture at physiological oxygen tension and antioxidant supplementation, resulting in an improvement in the calcium transient to the same degree as PGC-1α knockdown. Based on this data, this should possibly become a standard aspect of PSC-derived cardiomyocyte culture, at least at later stages of differentiation. So while an increase in mitochondrial mass is a feature of maturity, if the ROS they produce are not controlled this may be restrictive to overall function and contractility. Further work will be required to determine precisely how ROS should be manipulated temporally to control the balance between maturation-related mitochondrial biogenesis and wider cell function.

Both raising the energetic demands of the cardiomyocyte through stimulation with fetal calf serum (FCS), or exerting physiologically relevant chronotropic stimulation through beta-adrenergic receptor activation, exposed shortcomings in PGC-1α knockdown cardiomyocytes. FCS induced a consistent hypertrophic growth, and in cells with repressed PGC-1α or uncoupled by DNP, this resulted in sarcomeric disorganization, presumably as a multi-factorial response to inadequate energy supply. The mechanism for the PGC-1α-dependent loss of automaticity on isoproterenol stimulation is unknown, as is the lower AP frequency under basal conditions. They may relate to the longer action potential we observed in these cells, the chronic energy disturbance, or an unknown connection between PGC-1α and automaticity. In line with this, both PGC-1α and PGC-1β knockout mouse hearts show blunted responses to beta-adrenergic stimulation with dobutamine, and it has been suggested they may be required for maximal automaticity of pacemaker cells, although a mechanism is lacking [27,32,33].

We set out to develop a human model of acquired heart disease by diminishing mitochondrial function. The rationale stemmed from the large body of literature reporting down-regulation of mitochondrial pathways during heart failure, but without explanation of how and why this occurs or the consequences for individual heart cells [7]. We have confirmed that hESC-derived cardiomyocytes are a valuable tool for exploring functional relationships relating to mitochondria and heart disease, revealing perhaps unexpected outcomes, and these studies go hand in hand with the goal of producing more adult-like cells for these and other applications. For maximizing their potential function, the current study emphasizes the importance of controlling ROS, since the mitochondrial biogenesis program is activated in PSC-derived cardiomyocytes.
**Experimental procedures**

**Cell culture and differentiation**

NKX2-5<sup>eGFP</sup>/hESCs generated previously [15], were maintained on mouse embryonic fibroblasts and passaged using TrypLE Select (Invitrogen). Differentiations were performed in serum-free “BPEL” media as previously described [39]. The following growth factors were present for the first 3 days of differentiation: 35ng/ml BMP4, 30ng/ml activin A, 30ng/ml VEGF, 40ng/ml SCF plus the GSK-3β inhibitor ChiR 99021 (1.5 μM). The Tankyrase inhibitor XAV 939 (1 μM) was present days 3-6. Co-culture differentiations were performed as previously described [40].

EBs were typically dissociated on day 12 of differentiation using TrypLE Select and plated onto Matrigel-coated (Invitrogen) plastic or glass.

**Lentiviral transduction**

PGC-1α or PGC-1β were overexpressed from a pLenti CMV/TO Puro DEST [41] (Addgene plasmid: 17452). The empty vector was used as the control. shR-NAs against PGC-1α were obtained from Open Biosystems in the pLKO Puro vector (TRCN0000001167 (#1) and TRCN0000001166 (#2)). A scrambled control shRNA was used as control (Addgene plasmid: 1864). The vectors used for luciferase measurements are described in supplementary experimental procedures.

**Respiration and acidification rates measured using Seahorse XF-24 analyzer**

Respiration and acidification rates were measured on adherent cells using a Seahorse XF-24 analyser (Seahorse Bioscience, North Billerica, MA) as previously described [17]. When combined with shRNA transductions, virus was added on cell plating at an appropriate titre to infect >90% of cells.

Basal acidification rates were taken as the mean rate from the second and third baseline readings, and “Max/stimulated” rates were taken after oligomycin addition. ATP production rates were calculated as previously described [17]. Maximal ATP production rates (“Max”) were calculated from the oxygen consumption rate difference between the oligomycin rate and the FCCP rate, and from the maximum ECAR rate with oligomycin. At least 3 separate experiments were performed for each cell population.

For ATP demand calculations, normal untransduced cardiomyocytes were used with the experiment set up as above. After four baseline measurements, blebbi-
statin (5 μg/ml) or nifedipine (10 μM) plus blebbistatin, or DMSO were injected and the next measurement was used for the “process inhibited” state. This was followed by oligomycin, FCCP, and rotenone and antimycin A injections. The fraction of oligomycin-sensitive respiration responsive to the drugs was calculated and the effect of buffer-alone injection was subtracted.

Confocal imaging of mitochondrial volume ratios
Cells were loaded with 40 nM MitoTracker Deep Red (Invitrogen) and imaged live in the presence of 10 μM nifedipine on a Leica SP5 confocal. hESCs and GFPneg cells were also loaded with Calcein-AM (1 μM) for imaging the cell volume. Image acquisition and data analysis were as previously described [17].

Confocal imaging for calculation of cell volume
Cells were imaged live in the presence of 10 μM nifedipine on a Leica SP5 confocal within a 37°C chamber using a Plan-Apochromat 40x/1.25 oil lens. Sequential imaging stacks were taken through the entire thickness of Calcein-AM-loaded cardiomyocytes and their volumes calculated by calibration to 4 μm TetraSpek fluorescent microsphere standards (Invitrogen) imaged in the same way. Further details are described in supplemental experimental procedures.

Reactive oxygen species and TMRM measurements
For reactive oxygen species measurement cell cultures were dissociated using TrypLE Select and labeled for 30 minutes at 37°C with 20 μM dihydroethidium (DHE) (Molecular Probes) and washed twice with buffer before being measured immediately by FACS. For TMRM measurements, 5 nM TMRM (Invitrogen) was added in BPEL media the day before measurement. Cells were dissociated as above but with TMRM present throughout and also during measurement.

Electrophysiological characterization
Action potentials (AP) were measured 7-15 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.

APs were recorded at 37°C 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 from single as well as from small cluster of cardiomyocytes. The clusters were spontaneously active, while in single cardiomyocytes APs were elicited by 3 ms 1.2x threshold current pulses through the patch pipette at 1 Hz. AP parameter values obtained from 10 consecutive APs were averaged and data were collected from at least 3 independent differentiations per condition.

Calcium imaging
Intracellular Ca^{2+} ([Ca^{2+}]) was measured at 37°C in indo-1 loaded clusters of cardiomyocytes. In brief, cardiomyocytes were loaded with 5 μM of the fluorescent dye Indo-1-AM (Molecular Probes, Eugene, OR, USA) for 50 min at 37°C in Tyrode’s solution. The cardiomyocyte clusters were stimulated at 0.5 Hz using field stimulation. Dual wavelength emission of Indo-1 upon excitation at 340 nm was recorded at 405–440 and 505–540 nm using photomultiplier tubes, and, after correction for background fluorescence, free [Ca^{2+}], was calculated as previously described [42].

Sarcoplasmic reticulum (SR) calcium content was analyzed using rapid cooling (RC) with ice-cold (0–1°C) Tyrode’s solution, and typically results in depletion of calcium from SR and calcium released remains confined to the cytoplasm [43]. Data were collected from at least 3 independent differentiations per condition.

Acknowledgments: This research was support by grants from: Human Frontiers Research Program (MB); European Research Council ERC 323182 (to CM); ZonMW Animal Alternatives, 114000101 (SC); Netherlands Institute of Regenerative Medicine (MB); Rembrandt Institute of Cardiovascular Science RICS (CM and GK). Netherlands Genomics Initiative NGI/NWO 05040202 (PM); Marie Curie IRG 247918 (PM). The Seahorse Extracellular Flux Analyzer has been purchased with the generous contribution of the Dorpmans-Wigmans Stichting.

We thank Richard Davis, Stefan Braam and Dorien Ward for advice and protocols for the spin EB differentiation method and for discussion on the data. We thank Arie Verkerk for help with manuscript preparation. We thank David Hood (York University, Ontario, Canada) for the PGC-1α promoter plasmid.

CLM is co-founder and consultant of Pluriomics bv.
Supplemental information

Figure S1

**Figure S1.** GFPneg population identity, and differentiation to beating NKX2-5pos (GFPpos) cardiomyocytes without mitochondrial ATP production.

(A) FACS plots of a differentiated unsorted population (day 20) with surface makers: NG2, PDGFRα, PDGFRβ and CD146 which represent pericyte/smooth muscle cell makers; CD105, CD73, CD90 which represent mesenchymal stem cell makers; and CD31 which marks endothelial cells. The x-axis shows GFP fluorescence. (B) Immunocytochemistry of a similar mixed population for smooth muscle/fibroblast markers: vimentin, SM22α and calponin, and the endodermal marker AFP. A green troponin I label was added to clearly identify the GFPpos cardiomyocytes. The scale bar is 100 μm. (C) Embryoid bodies exposed to the indicated inhibitors at day 3, refreshed every 3 days and imaged here at day 12 with phase contrast overlayed with GFP. 21% O₂ indicates the differentiation was at ambient O₂ throughout. 3% O₂ indicates that the differentiation was transferred to 3% O₂ at day 3 and maintained thereafter. The + indicates that some of the EBs under this condition were beating. The - sign indicates that none of the EBs were beating. Rotenone caused high levels of toxicity presumably due to stimulated ROS production. The inhibitory effect of antimycin A could be rescued at low oxygen also suggesting an involvement of ROS. 0.5 μg/ml oligomycin is sufficient to achieve maximum inhibition of the ATP synthase and still permitted differentiation to beating cells. The smaller size of the EBs under low oxygen or in the presence of mitochondrial inhibitors was representative.
Figure S2. Expression of mitochondrial regulator genes changes during human heart development; PGC-1α shRNA validation; and PGC-1α isoform-specific PCR during cardiac differentiation.

(A) A reproduction of microarray data from Elliott et al., 2011 showing relative mRNA levels in hESCs, 9 week human fetal heart (9w), 10 week fetal heart (10w), 12 week fetal heart (12w) and adult human heart. For adult human heart the data are represented by the mean ± SEM from 3 independent tissue samples. (B) Real-time PCR amplification traces showing the housekeeping gene hARP with PGC-1α1 and PGC-1α4 in day 3 and day 12 embryoid bodies (EBs). (C) GFPpos cardiomyocytes were isolated by FACS 7 days after transduction (at day 12 of differentiation) with the scrambled control shRNA, PGC-1α shRNA #1 or PGC-1α shRNA #2 including a puromycin selection step. RNA was isolated immediately and expression of PGC-1α assessed by real-time PCR. Expression was normalized to the housekeeping gene hARP and is shown relative to the scrambled shRNA control. Data are represented as mean ± SEM from 3 independent experiments.
Figure S3. The effects of reactive oxygen species and energetic stress on mitochondrial biogenesis in cardiomyocytes, and gene expression changes with differentiation at 21% or 3% O$_2$.

(A) Mitochondria-to-cell volume ratios in cardiomyocytes transferred at day 12 to low oxygen conditions (3% O$_2$) compared to ambient control conditions (21% O$_2$). Replicated time-points are represented by the mean ± range. The data was accumulated from 5 independent experiments. (B) Mitochondria-to-cell volume ratios in cardiomyocytes measured at day 45 following 33 days under test conditions while maintained under ambient or low oxygen. Low concentrations of H$_2$O$_2$, rotenone or antimycin A should all increase ROS levels and DNP will induce energetic stress by uncoupling mitochondria and leading to ATP hydrolysis by the ATP-synthase. H$_2$O$_2$ = hydrogen peroxide, Rot = rotenone, AMA = antimycin A, DNP = 2,4-dinitrophenol. Data are represented as mean ± SEM from 3-5 independent experiments. Statistical significance was calculated using a one-way ANOVA with Dunnett’s correction. *p<0.05. (C) GFPpos cardiomyocytes were isolated by FACS at day 12 of differentiation with days 6-12 of differentiation at either 21% or 3% O$_2$ culture. Expression was normalized to the housekeeping gene hARP and is shown relative to the hESC population.
Figure S4. Contraction rates of beating cardiomyocyte clusters on exposure to oligomycin.

(A) Contraction rates of individual beating clusters under basal conditions and after exposure to 0.5 μg/ml oligomycin (measured again after approximately 15 minutes). (B) The data from A presented as a box and whisker plot. Experiments were performed in “BPEL” media with 5% CO₂. The differences were not significantly different when tested with a paired t test.
Figure S5. Calcium transient characteristics with PGC-1α knockdown in clusters of cardiomyocytes paced at 1Hz.

Quantifications of calcium transients from four different treatment groups showing (A) diastolic concentration, (B) systolic concentration, and (C) transient amplitude. Statistical significance was calculated using an unpaired t test. *p<0.001 (data are from 3 independent experiments).

Extended experimental procedures

Cell culture and differentiation

NKX2-5eGFP/wt hESCs generated previously [15] were maintained on mouse embryonic fibroblasts in DMEM/F12 containing 20% Knockout Serum Replacement and 10ng/ml FGF2. Cultures were passaged using TrypLE Select (Invitrogen). Differentiations were performed in serum-free “BPEL” media composed of: IMDM/F12, 5% Protein-free hybridoma mixture II, 0.25% deionized BSA, 0.125% PVA, 400 μM α-monothioglycerol, 1X chemically defined lipids, 50 μg/ml ascorbic acid 2-AP, 0.1X Insulin/Transferrín/Selenium (ITS-X) and 2 mM Glutamax. This media has been previously described [39]. After feeder depletion 3000 cells are deposited in individual wells of an uncoated V-shaped 96 well plate (Greiner) and centrifuged to aid aggregation. The following growth factors were present for the first 3 days of differentiation: 35ng/ml BMP4, 30ng/ml activin A, 30ng/ml VEGF, 40ng/ml SCF plus the GSK-3β inhibitor ChiR 99021 (1.5 μM). The Tankyrase inhibitor XAV 939 (1 μM) was present days 3-6. Co-culture differentiations were performed as previously described [40].

EBs were typically dissociated on day 12 of differentiation using TrypLE Select and plated onto tissue culture plastic. For imaging, cells were plated onto 35 mm glass-bottomed dishes or 24-well glass bottomed plates (MatTek). All surfaces were pre-coated with Matrigel (Invitrogen).
Cell sorting based on GFP was performed using a FACS Aria III (Becton Dickinson) using an 85 μm nozzle. Cells were collected into BPEL media and then plated or immediately processed for RNA as below.

**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 CFX96 machine. Primer sequences were described previously [17]. Gene expression changes were normalized to the housekeeping gene human acidic ribosomal phosphoprotein (hARP).

**Immunocytochemistry**

Cells were fixed with 2% paraformaldehyde for 20 minutes and permeabilised with 0.1% Triton X. Blocking was achieved with 4% normal goat serum. Primary antibody incubations were overnight in 4% normal goat serum at 4°C using the following antibodies at the following dilutions: anti-troponin I (1:500; clone H170, Santa Cruz), anti-α actinin (sarcomeric) (1:800; clone EA53, Sigma), anti-vimentin (1:300; Sigma V6630), anti-SM22α (1:300; Abcam ab14106), anti-calponin (1:300; Sigma C2687), anti-alpha-fetoprotein (AFP) (1:200; sc-15375). For surface stainings live cells were labeled with the following conjugated antibodies for 20 mins on ice followed by washing and measuring by FACS: mouse IgG1-PE isotype control (Miltenyi Biotec 130-098-845), anti-NG2-PE (R&D FAB2585P), anti-PDGFRa-PE (BD 556002), anti-PDGFRb-PE (BD 558821), anti-CD146-PE (BD 550315), anti-CD105-PE (Life Technologies MHCD10504), anti-CD73-PE (BD 550257), anti-CD90-PE (BD 555596), anti-CD31-APC (eBioscience 17-0319-42, clone: WM59).

**Lentiviral transduction**

cDNA for PGC-1α was obtained from a previously generated plasmid [44] (Addgene plasmid: 10974) and cDNA for PGC-1β was obtained from Open Biosystems (clone ID: 40146993). These were cloned into a pLenti CMV/TO Puro DEST [41] (Addgene plasmid: 17452). The empty vector was used as the control. shRNAs against PGC-1α were obtained from Open Biosystems in the pLKO Puro vector (TRCN0000001167 (#1) and TRCN0000001166 (#2)). A scrambled control shRNA was used as control [45] (Addgene plasmid: 1864).

For luciferase experiments, the 2.2 kb promoter of PGC-1α was obtained from a previously described vector (Ircher et al., 2008, 2009), and a 0.6 kb EcoRI-
5’-digested version (cuts at -633 bp from start site) was also generated. These were cloned into pENTR-LUC (Addgene plasmid: 17473) and recombined with pLenti X1 Puro DEST (Addgene plasmid: 17297). An empty pENTR-LUC vector was recombined and used for the 0 kb control. All generated constructs were confirmed by sequencing. NKX2-5<sup>eGFP</sup>/w hESCs were transduced at low titer with the aim of achieving no more than one integration per cell and then selected with Puromycin to generate stable lines.

For packaging lentiviruses, 293FT cells were used (Invitrogen). Lentiviral supernatants were concentrated using ultracentrifugation and titers were used to infect around 80-90% of the cells based upon a CMV-GFP control virus. Cells were selected with Puromycin for 3 days and allowed to recover before analysis.

**Luciferase measurements**

Suspension EBs or hESCs on Matrigel were lysed on the indicated day and luciferase was measured on a luminometer (PerkinElmer VICTOR3 V Multilabel counter model 1420) following substrate addition (Promega).

**Respiration and acidification rates measured using Seahorse XF-24 analyzer**

Respiration and acidification rates were measured on adherent cells using a Seahorse XF-24 analyser (Seahorse Bioscience, North Billerica, MA). The XF24 V7 assay plates were coated with Matrigel (1:100) prior to use. hESCs were seeded 24 hours before measurement at a density of 3x10<sup>4</sup> cells/well, and feeder only wells were used to deduct the feeder carry-over contribution; GFPpos cells (cardiomyocytes) and GFPneg cells were seeded at a density of 8x10<sup>4</sup> cells/well and 5x10<sup>4</sup> cell/well respectively, and measured after 7 days. When combined with shRNA transductions, virus was added on plating at an appropriate titre to infect >90% of cells. FACS-sorted GFPpos and GFPneg populations were used to generate the data in Figure 1, whereas manually selected high percentage GFPpos EBs were used for the data in Figure 3.

The assay was performed in bicarbonate-free DMEM (Sigma D-5030) supplemented with 15 mM glucose, 2 mM L-glutamine, 0.5 mM sodium pyruvate, 10 mM NaCl and 0.4% Bovine Serum Albumin. Cells were washed twice and preincubated in this medium for 1 hour before measurement. Oligomycin was used at 0.5 μg/ml; FCCP was titrated in 2/3 injections to 1.5 μM for hESCs and 2.5 μM for GFPpos and GFPneg populations; rotenone and antimycin A were added at 1 μM and 2 μM respectively. hESCs cannot reach maximum respiratory rate when uncoupling is performed after oligomycin addition, so with these cells,
separate wells were used for the oligomycin measurement and the uncoupled rates as performed previously [17]. Oxygen consumption values were calculated from 3-minute measurement cycles by the XFReader software Version 1.4 updated with a recent correction [46]. Basal acidification rates were taken as the mean rate from the second and third baseline readings, and “Max/stimulated” rates were taken after oligomycin addition. After the assay a standard protein assay was performed. ATP production rates were calculated as previously described [17]. Maximal ATP production rates (“Max”) were calculated from the oxygen consumption rate difference between the oligomycin rate and the FCCP rate, and from the maximum ECAR rate with oligomycin. At least 3 independent experiments were performed for each cell population.

For ATP demand calculations, normal untransduced cardiomyocytes were used with the experiment set up as above. After four baseline measurements, blebbistatin (5 μg/ml) or nifedipine (10 μM) plus blebbistatin, or DMSO were injected and the next measurement was used for the “process inhibited” state. This was followed by oligomycin, FCCP, and rotenone and antimycin A injections. The fraction of oligomycin-sensitive respiration responsive to the drugs was calculated and the effect of buffer-alone injection was subtracted.

Confocal imaging of mitochondria-to-cell volume ratios
Cells were loaded with 40 nM MitoTracker Deep Red (Invitrogen) for 20 mins in the ‘experimental buffer’ described above and imaged in the same media with the addition of 10 μM nifedipine on a Leica SP5 confocal. hESCs and GFPneg cells were also loaded with Calcein-AM (1 μM) for imaging the cell volume. Single planes of 1024x1024 pixel images at 44 nm/pixel resolution were recorded using a Plan-Apochromat 100x/1.4 oil lens. GFP/Calcein-AM and MitoTracker Deep Red were excited simultaneously using the Argon 488 nm and HeNe 633 nm laser lines at high intensity and fluorescence emission was collected in the range of 500-530 nM and above 640 nM, respectively. Image acquisition approach and data analysis were performed as previously described using Image Analyst MKII (Novato, CA, USA) [17,46]. At least 25 images (from different cells) were acquired for each experimental replicate.

Confocal imaging for calculation of cell volume
Cells were incubated with “experimental buffer” plus 10 μM nifedipine and imaged live on a Leica SP5 confocal within a 37°C chamber using a Plan-Apochromat 40x/1.25 oil lens. Around 25 single GFPpos cells were first identified in each experiment and their co-ordinates saved. Wide field GFP images of these
co-ordinates were first recorded for reference. The cells were then loaded with Calcein-AM (200 nM) and incubated for 20 minutes to ensure all cells were brightly and uniformly fluorescent. Sequential imaging stacks were taken through the entire thickness of each cell at 0.5 μM steps with 1024x1024 pixel images at 378 nm/pixel resolution. To calibrate the volume calculation 4 μm TetraSpek fluorescent microsphere standards (Invitrogen) were imaged in the same way. Image analysis was performed using Image Analyst MKII (Novato, CA, USA). A cut-off threshold of 12.5% of pixel intensity was set to exclude obviously out of plane light and the data was binarized. The sum of pixel values through the z-stack was calculated and calibrated to our microsphere standards to give the final cell volume.

**Reactive oxygen species and TMRM measurements**

For reactive oxygen species measurement cell cultures were dissociated using TrypLE Select, diluted in appropriate culture media and centrifuged, then labeled for 30 minutes at 37°C with 20 μM dihydroethidium (DHE) (Molecular Probes) in “experimental buffer” as described above. The cells were washed twice with buffer before being measured immediately by FACS. For TMRM measurements, 5 nM TMRM (Invitrogen) was added in BPEL media the day before measurement. Cells were dissociated as above but with TMRM present throughout and also during measurement. FL-1 and FL-2 channels were recorded for GFP and DHE or TMRM respectively.

**Electrophysiological characterization**

Action potential (AP) were measured 7-15 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.

APs were recorded at 37°C 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 from single as well as from small cluster of cardiomyocytes. The clusters were spontaneously active, while in single cardiomyocytes APs were elicited by 3 ms 1.2x threshold current pulses through the patch pipette at 1 Hz. APs were characterized by duration at 50 and 90% repolarization (APD₅₀,
and APD\textsubscript{90}, respectively), maximal diastolic potential (MDP), AP amplitude, maximal upstroke velocity, and, in case spontaneously active, frequency. AP parameter values obtained from 10 consecutive APs were averaged and data were collected from at least 3 independent differentiations per condition.

**Calcium imaging**

Intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) was measured at 37°C in indo-1 loaded clusters of cardiomyocytes. In brief, cardiomyocytes were loaded with 5 μM of the fluorescent dye Indo-1-AM (Molecular Probes, Eugene, OR, USA) for 50 min at 37°C in Tyrode’s solution. The cardiomyocyte clusters were stimulated at 0.5 Hz using field stimulation. Dual wavelength emission of Indo-1 upon excitation at 340 nm was recorded at 405–440 and 505–540 nm using photomultiplier tubes, and, after correction for background fluorescence, free [Ca\textsuperscript{2+}]\textsubscript{i}, was calculated as previously described [42].

Sarcoplasmic reticulum (SR) calcium content was analyzed using rapid cooling (RC). RC was performed by fast superfusion (<200 ms) with ice-cold (0–1°C) Tyrode’s solution, and typically results in depletion of calcium from SR and calcium released remains confined to the cytoplasm (Bers, 1987). We analyzed diastolic, systolic and Ca\textsuperscript{2+} transient amplitudes. Data were collected from at least 3 independent differentiations per condition.

**Statistical methods**

Data are mean ± S.E.M. Data were statistically analyzed using GraphPad Prism version 5.0 for Windows. \textit{p}<0.05 defined statistical significance.
References


