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Supplemental Information

PGC-1α and Reactive Oxygen Species Regulate Human Embryonic Stem Cell-Derived Cardiomyocyte Function

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Supplemental information – Inventory

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Supplemental information

Figure S1

A

B

C

Vehicle

1 µM rotenone

1 µM antimycin A

1 µg/ml oligomycin

21% O₂

3% O₂

200 µm

200 µm

200 µm

200 µm
Figure S3

A

Mitochondria-to-cell volume ratio

- 21% O₂
- 3% O₂

Differentiation (days)

0 10 20 30 40 50 60 70

B

Mitochondria-to-cell volume ratio

21% O₂ Control
21% O₂ + 10 mM H₂O₂
21% O₂ + 15 mM Rot
21% O₂ + 7.5 mM AMA
21% O₂ + 100 μM DNP
3% O₂ Control
3% O₂ + 10 mM H₂O₂
3% O₂ + 15 mM Rot
3% O₂ + 7.5 mM AMA
3% O₂ + 100 μM DNP

C

Gene expression changes with differentiation at 21% O₂

PGC-1α
PGC-1β
RIP140

Relative gene expression

hESC GFP+

Gene expression changes with differentiation at 3% O₂

PGC-1α
PGC-1β
RIP140

Relative gene expression

hESC GFP+
Figure S4

A (n=19)

B (n=19)
Figure S5

A) Diastolic $[\text{Ca}^{2+}]$

B) Systolic $[\text{Ca}^{2+}]$

C) Transient amplitude

[Graphs showing data with symbols and error bars for different conditions: Scr shRNA, PGC-1α shRNA, 3%O₂+6mM NAC, and Control. Significant differences indicated by asterisks (*).]
**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% O2.** (A) Mitochondria-to-cell volume ratios in cardiomyocytes transferred at day 12 to low oxygen conditions (3% O2) compared to ambient control conditions (21% O2). 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 H2O2, 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. H2O2 = 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% O2 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/w hESCs generated previously (Elliott et al., 2011) 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/Transferrin/Selenium (ITS-X) and 2 mM Glutamax. This media has been previously described (Ng et al., 2008).

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 (Mummery et al., 2007).

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 (Birket et al., 2011). 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-PDGFRα-PE (BD 556002), anti-PDGFRβ-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 (Ichida et al., 2002) (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 (Campeau et al., 2009) (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 (Sarbassov et al., 2005) (Addgene plasmid: 1864).
For luciferase experiments, the 2.2 kb promoter of PGC-1α was obtained from a previously described vector (Irrcher 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-eGFP/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 3x10⁴ 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 8x10⁴ cells/well and 5x10⁴ 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 GFP pos 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 GFP pos and GFP neg 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 (Birket et al., 2011). Oxygen consumption values were calculated from 3-minute measurement cycles by the XFReader software Version 1.4 updated with a recent correction (Gerencser et al., 2009). 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 (Birket et al., 2011). 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) (Birket et al., 2011; Gerencser et al., 2012). 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 APD₉₀, 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\(^{2+}\) ([Ca\(^{2+}\)]_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\(^{2+}\)]_i was calculated as previously described (Baartscheer et al., 1996).

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\(^{2+}\) 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. \(p<0.05\) defined statistical significance.
References

Baartscheer, A., Schumacher, C.A., Opthof, T., and Fiolet, J.W.T. (1996). The Origin of Increased Cytoplasmic Calcium upon Reversal of the Na+/Ca2+-Exchanger in Isolated Rat Ventricular Myocytes. J. Mol. Cell. Cardiol. 28, 1963–1973.

Bers, D.M. (1987). Ryanodine and the calcium content of cardiac SR assessed by caffeine and rapid cooling contractures. Am. J. Physiol. Cell. Physiol. 253, C408–C415.

Birket, M.J., Orr, A.L., Gerencser, A.A., Madden, D.T., Vitelli, C., Swistowski, A., Brand, M.D., and Zeng, X. (2011). A reduction in ATP demand and mitochondrial activity with neural differentiation of human embryonic stem cells. J. Cell. Sci 124, 348–358.

Campeau, E., Ruhl, V.E., Rodier, F., Smith, C.L., Rahmberg, B.L., Fuss, J.O., Campisi, J., Yaswen, P., Cooper, P.K., and Kaufman, P.D. (2009). A versatile viral system for expression and depletion of proteins in mammalian cells. PLoS One 4, e6529.

Elliott, D.A., Braam, S.R., Koutsis, K., Ng, E.S., Jenny, R., Lagerqvist, E.L., Biben, C., Hatzistavrou, T., Hirst, C.E., Yu, Q.C., et al. (2011). NKX2-5GFP/w hESCs for isolation of human cardiac progenitors and cardiomyocytes. Nat. Meth. 8, 1037–1040.

Gerencser, A.A., Neilson, A., Choi, S.W., Edman, U., Yadava, N., Oh, R.J., Ferrick, D.A., Nicholls, D.G., and Brand, M.D. (2009). Quantitative Microplate-Based Respirometry with Correction for Oxygen Diffusion. Anal Chem. 81, 6868-6878

Gerencser, A.A., Chinopoulos, C., Birket, M.J., Jastroch, M., Vitelli, C., Nicholls, D.G., and Brand, M.D. (2012). Quantitative measurement of mitochondrial membrane potential in cultured cells: calcium-induced de- and hyperpolarization of neuronal mitochondria. J. Physiol. (Lond.) 590, 2845–2871.

Ichida, M., Nemoto, S., and Finkel, T. (2002). Identification of a specific molecular repressor of the peroxisome proliferator-activated receptor gamma Coactivator-1 alpha (PGC-1alpha). J. Biol. Chem. 277, 50991–50995.

Irrcher, I., Ljubicic, V., Kirwan, A.F., and Hood, D.A. (2008). AMP-activated protein kinase-regulated activation of the PGC-1alpha promoter in skeletal muscle cells. PLoS ONE 3, e3614.

Irrcher, I., Ljubicic, V., and Hood, D.A. (2009). Interactions between ROS and AMP kinase activity in the regulation of PGC-1alpha transcription in skeletal muscle cells. Am. J. Physiol., Cell Physiol 296, C116–123.

Mummery, C.L., Ward, D., and Passier, R. (2007). Differentiation of human embryonic stem cells to cardiomyocytes by coculture with endoderm in serum-free medium. Curr. Protoc. Stem Cell Biol. Chapter 1, Unit 1F.2.

Ng, E.S., Davis, R., Stanley, E.G., and Elefanty, A.G. (2008). A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat. Protocols 3, 768–776.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307, 1098–1101.