Distinct profiles of human embryonic stem cell metabolism and mitochondria identified by oxygen

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Abstract

Oxygen is a powerful regulator of cell function and embryonic development. It has previously been determined that oxygen regulates human embryonic stem (hES) cell glycolytic and amino acid metabolism, but the effects on mitochondria are as yet unknown. Two hES cell lines (MEL1, MEL2) were analyzed to determine the role of 5% (physiological) and 20% (atmospheric) oxygen in regulating mitochondrial activity. In response to extended physiological oxygen culture, MEL2 hES cells displayed reduced mtDNA content, mitochondrial mass and expression of metabolic genes *TFAM*, *NRF1*, *PPAR α* and *MT-ND4*. Furthermore, MEL2 hES cell glucose consumption, lactate production and amino acid turnover were elevated under physiological oxygen. In stark contrast, MEL1 hES cell amino acid and carbohydrate use and mitochondrial function were relatively unaltered in response to oxygen. Furthermore, differentiation kinetics were delayed in the MEL1 hES cell line following BMP4 treatment. Here we report the first incidence of metabolic dysfunction in a hES cell population, defined as a failure to respond to oxygen concentration through the modulation of metabolism, demonstrating that hES cells can be perturbed during culture despite exhibiting the defining characteristics of pluripotent cells. Collectively, these data reveal a central role for oxygen in the regulation of hES cell metabolism and mitochondrial function, whereby physiological oxygen promotes glucose flux and suppresses mitochondrial biogenesis and gene expression.

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Introduction

Currently, the characterization of human embryonic stem (hES) cells focuses on examining the pluripotency, renewal capacity, differentiation ability and genome integrity of the cell. In contrast, the metabolic state of the cell is rarely considered. An assessment of hES cell function does not necessarily reflect underlying cell metabolism, and it has been shown that culture components can impact hES cell metabolism with no effect on self-renewal (Rathjen *et al.* 2013, Harvey *et al.* 2014). Appropriate modulation and regulation of metabolism is an essential aspect of cellular function due to the interaction of metabolism with the epigenome (Panopoulos *et al.* 2011, Shiraki *et al.* 2014). Through epigenetic modulation, abnormal patterns of metabolic function can lead to heritable alterations in the DNA landscape and in gene expression. The projected applications of hES cells in clinical medicine, as models of disease, components of cell-based devices and, ultimately, a source of cells for transplantation into humans, demand that the standards used to determine cell function in hES cells need to be higher than those used for other cultured cells. It is not sufficient to limit the focus of hES cell characterisation to potency, self-renewal and genomic integrity.

Embryonic stem (ES) cell metabolism is characterized by a heavy dependency on glycolysis, with 50–70% of glucose being converted to lactate (Kondoh *et al.* 2007, Varum *et al.* 2011, Zhang *et al.* 2011, Zhou *et al.* 2012, Rathjen *et al.* 2013, Harvey *et al.* 2014). Like many tumor cells and mammalian blastocysts, aerobic glycolysis is used preferentially by embryonic stem cells (Warburg 1956, Gott *et al.* 1990, Wu *et al.* 2007, Gardner 2015). Oxidative phosphorylation (OXPHOS) occurs at relatively low levels in ES cells compared to their differentiated counterparts, and ES cells contain comparatively lower mitochondrial DNA (mtDNA) copy numbers and mitochondrial mass (Cho *et al.* 2006, Varum *et al.* 2011). Although utilized at reduced levels, OXPHOS is required for ES cell function; its inhibition impacts self-renewal and the expression of pluripotency markers (Facucho-Oliveira *et al.* 2007, Varum *et al.* 2009, Todd *et al.* 2010, Mandal *et al.* 2011).

Oxygen, as a nutrient in cell culture, is an important regulator of cell metabolism and cell state (Simon & Keith 2008). The mammalian preimplantation embryo, from which ES cells are derived, develops under relatively low oxygen conditions *in vivo*, ~2–9% oxygen (Fischer & Bavister 1993). Embryo culture in atmospheric oxygen (20%), compared to physiological

concentrations, is associated with perturbed gene expression (Harvey *et al.* 2004, Rinaudo *et al.* 2006) changes to the proteome (Katz-Jaffe *et al.* 2005), alterations in metabolic activity (Lane & Gardner 2005, Wale & Gardner 2012, Wale & Gardner 2013) and retarded development in several species, including the human (Thompson *et al.* 1990, Gardner & Lane 1996, Meintjes *et al.* 2009, Waldenström *et al.* 2009). In previous reports, hES cell lines showed changes in metabolism (Forristal *et al.* 2013, Christensen *et al.* 2014, Harvey *et al.* 2014), epigenetics (Petrucelli *et al.* 2014), transcription (Forsyth *et al.* 2008, Westfall *et al.* 2008), self-renewal capacity (Prasad *et al.* 2009), clonal recovery (Forsyth *et al.* 2006, Hewitt *et al.* 2006) and pluripotency (Ezashi *et al.* 2005) in response to changes in environmental oxygen concentration. For example, culture of hES cells in physiological oxygen (~5%) results in increased glucose consumption and increased lactate production when compared to cells cultured in 20% oxygen (Forristal *et al.* 2013, Harvey *et al.* 2014, Turner *et al.* 2014). Increased glucose consumption in physiological oxygen is accompanied by increased glycolytic gene expression (Westfall *et al.* 2008, Harvey *et al.* 2014). Although some variation exists in the literature, it is now possible to describe with confidence the metabolism of hES cells in culture and the changes that occur within the cells as oxygen concentrations are varied.

hES cells predictably increase their uptake of glucose in response to low oxygen consistent with the mammalian blastocyst (Wale & Gardner 2012). This suggests that mitochondrial function is concomitantly regulated by oxygen in the culture environment. Somatic cell mitochondrial activity and biogenesis have been shown to be regulated by oxygen (Piruat & Lopez-Barneo 2005). The effects of oxygen on mitochondrial activity and biogenesis, and on mitochondrial gene transcription, have not been examined in hES cells. Mitochondrial regulators influence hES cell differentiation (Prowse *et al.* 2012) and cell survival (Todd *et al.* 2010), and generation of iPS cells requires remodeling of mature, somatic mitochondria into a pluripotent mitochondrial state (Folmes *et al.* 2011). Here, we determined the role of oxygen in the regulation of mitochondria in hES cells in culture. Physiological oxygen was shown to significantly alter MEL2 hES cell mitochondrial function and metabolite turnover. MEL2 is a well-characterized hES cell line that shows the expected hES cell metabolism in culture (Forristal *et al.* 2013, Christensen *et al.* 2014, Harvey *et al.* 2014, Turner *et al.* 2014). As such, these data confirm that oxygen is a regulator of hES cell mitochondrial activity. In contrast, oxygen failed to elicit a response in mitochondrial function in a second hES cell line examined, MEL1 hES cells. Comparison of the metabolism of MEL1 with MEL2 cells revealed significant differences, suggesting that the population of MEL1 cells examined here is metabolically aberrant despite demonstrating the defining

characteristics of a pluripotent cell. The pluripotency of these lines was indistinguishable, with both able to form the three primary germ lineages. However, differences in differentiation kinetics were detected, specifically delays in differentiation from MEL1 hES cells. The findings reported here suggest that human ES cells in culture can lose the ability to respond to physiological stimuli, or lose metabolic fidelity, in the absence of overt changes in standard hES cell characteristics, and identify a need for metabolic screening of hES cell lines before use in research or clinical applications.

Materials and methods

hES cell culture

hES cells MEL1 and MEL2 (Australian Stem Cell Centre, Monash, Vic., Australia) were maintained in mTeSR1 medium (Stem Cell Technologies, Tullamarine, Vic., Australia; Ludwig *et al.* 2006) on ES-cell qualified Matrigel (BD Biosciences, Scoresby, Vic., Australia) coated tissue culture plates (Becton Dickinson, Scoresby, Vic., Australia). Cells were maintained at 37 °C in an atmosphere of 5% CO₂ and either 5% or 20% oxygen. Cells were passaged every 5–7 days using Dispase (Stem Cell Technologies), with medium refreshed every 24 h in a standard biosafety cabinet. To minimize exposure to atmospheric oxygen conditions, medium was pre-equilibrated under respective oxygen conditions. All cell cultures were acclimated to 5% or 20% oxygen conditions for a minimum of two passages before use. MEL1 hES cells were analyzed between passages 28 and 33, and MEL2 hES cells between passages 30 and 35. Images of hES cells were captured on a Nikon Eclipse Ti-U inverted microscope using a Photometrics Coolsnap HQ² camera and acquired using NIS-Elements BR 3.0 software (Nikon Instruments Inc., Melville, NY, USA).

RNA isolation and reverse transcription

Total RNA was isolated from MEL1 and MEL2 cell lines using Absolutely RNA Miniprep kit (Agilent Technologies, Mulgrave, Vic., Australia). DNase-treated RNA was quantified using a 2000c Thermo Nanodrop (Thermo Fisher Scientific, Scoresby, Vic., Australia) spectrophotometer. cDNA was synthesized from 1 µg of RNA using M-MLV Reverse Transcriptase and oligo(dT) primers (Promega). Triplicate reactions of each sample were performed using EvaGreen Master Mix (Solis BioDyne, Tartu, Estonia) on a ViiA7 thermocycler (Applied Biosystems, Foster City, CA, USA) according to the following sequence: 50 °C for 5 min, 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, final extension at 72 °C for 5 min followed by melt curve analysis. Primers were designed using Primer3Plus (Untergasser *et al.* 2012) and verified using NCBI-Primer Blast (Ye *et al.* 2012) or were sourced from the literature as listed in Table 1. The relative concentration of each gene was normalized to that of *RPLP0* and analyzed using Q-Gen (Muller *et al.* 2002), which is based on the $\Delta\Delta C_t$ method. All PCR products were verified by sequencing.

Table 1 Human nuclear and mitochondrial PCR primers used to determine mitochondrial metabolism, pluripotency and differentiation.

Gene	Size	Forward	Reverse	Reference
<i>PGC1a</i>	116	CAGAGAACAGAAACAGCAGCA	TGGGGTCAGAGGAAGAGATAAA	^{-b}
<i>NRF2</i>	131	GGATACAGTGCAGCGGAGTT	TCCGTTTGACAGAGAAGCAGT	^{-b}
<i>PPARa</i>	106	GCAATCCATCGGCGAGGATA	TGAAAGCGTGTCCTGATGA	^{-b}
<i>UCP2</i>	100	TGTCCGCATCGGCCTGTAT	ACCTGTGGTGCTGCCTGCTA	^{-b}
<i>AMPKa1</i>	95	CGACAAGCCCCACCTGATTCT	TGCCCTTGGTGTTTCAGCAA	^{-b}
<i>AMPKa2</i>	105	GGCACCTCCCATTTGATGA	GAGAGTGGCGACAGAACGAT	^{-b}
<i>TFAM</i>	83	AAGATTCCAAGAAGCTAAGGGTGA	CAGAGTCAGACAGATTTTCCAGTT	Li <i>et al.</i> (2005)
<i>POLGa</i>	118	CAACCCCTAGCTCTGACTGC	GAGGCAGCTTGAAAAACCAAG	Armstrong <i>et al.</i> (2010)
<i>NRF1</i>	102	AGGAACACGGAGTGACCCAA	TATGCTCGGTGTAAGTAGCCA	Primerbank: 93141038c1
<i>DNMT1</i>	213	GCACAACTGACCTGCTTCA	GCCTTTTACCTCCATCAAA	Reichard <i>et al.</i> (2007)
<i>COX4-1</i>	129	GTTTCACCGCGCTCGTTATC	CCTGGATGGGGTTCACCTTC	^{-b}
<i>COX4-2</i>	169	ACTACCCCATGCCAGAAGAG	TCATTGGAGCGACGGTTCATC	Primerbank: 17999525c1
<i>MT-ND1^a</i>	140	GAGCAGTAGCCCAACATCTC	GGGTCTAGTGGCAGGAGTAAT	Nagao <i>et al.</i> (2008)
<i>MT-ND2^a</i>	129	CGGCCTGCTTCTTCTACAT	CCACCTCAACTGCCTGCTAT	^{-b}
<i>MT-ND4^a</i>	151	GCCCAAGAACTATCAAACCTCTGA	CGGCAAGTACTATTGACCCAGC	Lu <i>et al.</i> (2007)
<i>MT-ND5^a</i>	272	ACATCTGTACCCACGCCCTC	CAGGGAGGTAGCGATGAGAG	Armstrong <i>et al.</i> (2010)
<i>MT-COX1^a</i>	132	ATACCAACGCCCCCTCTTCG	GGGTCCGAAGAAGGTGGTGTT	^{-b}
<i>MT-ATP6^a</i>	149	CGTACGCCTAACCGCTAACATTA	CGACAGCGATTCTAGGATAGTCA	^{-b}
<i>MT-ATP8^a</i>	151	CATACTCCTTACACTATTCCTCATCACC	GGGCAATGAATGAAGCGAAC	Lu <i>et al.</i> (2007)
<i>RPLP0</i>	106	GGAGGGTGTCCGCAATGTT	CAAGGCCAGGACTCGTTTGT	^{-b}
<i>GAPDH</i>	302	AGCCACATCGCTCAGACACC	GTACTCAGCGGACGATCG	^{-b}
<i>NANOG</i>	158	CAAAGGCAAAACAACCCACTT	TCTGCTGGAGGCTGAGGT	^{-b}
<i>OCT4</i>	142	AGCGAACCAAGTATCGAGAAC	TTACAGAACCACACTCGCAC	^{-b}
<i>MIXL1</i>	270	GCTTTCAGTTACCCTCCAGATAAC	GCACAGGAAGTACAATAACAAGTGC	^{-b}
<i>GATA4</i>	275	CTAGACCGTGGGTTT	TGGGTAAAGTGCCCC	^{-b}
<i>PAX6</i>	66	CTGGGTAGCGAAAAGCAACAG	CCCCTTCAACATCCTTAGTTTATCA	Oldershaw <i>et al.</i> (2010)
<i>OTX2</i>	190	GAGAGGAGGTGGCACTGAAAATC	AATTGGCCACTTGTTCCACTCTC	^{-b}
<i>PDGFRβ</i>	64	TGGCAGAAGAAGCCACGTT	GGCCGTCAGAGCTCACAGA	Oldershaw <i>et al.</i> (2010)
<i>T</i>	163	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTGCATCAAG	^{-b}
<i>GATA6</i>	66	GAAGCGCGTGCCTTCATC	GTGGTAGTTGTGGTGTGACAGTTG	^{-b}
<i>SOX17</i>	155	GAGTTGAGCAAGATGCTGG	AGCCGCTTCACCTGCTTG	^{-b}
<i>CDX2</i>	211	AGTCGCTACATCACCATCC	CACCTCTCAGAGGACCTGG	^{-b}

^aGenes encoded by mtDNA. ^bPrimers were designed using Primer3Plus and validated using Primer-BLAST and sequencing. Primerbank is an online, free public resource for PCR primers (Spandidos *et al.* 2010).

mtDNA copy number

mtDNA copy number was determined in duplicate reactions using the NovaQUANT Human Mitochondrial to Nuclear DNA Ratio kit (Novagen, Frenchs Forest, NSW, Australia) using 1 ng of DNA. MEL1 and MEL2 hES cells were harvested using TrypLE Select (Invitrogen) and cell numbers determined from an aliquot prior to DNA isolation using a QIAamp DNA Mini Prep kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 200 µl of the buffer provided, quantified using a 2000c Thermo Nanodrop spectrophotometer and stored at -20 °C until analysis. Thermocycler settings were the same as described for real-time PCR. The relative copy number method was used to calculate mtDNA copy number, where the ratio of the mitochondrial genes *ND1* and *ND6* were compared to the nuclear genes *BECN1* and *NEB* respectively and the average was taken.

ATP quantification

ATP levels were determined using the ATPlite luminescence detection kit (Perkin-Elmer, Boston, MA, USA) (Prigione *et al.* 2010). hES cells were treated with Y-27632 (ROCK inhibitor; 10 µM; AdipoGen) for 1 h, dissociated with TrypLE Select, and seeded as single cells at a density of 30 000 cells/well in a 96-well white/opaque opti-plate (Perkin Elmer) in mTeSR+Y-27632

(10 µM). For cell number determination a parallel seeding of 30 000 cells/well of a 96-well clear bottom plate (Falcon) in mTeSR+Y-27632 (10 µM) was performed. After 24 h of culture, Y-27632 was removed from all wells. After a further 24 h cells in the opaque plate were lysed and the ATP concentration was determined from a standard curve using a FLUOstar Omega plate reader (BMG Labtech, Cary, NC, USA). Cells seeded on the clear plate were fixed in 70% ethanol and stained with DAPI to determine cell number after adjusting for well surface area.

Quantitation of mitochondrial membrane potential

hES cells cultured in mTeSR1 on matrigel-coated 12-well plates were trypsinized using TrypLE Select (day 5) and stained using a MitoProbeJC-1 Assay Kit for Flow Cytometry (Invitrogen) with the addition of a non-stained control for fluorescence baseline identification. A minimum of 10 000 live cell events selected using DAPI gating were acquired for each sample using a Beckman Coulter CyAn analyzer (Beckman Coulter, Mount Waverly, Vic., Australia). Flow density plots were generated using Summit 4.3 Software (Beckman Coulter). Mean fluorescence data values from 3 (MEL1) or 4 (MEL2) independent biological replicates, each with three technical replicates at each oxygen concentration, were combined and normalized for statistical analysis. Individual fluorescence channels

give separate information; red=high membrane potential, green=low membrane potential. Mitochondrial membrane potential (MMP) was calculated as the ratio of red to green fluorescence.

Protein quantification

As an additional measure of mitochondrial mass, western blotting of TOM20 and citrate synthase was performed. Total protein was extracted from hES cells using RIPA buffer containing protease inhibitor (P8350, Sigma) and phosphatase inhibitor (04906845001, Roche) on day 5 of culture. A Bradford assay was used to normalize the protein levels and a 1:1 solution was made with Lammeli buffer (BioRad) containing β -mercaptoethanol (Sigma). Samples were incubated at 100 °C for 10 min and separated using a 12.5% SDS polyacrylamide gel using 10 μ g of total protein per well. Standard immunoblot procedures were followed and proteins were visualized using the Clarity Western ECL blotting substrate (BioRad) and a Bio-Rad ChemiDoc XRS. Anti-TOM20 (ab186734; Abcam, Cambridge, UK) and anti-Citrate Synthase antibodies (ab85669; Abcam) were used at a 1:1000 dilution. Anti- β -Tubulin antibody (Sigma) was used at 1:10 000 dilution. Quantitative densitometry was performed using ImageLab software (BioRad).

Proliferation assay

Proliferation was assessed on days 3 and 5 of culture through incorporation of bromodeoxyuridine (BrdU; Sigma–Aldrich). Cells were seeded into four-well Nunc Lab-Tek Chamber slides on permaxox (Sigma–Aldrich) and cultured as above. On day 3 or 5, cells were incubated with 10 μ M BrdU for 1 h at 37 °C in culture medium, fixed with ice-cold 70% ethanol for 30 min and washed with PBS (Invitrogen). DNA was denatured with 1 M HCl for 20 min and non-specific antibody binding was blocked with PBS/0.1% Triton X-100/1% FCS for 30 min at room temperature. Cells were incubated with anti-BrdU antibody (1:200; Bioclone, Marrickville, NSW, Australia) overnight at 4 °C followed by Alexa-Fluor 488 conjugated secondary (1:1000; Invitrogen) for 1 h at room temperature. DAPI was applied for 5 min before mounting with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Nine random colonies were selected from each biological replicate and the number of proliferating cells (BrdU⁺) and total cells (DAPI⁺) were determined.

Spent medium metabolite extraction for NMR spectroscopy

For the determination of hES cell metabolite use by NMR spectroscopy, three independent biological replicates of hES cells (MEL1 and MEL2) were cultured in matrigel-coated 12 well plates in 5% or 20% oxygen as described above. To determine metabolite consumption and production, one well containing matrigel with medium only was used as an unspent medium control for each replicate. Precisely 24 h after feeding on day 4, spent medium was collected, centrifuged to remove cellular debris (1000 *g* for 2 min), then transferred to rubber

o-ring vials to prevent gas exchange and stored at –80 °C until metabolite extraction. To determine cell numbers for each replicate well, cells were trypsinized using TrypLE Select and counted on an hemocytometer. Two replicate wells of both 5% and 20% oxygen and a blank control from each independent biological replicate were selected for NMR analysis (*n*=6 samples per treatment per cell line). Metabolite extraction was performed on 200 μ l of sample. Each sample was centrifuged in a three-times H₂O-washed 3 kDa Amicon Ultra-15 Centrifugal filter unit (Merck-Millipore, Frenchs Forest, NSW, Australia) at 4000 *g* for 60 min. Subsequently, 100 μ l of the filtrate was added to 200 mM Na₃PO₄ in D₂O (trisodium phosphate in deuterium oxide) titrated to pH 7 using deuterium chloride and sodium deuterium oxide, supplemented with 5 mM 2,2-dimethylsilapentane-5-sulfonate (DSS) in D₂O as an internal reference, and 5 mM imidazole in D₂O as an internal standard controlling for instrument error and sample pH, for a total sample volume of 600 μ l.

NMR spectroscopy, spectral identification and metabolite quantification

Samples were analyzed on a 600 MHz NMR spectrometer (Bruker Biospin, Alexandria, NSW, Australia) and quantified following the procedure outlined in (Sheedy *et al.* 2010). The following parameters were used: 1D Nosey pulse sequence (for ¹H) with presaturation for water suppression (90° pulse width = 20 μ s, d1 = 1.5 s, d8 = 0.5 s, d11 = 0.03 s, d12 = 0.00002 s), 256 scans, sample temperature = 25 °C. Fourier transformation, an exponential window function and baseline/phase correction were performed using Topspin 3.1 (Bruker, Karlsruhe, Germany) followed by amplitude normalization of all spectra to the internal standard (DSS). Signals were identified and quantified using Chenomx NMR Suite software (Chenomx, Edmonton, AB, Canada). Validation of metabolite identity was performed by 2D ¹H-¹H Total Correlation Spectroscopy (TOCSY) NMR experiment using the phase sensitive DIPSI2 sequence and 3-9-19 WATERGATE for water suppression with presaturation during the relaxation delay. Thirty-two scans were employed with mixing time of 50 ms and 4096 *T*₂ and 1024 *T*₁ data points. States-time proportional phase incrementation was used for quadrature detection and processed using a qsin window function, and free induction decays multiplied by 1 Hz line broadening for *T*₂ data and 0.3 Hz for *T*₁ data. 2D spectra were manually phase-corrected and automatically baseline-corrected in Topspin. All spectra were referenced to the trimethylsilyl singlet of DSS at 0.00 ppm. Metabolite identification and quantification were performed using the Chenomx 6.1 NMR Suite Profiler module and the 600 MHz compound libraries (for samples in the pH range of 6–8). The chemical shifts of the imidazole signals at 7.34 and 8.34 ppm confirmed a pH of 7 for all samples. The chemical shifts of identified metabolites from 1D spectra were validated by the 2D NMR spectrum, and by comparing chemical shift values to experimental and predicted 1D and 2D ¹H-NMR spectra from the Human Metabolome Database (<http://www.hmdb.ca>).

For amino acid and carbohydrate multivariate data analysis, the spectra were divided into bin regions of 0.04 ppm and

absolute bin values were normalized to cell number and log transformed. To visualize the cluster pattern of the data set, principle component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were then applied using Unscrambler X Software (Camo, Oslo, Norway). To quantitate metabolites, univariate amino acid and carbohydrate data were normalized to the internal standard imidazole and to cell number. Control values (spent medium without cells) were subtracted from their matching samples to give consumption and production values. Amino acid turnover was calculated as the sum of amino acids consumed and produced. Glycolytic rate was calculated on the basis that one mole of glucose would give rise to two moles of lactate, where glycolytic rate = (# moles of lactate)/(# moles of glucose × 2) (Lane & Gardner 1996, Harvey *et al.* 2014).

Karyotyping

Passage 32 MEL1 and MEL2 hES cell lines were karyotyped by G-banding chromosome analysis (Southern Cross Pathology Australia, Clayton, Vic., Australia).

Differentiation assays

To form embryoid bodies (EBs) hES cells were dissociated using TrypLE Select, counted, and 10 000 cells were suspended in ~50 hanging drops of mTeSR1 over a layer of PBS in 5 and 20% oxygen conditions (Mitalipova & Palmarini 2006). hES cells were collected and stored for RNA analysis (day 0). After 48 h EBs were transferred to ultra-low attachment Costar 96-well plates (1 drop/well; Corning, NY, USA) and cultured for a further 8 days in mTeSR+20% fetal bovine serum under respective 5% or 20% oxygen conditions. Medium changes occurred every 48 h. At days 5 and 10, ~25 EBs from each treatment were pooled and harvested for RNA ($n=3$ biological replicates). To induce mesendoderm differentiation, adherent colonies of MEL1 and MEL2 hES cells were seeded into organ culture dishes for immunofluorescence and RNA harvesting and cultured for 24 h in mTeSR, then treated for 72 h with 30 ng/ml recombinant human BMP4 (R&D Systems, Abingdon, UK) in mTeSR (Rathjen *et al.* 2013). Medium and growth factor were replenished daily. Cells were harvested for RNA or fixed (4% PFA) for immunofluorescence imaging.

Immunofluorescence

To examine differentiation marker expression following BMP4 treatment, fixed cells were permeabilized using PBS/0.25% Triton X-100 and blocked with PBS/0.1% Triton X-100/1% FCS for 30 min. Antibodies against MIXL1 (MAB2610; R&D Systems) and T (sc-17743; Santa Cruz Biotechnology) were used concurrently at a 1:400 dilution. Secondary antibodies AF488 (A100059; Invitrogen) and AF568 (A11057; Invitrogen) were used at a 1:1000 dilution. Images of hES cell were captured on a Nikon Eclipse Ti-U inverted microscope using a Photometrics Coolsnap HQ² camera and acquired using NIS-Elements BR 3.0 software (Nikon). Colour was overlaid using ImageJ (<http://imagej.nih.gov/ij/>).

Statistical analysis

Data were analyzed using R (Pinheiro *et al.* 2013), Prism Software (GraphPad Prism 5, San Diego, CA, USA) and SPSS (IBM). Gene expression data were analyzed using an unpaired, two-tailed Student's *t*-test comparing 5% and 20% oxygen treatments. Variances in mtDNA copy number, ATP, amino acid and carbohydrate utilization, percentage glycolysis and differentiation gene expression analysis were analyzed using a two factor ANOVA; factor 1: oxygen (5%; 20%), factor 2: cell line (MEL1; MEL2). Proliferation rate was assessed using a three factor ANOVA; factor 3: day (3; 5). Significant ($P<0.05$) main effects and interactions were further analyzed using simple main effects calculated using the MS_{residual} from the initial ANOVA. Alternatively, a planned comparison between 5% and 20% oxygen treatments was performed. All data were analyzed for normality and scedasticity of variance using box and density plots in R. Non-normal data were log transformed. Results were considered statistically significant at $P<0.05$. # designates $P<0.06$. All data are presented as mean $\pm$ S.E.M.

Ethics approval

All methods used were approved by the University of Melbourne Human Ethics Committee. Approval number 0722502.1.

Results

Mitochondrial mass is increased at high oxygen culture

To investigate the effect of oxygen on hES cell mitochondrial activity and mass, MMP was quantified using JC-1 staining. hES cell MMP (given as the ratio of PE to FITC staining) was unaltered by oxygen concentration in either cell line (Fig. 1A, B, C, D, E and F). Cell density plots of JC-1 fluorescence in MEL2 hES cells (Fig. 1E and F) displayed a significant increase ($P<0.001$) in the PE^{High}, FITC^{High} quartile from 33% $\pm$ 2.7 at 5% oxygen to 60% $\pm$ 4.9 at 20% oxygen. Total mitochondrial fluorescence was ~25% higher in the MEL2 line at 20% compared to 5% oxygen ($P<0.001$; Fig. 1B) corresponding to a greater mitochondrial volume without a change in MMP. Unlike MEL2 hES cells, MEL1 total mitochondrial fluorescence was unaltered by oxygen (Fig. 1A).

To supplement the flow cytometric analysis, the expression of three mitochondrial proteins by MEL1 and MEL2 cells was estimated by western blot. Protein levels of mitochondrial translocase 20 (TOM20), the TCA cycle rate limiting enzyme, citrate synthase and cytochrome c oxidase subunit IV of the electron transport chain (ETC) were unchanged by oxygen treatment in either cell line (Fig. 2A). Considerable variation was observed in TOM20 and COXIV protein levels, however relative equivalence across both oxygen and cell line for all proteins is shown in Fig. 2A.

Figure 1 Mitochondrial membrane potential (JC-1) flow cytometry plots of MEL1 and MEL2 hES cells cultured at 5% or 20% oxygen. (A) MEL1 and (B) MEL2 JC-1 fluorescence at 20% oxygen expressed relative to fluorescence at 5% oxygen represented by the line at $y=1$. Overall mitochondrial membrane potential (MMP) is calculated as the ratio of high MMP to low MMP. Total fluorescence is an average of both channels. (C, D, E and F) MEL1 and MEL2 JC-1 flow density plots of hES cells cultured at 5% and 20% oxygen. High MMP fluorescence is presented on the vertical axis (PE); low MMP fluorescence is presented on the horizontal axis (FITC). Representative percentages of cells in each quadrant are given. Cross hairs remain static within cell lines. (C and D) MEL1 hES cells cultured at 5% and 20% oxygen respectively. (E and F) MEL2 hES cells cultured at 5% and 20% oxygen respectively. Data are representative percentages; $n=3$ (MEL1), $n=4$ (MEL2). *** $P<0.001$ Significant change in mean JC-1 fluorescence (A and B) or percentage of cells in a quadrant (C, D, E and F) comparing 5% and 20% oxygen treatments.

Oxygen alters mtDNA copy number and ATP in MEL2 but not MEL1 hES cells

To investigate the effect of oxygen on hES cell mitochondrial biogenesis, mtDNA copy number was examined in MEL2 and MEL1 hES cells. MEL2 hES cells cultured at 20% oxygen displayed a 1.5 fold higher level of mtDNA copy numbers (213 ± 11 ; $P \leq 0.001$) compared to MEL2 hES cells cultured at 5% oxygen (142 ± 19 ; Fig. 2B; Supplementary Table 1, see section

on supplementary data given at the end of this article). In contrast, mtDNA copy number was not affected by oxygen concentration in MEL1 hES cells ranging from 265 ± 19 copies at 5% oxygen to 296 ± 31 copies at 20% oxygen. Overall, MEL1 hES cell mtDNA copy number was significantly higher ($P<0.001$) than that of the MEL2 hES cells.

Steady-state intracellular ATP levels were measured in both cell lines. MEL2 hES cells exhibited a 1.4 fold increase ($P<0.05$) in intracellular ATP when cultured at 20% oxygen (1.04 ± 0.08 nM/cell) compared to 5% (0.75 ± 0.1 nM/cell), while MEL1 ATP levels remained constant (0.84 ± 0.08 and 0.88 ± 0.1 nM/cell at 5% and 20% oxygen respectively; Fig. 2C). Overall, the two cell lines did not differ significantly in their ATP content.

hES cell mitochondrial gene expression is altered by high oxygen

To investigate the effect of oxygen on regulators of mitochondrial biogenesis and function, nuclear- and mitochondrial-encoded metabolic gene expression was examined by real-time PCR. MEL2 expression of mitochondrial-encoded *MT-ND2* ($P<0.05$), *MT-ND4* ($P<0.05$), *MT-ATP6* ($P \leq 0.01$) and *MT-ATP8* ($P<0.01$)

Figure 2 Protein expression, mtDNA copy number and ATP levels in MEL1 and MEL2 human ES cells cultured at 5% and 20% oxygen. (A) Mitochondrial protein levels and densitometry normalized to β -tubulin. (B) mtDNA copy number measured as the ratio of the mitochondrial genes *ND1* and *ND6* to the nuclear genes *BECN1* and *NEB* respectively using the relative copy number method. (C) Steady state intracellular ATP levels (nM/cell). Data are presented as mean $\pm$ S.E.M.; $n=4$ (protein and mtDNA), $n=8$ (ATP). *** $P<0.001$, * $P<0.05$.

was significantly higher when cultured at 20% oxygen compared with culture at 5% (Fig. 3C). MEL2 hES cells cultured under 20% oxygen displayed significantly higher expression levels of the nuclear encoded mitochondrial regulators mitochondrial transcription factor A (*TFAM*; $P < 0.001$), DNA methyltransferase 1 (*DNMT1*; $P < 0.05$), peroxisome proliferator-activated receptor α (*PPARA*; $P < 0.01$) and nuclear respiratory factor 1 (*NRF1*; $P < 0.05$; Fig. 3D) when compared with MEL2 hES cells cultured at 5% oxygen. Culture at 20% oxygen also resulted in significantly lower levels of cytochrome c oxidase subunit IV-2 (*COX4-2*) expression ($P < 0.01$), the oxygen-regulated subunit of COX4 (Fukuda *et al.* 2007).

No statistically significant differences in the expression of mitochondrial-encoded genes were detected in MEL1 hES cells cultured at 5% or 20% oxygen (Fig. 3A). Nuclear

Figure 3 Metabolic gene expression of MEL1 and MEL2 hES cells cultured at 5 and 20% oxygen. (A, B, C and D) Mitochondrial encoded genes, nuclear encoded metabolic genes and regulators of mitochondrial biogenesis and activity were examined in hES cells in response to oxygen. Expression was normalized to the housekeeper *RPLP0* and expressed relative to the expression of 5% oxygen samples, represented by the line at $y = 1$. Data are presented as mean $\pm$ S.E.M.; $n = 5$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

encoded regulators of mitochondrial function were also not altered by oxygen in MEL1 hES cells (Fig. 3B), with the exception *COX4-2*. *COX4-2* expression was significantly lower following culture at 20% oxygen ($P \leq 0.001$) compared to hES cells cultured at 5%.

MEL1 and MEL2 hES cells satisfy the standard measures of pluripotency

To ensure MEL1 and MEL2 hES cells had retained pluripotency in culture, markers of pluripotency and differentiation were assessed. No discernible differences were observed in cell morphology between cell lines or between oxygen treatments, with all treatment groups exhibiting a characteristic flat, compact hES cell colony morphology with no detectable signs of differentiation (Fig. 4A). qPCR showed MEL1 and MEL2 cell lines expressed the pluripotency markers *OCT4* and *NANOG* (Fig. 4B). Mesoderm lineage marker *MIXL1* mRNA was expressed at much lower levels in both cell lines (Fig. 4B), as were lineage markers *TBX5*, *GATA4* and *OTX2* (data not shown). The culture of cells in 20% oxygen significantly increased the transcript levels of *MIXL1* in MEL1 and MEL2 cell lines by ~ 1.5 to twofold ($P < 0.05$), and *NANOG* in MEL2 (~ 1.5 fold higher in 20% oxygen; $P < 0.05$). *OCT4* was unaltered by oxygen in either cell line. Lineage markers *GATA4* (endoderm) and *OTX2* (ectoderm) displayed higher mean mRNA levels at 20% oxygen in both cell lines, although this was not significant (data not shown).

To exclude chromosomal instability as a possible underlying mechanism modulating the different responses of MEL1 and MEL2 hES cells to oxygen, karyotypic analyses were performed. MEL1 and MEL2 hES cells at passage 32 (representing six and five passages respectively under each oxygen condition) displayed a normal 46,XY and 46,XX karyotype (Fig. 4C) respectively.

The potency of MEL1 and MEL2 hES cells was assessed using EBs. MEL1 and MEL2 hES cells at 5% and 20% oxygen gave rise to EBs (Fig. 4D) and upregulated transcripts diagnostic of the three germ layers (Fig. 3F). EBs were assessed on days 5 and 10 for their expression of ectoderm (*PAX6* and *OTX2*), mesoderm (*PDGFR β* and *CDX2*) and endoderm (*GATA6* and *SOX17*) lineage markers; gene expression was compared to hES cells collected on day 0. All treatments resulted in similar transcript profiles with no significant effect of cell line or oxygen.

Asynchronous lineage priming during mesendoderm differentiation

The early stages of differentiation from MEL1 and MEL2 hES cells were examined. Mesendoderm differentiation was induced through the addition of 30 ng/ml of rhBMP4 to hES cells for 72 h under 5% and 20% oxygen (Fig. 5A). By immunofluorescence, the expression of primitive streak markers *MIXL1* and *T* (*BRACHYURY*; Fig. 5B) was detected in all conditions.

Figure 4 MEL1 and MEL2 hES cells display typical pluripotent characteristics. (A) Morphology of MEL1 and MEL2 hES cells cultured at 5% oxygen and 20% oxygen. From left to right: MEL1 5%, MEL1 20%, MEL2 5%, MEL2 20%. Scale bar: 200 μ m. (B) Ct values from a qPCR of MEL1 and MEL2 pluripotency and differentiation genes after culture at 5% and 20% oxygen. Pluripotency markers *OCT4* and *NANOG* appear from cycles 18 to 23. Differentiation marker *MIXL1* appears from cycles 31 to 34. (C) Karyotypes of MEL1 hES cells at passage 32 (46,XY) and MEL2 hES cells at passage 32 (46,XX). (D) Representative EBs. From left to right: MEL1 5%, MEL1 20%, MEL2 5%, MEL2 20%. Scale bar: 200 μ m. (E) Gene expression of lineage markers in EBs formed from MEL1 and MEL2 hES cells over 10 days. Gene expression is presented as fold change relative to ES cells at day 0. Data are presented as mean $\pm$ S.E.M.; (B) $n=5$. (E) $n=3$; y-axis is log₁₀ scale. Only positive S.E.M. values are given for clarity.

The pluripotency marker *OCT4* was downregulated in MEL2 hES cells after 72 h of BMP4 treatment but upregulated in MEL1 hES cells (Fig. 5C; MEL2: ~ 0.4 fold; MEL1: approximately two- to sixfold; $P<0.06$). *NANOG* expression was also dissimilar between lines (MEL2: ~ 0.5 fold decrease; MEL1: approximately three- to fourfold increase); due to the highly variable expression of *NANOG* in differentiating MEL1 hES cells, this difference was not statistically significant. *T* was more highly expressed in the MEL1

line than the MEL2 ($P<0.06$). Mesoderm lineage marker *CDX2* and endoderm lineage marker *GATA4* were more highly expressed in the MEL2 line than the MEL1 ($P<0.05$). Notably, *GATA4* gene expression in BMP4-treated MEL1 hES cells was 0.85 fold lower compared to their pluripotent state. Endoderm and primitive streak markers *SOX17* and *MIXL1*, showed no difference due to cell line. No impact of oxygen concentration was observed in any of the mesendoderm differentiation transcript profiles.

MEL1 and MEL2 hES cells have distinct metabolite profiles in response to oxygen

To examine further the metabolism of MEL1 and MEL2 hES cells, amino acid and carbohydrate use were

Figure 5 BMP4 induced differentiation of MEL1 and MEL2 hES cells at 5% and 20% oxygen. (A) Bright field (BF) and (B) immunostaining (*MIXL1* and *T*) of BMP4-induced mesendoderm differentiation. BF colonies are different from immunostained colonies. Scale bars: 200 μ m. (C) Gene expression of pluripotency and lineage markers in BMP4-treated hES cells. Gene expression is presented as fold change relative to control ES cells without BMP4 treatment. $n=3$. $T=BRACHYURY$. # $P<0.06$, * $P<0.05$.

profiled using ^1H -NMR spectroscopy and metabolite identity was confirmed using a 2D ^1H - ^1H TOCSY NMR experiment (Fig. 6B, C, D and E). PCA revealed a distinct difference in the metabolite profiles of the two hES cell lines. Further analysis of the data using PLS-DA (Fig. 6A) validated the differences between cell lines and showed differences in metabolite utilization due to oxygen

treatment in MEL2 hES cells. In contrast, the overall metabolite profile of MEL1 hES cells was not affected by oxygen concentration.

Oxygen significantly altered the production and consumption of individual amino acids (Fig. 7A, C; Supplementary Table 1). The consumption of serine by MEL2 hES cells was 3.5 times lower at 20% compared to

Figure 6 Partial least squares discriminant analysis (PLS-DA) of MEL1 and MEL2 metabolite profiles. (A) Each data point represents the entire spectral/metabolite profile for each MEL1 or MEL2 sample at either 5% or 20% oxygen. Samples closer together have similar metabolite profiles; samples diagonally opposite each other are more dissimilar. The variation explained by each eigenvector (axis) is given as the first percentage on that axis. $n=6$ in technical replicate. Ellipses are for illustrative purposes only and do not represent a statistically defined area. (B, C, D and E) The down-field signals (6.50–8.50 ppm) are annotated in the 1D ^1H -NMR spectrum (B) and confirmed by 2D ^1H - ^1H TOCSY NMR spectral analysis (D). The up-field signals (0.50–4.50 ppm) are annotated in the 1D ^1H -NMR spectrum (C) and confirmed by 2D NMR analysis (E). Referenced numbers are matched to annotated signals, and chemical shifts used for metabolite identification and quantification (δ , ppm). 1. Alanine ($\delta=1.47$ ppm, d); 2. Arginine ($\delta=1.65$ ppm, m); 3. Ascorbate ($\delta=4.02$ ppm, m); 4. Aspartate ($\delta=2.67$ ppm, m); 5. Choline ($\delta=3.20$ ppm, s); 6. Ethanol ($\delta=1.17$ ppm, t); 7. GABA ($\delta=2.29$ ppm, t); 8. Glucose ($\delta=3.46$ ppm, m); 9. Glutamate ($\delta=2.33$ ppm, m); 10. Glutamine ($\delta=2.43$ ppm, m); 11. Glycerol ($\delta=3.55$ ppm, m); 12. Glycine ($\delta=3.54$ ppm, s); 13. HEPES ($\delta=2.95$ ppm, m); 14. Histidine ($\delta=7.10$ ppm, s); 15. Imidazole ($\delta=7.34$ ppm, s); 16. Isoleucine ($\delta=0.93$ ppm, t); 17. Lactate (4.11 ppm, m); 18. Leucine ($\delta=0.96$ ppm, m); 19. Lysine ($\delta=1.51$ ppm, m); 20. Methanol ($\delta=3.35$ ppm, s); 21. Methionine ($\delta=2.64$ ppm, t); 22. Pantothenate ($\delta=0.89$ ppm, s); 23. Phenylalanine ($\delta=7.42$ ppm, t); 24. Proline ($\delta=2.06$ ppm, m); 25. Pyruvate ($\delta=2.36$ ppm, s); 26. Serine ($\delta=3.94$ ppm, m); 27. Threonine ($\delta=3.58$ ppm, d); 28. Tryptophan ($\delta=7.53$ ppm, d); 29. Tyrosine ($\delta=6.90$ ppm, d); 30. Valine ($\delta=1.03$ ppm, d). s, singlet; d, doublet; t, triplet; m, multiplet.

5% oxygen ($P<0.05$). Glycine consumption by MEL2 hES cells was also affected by oxygen and displayed an 80% reduction at 20% oxygen ($P<0.05$), while MEL2 hES cells produced asparagine at 20% oxygen and consumed it at 5% oxygen. Tyrosine consumption was decreased by ~60% in MEL2 and MEL1 hES cells cultured at 20% oxygen ($P<0.05$). Glutamate production in MEL1 hES cells cultured under 20% oxygen was 6.5 times higher ($P<0.001$) compared to 5% oxygen culture. MEL1 hES cells displayed a switch in their use of proline from production at 5% oxygen to consumption at 20% ($P<0.001$). Oxygen culture at 20% also reduced

Figure 7 Amino acid utilization profiles in MEL1 and MEL2 hES cells in response to oxygen measured by $^1\text{H-NMR}$. Spent media samples were collected after a 24-h period (days 4–5), normalized to cell number and an internal standard (imidazole) and analyzed for amino acid concentrations using $^1\text{H-NMR}$. Black bars: 5% oxygen; white bars: 20% oxygen. (A) MEL1 amino acid production and consumption. (B) MEL1 total amino acid production, consumption and turnover. (C) MEL2 amino acid production and consumption. (D) MEL2 total amino acid production, consumption and turnover. Data are presented as mean $\pm$ S.E.M.; $n=12$. * $P<0.075$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. * indicates a significant simple main effect of 5 and 20% oxygen treatment for individual amino acids or total amino acid use.

histidine consumption ($P<0.05$) in MEL1 hES cells. Oxygen concentration significantly affected total amino acid consumption and turnover (the sum of amino acids produced and consumed) in MEL2 hES cells (Fig. 7D). Amino acid consumption by MEL2 hES cells cultured at 20% oxygen was less compared to those cultured at 5% oxygen ($P<0.01$). Consequently, total amino acid turnover was reduced at 20% oxygen ($P\leq 0.01$). In contrast, MEL1 hES cell total amino acid production, consumption and turnover were not significantly altered by oxygen (Fig. 7B). The highest total production of amino acids was observed in MEL1 hES cells at 20% oxygen.

MEL1 and MEL2 hES cell lines differed significantly in the utilization of several amino acids (Fig. 7A and C, Supplementary Table 1). The most profound difference observed was that of serine utilization ($P<0.001$), which was produced in MEL1 hES cells and consumed in MEL2 hES cells. Furthermore, alanine production was higher in MEL2 cells compared with MEL1 cells ($P\leq 0.001$). The consumption of glutamine, lysine, methionine and the branched chain amino acids isoleucine and leucine was also different between cell lines ($P<0.05$, 0.05 , <0.0001 , <0.0001 , <0.001 respectively). Glutamine consumption was higher in MEL2 hES cells while lysine, methionine, isoleucine and leucine production was higher in MEL1 hES cells. An interaction between cell line and oxygen was observed for glutamate and proline ($P<0.05$ and 0.01 , respectively). MEL1 hES cell glutamate production was 6.5 fold higher at 20% oxygen compared to 5% oxygen ($P<0.001$) while MEL2 levels were unchanged. MEL1 proline production at 5% oxygen switched to consumption at 20% ($P<0.0001$) but was unaltered in MEL2 hES cells.

Oxygen alters carbohydrate use but not glycolytic rate in hES cells

$^1\text{H-NMR}$ facilitated the assessment of carbohydrate use and calculation of glycolytic rate in response to oxygen (Fig. 8A, B, C and D, Supplementary Table 1). Culture at 20% oxygen reduced the flux of carbohydrates in MEL2 hES cells (Fig. 8C). MEL2 hES cells cultured at 20% oxygen consumed less glucose ($P<0.0001$) and produced less lactate ($P<0.001$) compared to those cultured at 5% oxygen. Furthermore, MEL2 hES cells displayed a decrease in pyruvate consumption ($P<0.01$) at 20% oxygen. No difference in MEL1 glucose or pyruvate consumption or lactate production was observed in response to oxygen. MEL1 pyruvate consumption was approximately twice that of the MEL2 hES cells at both oxygen concentrations ($P<0.0001$). Glucose flux to lactate was not statistically different between oxygen treatments in either cell line, or between cell lines. A glucose to lactate conversion rate of 67–80% was observed for all groups, indicating that 20–33% of glucose is metabolized through other pathways, which may include the tricarboxylic acid

Figure 8 Carbohydrate utilization and percentage glycolysis in MEL1 (A and B) and MEL2 (C and D) hES cells in response to oxygen. (A and C) Glucose and pyruvate consumption and lactate production were quantified in spent medium samples following a 24 h incubation period (days 4–5) by ^1H -NMR and normalized to cell number. (B and D) Glucose to lactate flux (% glycolysis) for each cell line was calculated as the number of moles of lactate/2 $\times$ number of moles of glucose (Lane & Gardner 1996, Harvey *et al.* 2014). Data are presented as mean $\pm$ S.E.M.; $n=12$. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Significance indicates a significant simple main effect of 5% and 20% oxygen treatment for individual carbohydrate's consumption/production. (E) Proliferation rate of MEL1 and MEL2 hES cells on days 3 and 5, cultured at 5% and 20% oxygen. Data are presented as mean $\pm$ S.E.M.; $n=3$. **** $P<0.0001$. ^{a/b}Significantly different ($P<0.05$) from hES cells cultured at 20% oxygen on day 3.

(TCA) or pentose phosphate cycles. Even under conditions of high oxygen availability, hES cells maintain a high glucose/lactate flux.

Proliferation rates vary considerably between hES cell lines

The differences in MEL1 and MEL2 hES cell amino acid and carbohydrate utilization prompted us to investigate any resultant effects on proliferation rate as determined through BrdU incorporation on days 3 and 5 of culture. The MEL2 hES cell proliferation rate was 20% higher than that of MEL1 hES cells ($P<0.0001$; Fig. 8E;

Supplementary Table 1). Approximately 45% of MEL1 hES cells were observed to be BrdU⁺ with no observable impact of oxygen or day of culture. Approximately 65% of MEL2 hES cells were BrdU⁺ with no apparent impact of oxygen. However, a small but significant decrease ($P<0.05$) in proliferation was observed in MEL2 hES cells cultured at 20% oxygen on day 5 compared to day 3 ($67.55\%\pm0.02$ vs $62.9\%\pm0.16$ respectively), while those cultured at 5% oxygen maintained a constant proliferation rate.

Discussion

In concordance with what we and others have reported, atmospheric oxygen induced a more oxidative profile in MEL2 hES cells when compared to cells maintained in physiological oxygen (Forristal *et al.* 2013, Christensen *et al.* 2014, Harvey *et al.* 2014, Turner *et al.* 2014). In this study we have extended these observations to show that mtDNA, ATP, mitochondrial mass and genetic regulators of mitochondria respond to changes in the extracellular oxygen concentration in MEL2 cells. This is the first demonstration of the effect of oxygen on mitochondria in hES cells. Changes observed in mitochondrial function are likely to be representative of the hES cell response to oxygen in culture.

Surprisingly, atmospheric oxygen did not induce mitochondrial investment in MEL1 hES cells when compared to cells maintained in physiological oxygen. Moreover, the baseline metabolism of MEL1 hES cells in response to oxygen was significantly different to MEL2 hES cells and the preimplantation blastocyst (Wale & Gardner 2012). We interpret the difference between the MEL2 and MEL1 hES cell lines as evidence that the ability of a hES cell line to appropriately respond to oxygen, through the modulation of carbohydrate metabolism, mitochondrial regulation and alterations in amino acid use, can be corrupted during culture. We conclude from this that our MEL1 hES cell line exhibits a compromised metabolic state.

Oxygen concentration linked to oxidative investment

mtDNA copy number and ATP content per cell were significantly altered by oxygen in MEL2 hES cells, with higher levels of both detected in cells cultured in higher oxygen. Elevated copy numbers suggest an increased investment by MEL2 hES cells cultured under 20% oxygen in oxidative metabolism. mtDNA replication is observed in mouse (Facucho-Oliveira *et al.* 2007) and human (Cho *et al.* 2006) ES cells undergoing differentiation, suggests that differentiation of ES cells either requires, or initiates, higher levels of mitochondrial activity. Accompanying elevated levels of mtDNA and ATP, MEL2 hES cells cultured in 20% oxygen displayed more intense JC-1 staining. While overall MMP was not altered, the observed increase in total mitochondrial

fluorescence is indicative of elevated mitochondrial mass in MEL2 hES cells cultured at 20% oxygen.

Despite the increases in mitochondrial mass shown by mtDNA copy number, ATP production and mitochondrial staining in MEL2 hES cells with increased oxygen, no changes were observed in the levels of three mitochondrial-expressed proteins, TOM20, COXIV or Citrate Synthase. It is difficult to explain this result. Western blot is a semi-quantitative methodology and may have lacked the sensitivity to detect changes in protein levels. We note that we experienced considerable difficulty in determining protein levels due to inherent variability in the assay; we have chosen to show the most conservative interpretation of these data. Finally, the impact of oxygen may be manifest on other proteins and pathways in the mitochondria. Complex I, ATP Synthase and mtDNA replication machinery emerge as likely candidates.

Low levels of mtDNA, mitochondria and mitochondrial activity have been linked to the undifferentiated state of mouse and human ES cells (Rivolta & Holley 2002, St John *et al.* 2005, Varum *et al.* 2011). Therefore, physiological oxygen may maintain MEL2 hES cells in a stem-like state by enforcing low levels of mtDNA, fewer mitochondria and reduced oxidative activity.

MEL2 hES cells responded to altered oxygen conditions through changes in mitochondrial and metabolic gene expression, displaying higher transcript levels for genes encoding subunits of complexes I and V of the ETC at 20% oxygen. These two complexes have the most control over state 3 mitochondrial respiration (respiration in the presence of sufficient substrate levels; Doussiere *et al.* 1984, Moreno-Sanchez *et al.* 1991). Increases in transcript levels for complexes I and V, coupled with changes in mtDNA copy number, suggest a shift in the metabolic profile of MEL2 hES cells towards oxidative metabolism at 20% oxygen. This is supported by higher expression of *TFAM* and *NRF1*, key regulators of mtDNA transcription and replication (Fisher *et al.* 1992), in these cells. Elevated levels of *TFAM* and *NRF1* are consistent with the observed elevated mtDNA content. Collectively these results indicate an oxygen-dependent shift in the hES cell metabolic transcriptome, whereby atmospheric oxygen promotes OXPHOS gene transcription and mitochondrial biogenesis.

Amino acid utilization: linking metabolism to epigenetics

Amino acids are precursors for protein synthesis (Epstein & Smith 1973); their levels regulate ES cell proliferation (Shyh-Chang *et al.* 2013), differentiation (Washington *et al.* 2010, Tan *et al.* 2011), mouse and human embryo viability (Gardner & Lane 1996, Devreker *et al.* 2001) and epigenetic modifications (Shyh-Chang *et al.* 2013). The uptake and metabolism of L-proline induces the differentiation of mouse

embryonic stem (mES) cells into a later-stage pluripotent cell (early primitive ectoderm-like (EPL) cells) (Washington *et al.* 2010, Tan *et al.* 2011), and the regulation of intracellular L-proline concentrations has been linked to the maintenance of mES cells (D'Aniello *et al.* 2015). mES and EPL cells differ in their differentiation kinetics, with EPL cells upregulating mesendoderm markers earlier than ES cells (Washington *et al.* 2010). The use of L-proline by MEL1 and MEL2 cells differed. MEL2 cells increased the medium concentration of L-proline in 5% and 20% oxygen, consistent with the profiles of L-proline use in other reports. MEL1 also produced L-proline in 5% oxygen but consumed the amino acid when cultured in 20% oxygen, suggesting that the metabolic pathways regulating intracellular L-proline concentrations differed between the cells. It is tempting to speculate that the differences in L-proline metabolism by MEL1 and MEL2 cells could underpin their differences in differentiation kinetics.

The amino acids isoleucine, leucine, lysine and methionine were more highly consumed in MEL1 than MEL2 hES cells. These amino acids can act as precursors of the TCA cycle intermediates and mTOR substrates (Avruch *et al.* 2009), and may indicate a preference for oxidative metabolism in MEL1 hES cells (Schieke *et al.* 2006). In addition, methionine metabolism has been linked to the undifferentiated hES cell state and has been shown to be required for maintaining hES cell proliferation and to regulate the signaling of S-adenosylmethionine (SAM; Shiraki *et al.* 2014). SAM is a methyl donor crucial in DNA methylation and histone methylation (Goll & Bestor 2005, Shi 2007), one of the several links between amino acid metabolism and epigenetic regulation.

High levels of glutamine and glucose consumption are required to maintain a high α -ketoglutarate to succinate ratio, promoting histone and DNA demethylation and maintaining mES cell pluripotency (Carey *et al.* 2014). Glutamine consumption was significantly higher in the MEL2 line compared to the MEL1 line. Further analysis revealed 5% oxygen conditions resulted in the maximum level of MEL2 glutamine and glucose consumption. In MEL2 hES cells the increased consumption of serine and glycine at 5% oxygen may indicate increased use of the folate pathway (Locasale 2013). This pathway is essential for maintaining pluripotency and rapid proliferation in mES cells (Shyh-Chang *et al.* 2013). Moreover, this pathway provides the one-carbon units used in DNA methylation (Kim 2004). Methylation of H3K4 and H3K27 in mES cells has been linked to the undifferentiated state (Bernstein *et al.* 2006).

Targeted mesendoderm induction reveals asynchronous differentiation kinetics

MEL1 and MEL2 hES cells retain pluripotency in culture. The lines express *OCT4* and *NANOG* and show low

levels of lineage marker expression. Both lines demonstrate multilineage differentiation capacity when differentiated as EBs. Karyotypic analysis of MEL1 and MEL2 hES cells did not detect any gross chromosome instability in the cells. These standard ES cell measures of pluripotency do not distinguish between the lines or reflect the differences in the underlying physiologies of the populations. Both cell lines tested negative for mycoplasma and the mitochondrial common deletion (4977-bp; data not shown). While differentiation potential assessed through EB formation did not reveal any difference between the two cell lines, mesendoderm differentiation exposed a delayed transcriptional response by the MEL1 line. Treatment of hES cells with BMP4 has been shown to downregulate pluripotency markers within 48 h, induce transient primitive streak markers from 12 to 36 h, and then induce definitive mesendoderm markers from day 3 onwards (Zhang *et al.* 2008, Vallier *et al.* 2009). The gene expression profile of MEL2 hES cells after 3 days of BMP4 treatment was consistent with what has been reported in the literature. MEL1 cells, in contrast, continued to express pluripotency markers and primitive streak after 3 days of differentiation, and were yet to upregulate definitive mesendoderm markers; their differentiation appeared to be delayed. The maintenance of markers of pluripotency by differentiating MEL1 hES cell line could impact potential uses of these cells as the maintenance of pluripotency is linked to teratoma formation in transplanted cell populations. In mouse ES cells, attenuating mitochondrial function during differentiation resulted in cell populations that gave rise to teratomas on transplantation (100% of populations gave rise to tumors compared with 0% in untreated controls), suggesting that pluripotency had not been comprehensively lost during differentiation (Mandal *et al.* 2011). Potentially, the maintenance of pluripotency and delay in differentiation seen in this isolate of the MEL1 hES cell line are related to their aberrant metabolism.

MEL2 hES cells maintained at 20% oxygen did not display increased differentiation efficiency compared to those at 5% oxygen during EB or mesendoderm differentiation, despite increased mitochondrial investment as ES cells. Differentiation is associated with a significant increase in mitochondrial mass and mtDNA (Cho *et al.* 2006) that 20% oxygen conditions might reasonably be expected to support. Spontaneous differentiation of H1 hES cells is increased at 20% oxygen while contrarily, physiological oxygen results in better EB formation (Ezashi *et al.* 2005). Regulating the oxygen concentration noticeably improves the differentiation efficiency of several cell types from precursor populations, including mouse and human pancreatic β -cells (Cechin *et al.* 2014, Hakim *et al.* 2014), human endothelial cells (Kusuma *et al.* 2014) and human mesenchymal stem cells (Drela *et al.* 2014). It remains to be seen whether modulating oxygen concentrations

during directed differentiation of hES cells will increase differentiation efficiency.

hES cell metabolic dysfunction exposed

The analysis of MEL2 hES cells in 5% and 20% oxygen, coupled with the analyses by others on a range of hES cell lines, demonstrates that hES cells respond to oxygen concentrations in a predictable manner and that the physiological profile for MEL2 hES cells defined here is characteristic of hES cells more generally. The MEL2 response to oxygen is similar to those reported for the Shes3 and HuES7 hES cell lines (total amino acid turnover, individual amino acid profiles, carbohydrate use; Forristal *et al.* 2013, Christensen *et al.* 2014). Moreover, the response seen in hES cells is akin to that seen in the blastocyst (Wale & Gardner 2012). We have extended these analyses to show oxygen related changes in mtDNA content, ATP, JC-1 staining and metabolic gene expression. Our analysis of MEL1 hES cells has failed to show a response to oxygen (carbohydrate use, amino acid turnover) and we could not detect differences in the mitochondrial parameters (mtDNA content, ATP, JC-1 staining and metabolic gene expression). Furthermore, the MEL1 hES cell proliferation rate was considerably lower than that of MEL2 hES cells. The metabolic data collected on MEL1 cells developed here suggest a physiology that is distinct from other hES cell lines and from the embryo, and one which we consider to be aberrant.

It is not clear what event(s) may have impacted MEL1 hES cells' ability to respond appropriately to an environmental stimulus. Isolation technology or time in culture are unlikely to be contributing factors as the lines were isolated in the same laboratory (Adewumi *et al.* 2007) and were analyzed here after a similar time in culture. Both hES cell lines showed oxygen regulated expression of *COX4-2* (Fukuda *et al.* 2007), demonstrating that MEL1 hES cells can detect changes in oxygen. The failure of these cells to regulate other genes in response to oxygen suggests that they are unable to adapt through modulation of mitochondrial activity and biogenesis, regulation of gene expression or alterations in metabolism. Variables that may have impacted these cells include original embryo quality or unrecognized stressors being applied to the population during isolation and culture that has resulted in the selection of cells with an aberrant physiology. Whatever the cause, these analyses have identified metabolic dysfunction within this population of MEL1 hES cells. Most importantly, this study highlights the need to characterize the physiology and physiological responses of hES cells to environmental change in culture.

Conclusion

Oxygen was found to significantly impact hES cell metabolism, mitochondria and mitochondrial

transcripts. MEL2 hES cells displayed an amino acid and carbohydrate profile similar to that of other reported hES cell lines. When cultured at physiological oxygen, MEL2 hES cells displayed lower numbers of mtDNA, ATP and mitochondrial staining, reduced expression of genes associated with mitochondrial biogenesis and OXPHOS, elevated amino acid consumption and a higher glucose flux when compared to cells at 20% oxygen. This response suggests an investment of hES cells in oxidative metabolism under 20% oxygen, alterations to physiology which occurred in the absence of an altered colony morphology, karyotype, or loss of pluripotency marker expression. Significantly, MEL1 hES cells displayed none of these alterations in response to oxygen, indicative of an underlying metabolic dysfunction. Whether this dysfunction is related to the observed retarded differentiation kinetics remains to be tested. The MEL1 metabolic profile may indicate elevated basal expression of a nutrient sensing pathway (HIF, AMPK or mTOR), diminishing the impact of the oxygen treatment and retarding differentiation. For example, human mesenchymal stem cell differentiation requires repression of the HIF pathway, and differentiation is attenuated when HIF1 α is chemically stabilized under 20% oxygen conditions (Hsu *et al.* 2013). HIF1 α and HIF1 β activity exponentially increases as oxygen decreases from 7.0% to 0.5% (Jiang *et al.* 1996), therefore, MEL1 hES cells may respond to lower oxygen concentrations than 5%. Our data highlight the need for metabolic screening to establish stimulus-induced metabolic responsiveness as a standard measure of characterization. The reported contrast in metabolite profiles of MEL1 and MEL2 hES cells in a defined medium, demonstrates that oxygen, like serum or other medium components, can alter hES cell physiology. hES cells are anticipated to be used in drug discovery, disease modeling and therapeutics. Unidentified metabolic dysfunction in hES cell lines may affect their function when differentiated, cloud the results of drug and disease research or promote tumorigenesis when applied therapeutically.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0633>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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