

# Environmental Oxygen Tension Regulates the Energy Metabolism and Self-Renewal of Human Embryonic Stem Cells

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## Abstract

Energy metabolism is intrinsic to cell viability but surprisingly has been little studied in human embryonic stem cells (hESCs). The current study aims to investigate the effect of environmental O<sub>2</sub> tension on carbohydrate utilisation of hESCs. Highly pluripotent hESCs cultured at 5% O<sub>2</sub> consumed significantly more glucose, less pyruvate and produced more lactate compared to those maintained at 20% O<sub>2</sub>. Moreover, hESCs cultured at atmospheric O<sub>2</sub> levels expressed significantly less OCT4, SOX2 and NANOG than those maintained at 5% O<sub>2</sub>. To determine whether this difference in metabolism was a reflection of the pluripotent state, hESCs were cultured at 5% O<sub>2</sub> in the absence of FGF2 for 16 hours leading to a significant reduction in the expression of SOX2. In addition, these cells consumed less glucose and produced significantly less lactate compared to those cultured in the presence of FGF2. hESCs maintained at 5% O<sub>2</sub> were found to consume significantly less O<sub>2</sub> than those cultured in the absence of FGF2, or at 20% O<sub>2</sub>. GLUT1 expression correlated with glucose consumption and using siRNA and chromatin immunoprecipitation was found to be directly regulated by hypoxia inducible factor (HIF)-2 $\alpha$  at 5% O<sub>2</sub>. In conclusion, highly pluripotent cells associated with hypoxic culture consume low levels of O<sub>2</sub>, high levels of glucose and produce large amounts of lactate, while at atmospheric conditions glucose consumption and lactate production are reduced and there is an increase in oxidative metabolism. These data suggest that environmental O<sub>2</sub> regulates energy metabolism and is intrinsic to the self-renewal of hESCs.

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## Introduction

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst, the final stage of preimplantation embryo development. They proliferate through self-renewal and provide an excellent model to investigate developmental mechanisms since they have the potential to differentiate into all cells of the body [1]. However, if these cells are to be of therapeutic use it is imperative to ensure that a highly pluripotent population of hESCs are maintained which can then be directed down specific lineage pathways.

hESCs are notoriously difficult to maintain *in vitro* as the colonies have a propensity to spontaneously differentiate suggestive of suboptimal culture conditions; an effect which may be circumvented by the use of low environmental O<sub>2</sub> tensions [2]. Culture under atmospheric O<sub>2</sub> tensions has been found to decrease hESC proliferation and reduce pluripotency marker expression compared to culture under low (2–5%) O<sub>2</sub> tensions [3,4,5], an effect regulated by hypoxia inducible factors (HIFs), specifically HIF-2 $\alpha$  [5]. This promotion of an immature, stem cell like phenotype has also been observed in both malignant and non-malignant cells cultured under hypoxic conditions [6]. Despite these encouraging data, many of the biochemical and physiolog-

ical implications of hypoxic culture on hESCs remain to be elucidated.

Among the ~200 genes regulated by HIFs, metabolic genes feature extensively [7] suggesting that environmental O<sub>2</sub> tensions may also have a significant impact on hESC metabolism. However, the metabolic status of hESCs and the impact of environmental O<sub>2</sub> tension have received remarkably little attention. Significantly, our previous work has shown that metabolic activity is a central regulator of the phenotype and developmental potential of human preimplantation embryos [8,9] and highlights metabolism as a fundamental regulator of cellular function.

Morphologically, hESCs share many characteristics with ICM cells; a high nuclear to cytoplasmic ratio, low mitochondrial number, and expression of the same surface antigens [10,11,12,13]. It is therefore likely that the nutrition of the ICM may inform on the metabolism of hESCs. In terms of glucose utilisation, the murine ICM is wholly glycolytic compared to the differentiated trophectoderm where only 55% of the glucose consumed may be accounted for by lactate production [14]. Moreover, the ICM is also metabolically relatively quiescent in terms of mitochondrial activity, O<sub>2</sub> consumption and ATP production compared to the trophectoderm [15]. Knowledge of the metabolism of hESCs is still in its infancy but it has been

shown that pluripotency may be enhanced by inhibiting the mitochondrial respiratory chain [16]. This data was further supported by confirmation that hESCs and induced pluripotent stem cells rely on glycolysis for their energy requirements [17,18,40].

This study aims to investigate how environmental O<sub>2</sub> tension affects the regulation and energy metabolism of hESCs in terms of O<sub>2</sub>, glucose and pyruvate consumption and lactate production. Moreover, the effect of early hESC differentiation as demonstrated by the short term removal of FGF2 on hESC metabolism and pluripotency marker expression has also been investigated. These data suggest that environmental O<sub>2</sub> regulates glucose utilisation and is intrinsic to the energy metabolism and self-renewal of hESCs.

## Materials and Methods

### hESC Culture

Hues7 (D. Melton, Howard Hughes Medical Institute/Harvard University) [41] and Shef3 (Supplied by the UK Stem Cell Bank) hESCs were cultured at 20% O<sub>2</sub> in Knockout DMEM (Invitrogen) supplemented with 15% knockout serum replacement (Invitrogen), 100 µg/ml penicillin streptomycin (Invitrogen), 1 mM L-glutamine (Invitrogen), 1× non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol and 10 ng/ml FGF2 (Peprotech) on γ irradiated mouse embryonic fibroblasts (MEFs; a primary source derived in institutional facilities following University of Southampton ethical review committee approval and in accordance with UK Home Office regulations). hESCs were then transferred to Matrigel (BD Biosciences) coated plates and cultured in MEF-conditioned medium at both 20% and 5% O<sub>2</sub>. They were maintained for a minimum of 3 passages on Matrigel at both O<sub>2</sub> tensions prior to use.

### Measurement of Carbohydrate Utilisation

hESCs were passaged on to 12-well Matrigel coated plates and cultured in MEF conditioned medium. On day 2, 3 and 4 post-passage hESCs were pre-incubated in a defined metabolic medium [8] containing 1 mM glucose, 5 mM lactate, 0.47 mM pyruvate, 0.5% human serum albumin and amino acids [19] for 30 mins. The medium was then replaced with pre-determined quantities (300–500 µl) of defined medium for 1.5–3.5 h. At the end of the incubation period, all but 100 µl of medium was removed from each well and stored at –80°C prior to analysis of carbohydrate content and the number of cells in each well was determined using a haemocytometer. In subsequent experiments, the effect of removing FGF2 for 16 hours on hESC metabolism was monitored. FGF2 was removed from the medium prior to MEF conditioning and used to replace regular, MEF conditioned medium containing FGF2 on day 2 post-passage. Enzyme linked biochemical assays were used to measure the concentration of pyruvate, glucose and lactate in 180 µl of spent medium using a Konelab 20 autoanalyser (Thermo Scientific). The concentration of carbohydrates in cell containing wells was compared to cell-free control wells and the consumption of pyruvate and glucose and the production of lactate by hESCs calculated in pmol/cell/h.

### O<sub>2</sub> Assay

A 96-well O<sub>2</sub> biosensor plate (BD Biosciences) containing 3 wells of 200 mM sodium sulphite (0% O<sub>2</sub> control) and 3 wells of a defined metabolic medium (20% O<sub>2</sub> control) was incubated at 37°C in a fluorescence plate reader (BMG Labtech) for 30 mins. Hues7 hESCs on day 3 post-passage were pre-incubated with metabolic medium for 30 mins, harvested into 310 µl of fresh, pre-

warmed metabolic medium, added to a well of the O<sub>2</sub> biosensor plate and sealed using an adhesive PCR foil (Thermo Fisher) with care taken to ensure the absence of air bubbles. The fluorescence (excitation 485 nm and emission 612 nm) of each well was recorded every 2 minutes over a two hour period. After the final measurement, the protein content of each well was determined using the Bradford assay. O<sub>2</sub> consumption was calculated as µl O<sub>2</sub>/mg protein/h.

### RT-qPCR

RNA was isolated from Hues7 hESCs cultured under feeder-free conditions on Matrigel on day 3 post-passage using TriReagent (Sigma) and 2 µg reverse transcribed to cDNA using MMLV-reverse transcriptase (Promega). cDNA (4 µg) was amplified in 20 µl reactions containing 1 µl probes and primer mix (OCT4: Hs01895061\_u1; SOX2: Hs00602736\_s1; NANOG: Hs02387400\_g1; GLUT1: Hs00197884\_m1; UBC Hs00824723\_m1) and 10 µl 2× Taqman Universal PCR Master Mix (Applied Biosystems) using an ABI 7500 real time PCR system. The conditions used were 2 mins at 50°C, 10 mins at 95°C followed by 45 cycles of 95°C for 15 secs and 60°C for 1 min. Placental cDNA (0–10 ng) was used to produce a standard curve for each gene of interest as well as the endogenous control, UBC and used to quantify gene expression. All genes were analysed in duplicate and normalised to UBC.

### siRNA

siRNA was used to silence either *HIF-1α*, *HIF-2α* or *HIF-3α* in Hues7 hESCs cultured on Matrigel coated plates at 5% O<sub>2</sub>. The cells were passaged and the following day 50 nM siRNA (HIF-1α: Hs\_HIF1A\_5; HIF-2α: Hs\_EPAS1\_5; or HIF-3α: Hs\_HIF3A\_1), 12 µl HiPerfect transfection reagent (Qiagen) and 200 µl knockout DMEM were mixed, incubated at room temperature for 10 mins and added in a drop wise manner to hESCs. Allstars negative control siRNA (Qiagen) was used as a negative control. The ability of these siRNA to silence individual HIF-α isoforms has previously been validated [5]. The cells were harvested 48 h post-transfection and *GLUT1* mRNA quantified as above.

### Western Blotting

Protein was isolated from Hues7 hESCs cultured on Matrigel on day 3 post-passage by incubating in ice cold radio immunoprecipitation assay (RIPA) buffer for 30 mins followed by sonication for 30 secs. Protein (75 µg) was resolved on an 8% SDS bisacrylamide gel, transferred to nitrocellulose membrane and blocked in PBS containing 0.1% Tween-20 and 5% milk for 1 h at room temperature. The membrane was incubated in primary antibody (OCT4 (Santa Cruz) 1:1000; SOX2 (Millipore) 1:1000; NANOG (Abcam) 1:1000) diluted in blocking buffer overnight at 4°C. Membranes were washed and incubated in horse radish peroxidase-conjugated secondary antibodies (anti-mouse (GE Healthcare) 1:100,000 or anti-rabbit (GE Healthcare) 1:50,000) for 1 h at room temperature. The enhanced chemiluminescence advanced Western blotting detection kit (GE Healthcare) was used to develop the membranes prior to imaging on the Biorad Chemidoc XRS. Protein expression was quantified relative to β-actin (mouse anti-β-actin peroxidase conjugated antibody (Sigma) 1:50,000).

### Chromatin Immunoprecipitation (ChIP) Assays

Hues7 hESCs cultured in normoxic (20% O<sub>2</sub>), or hypoxic (5% O<sub>2</sub>) conditions were cross-linked with 1% formaldehyde for 10 min and the reaction blocked with 0.125 M glycine. ChIP

experiments were performed with HIF-2 $\alpha$  (Novus Biologicals) or immunoglobulin G (IgG) antibody (Santa Cruz) as previously described [20,21] except that immuno-complexes were washed using high-salt buffers as followed: 10 times with 600  $\mu$ l buffer A (0.1% SDS, 2 mM EDTA, 20 mM Tris HCl pH8, 1% Triton X-100, 500 mM NaCl), 8 times with 600  $\mu$ l buffer B (0.1% SDS, 2 mM EDTA, 20 mM Tris HCl pH8, 1% Triton X-100, 1 M NaCl), 3 times with 600  $\mu$ l TE (10 mM Tris HCl H8, 1 mM EDTA) buffer. Recovered DNA was amplified with custom Taqman Assays (Applied Biosystems) spanning a predicted hypoxia response element (HRE) site at -1691 bp of the GLUT1 proximal promoter (GLUT1 fwd: CAAATGTGTGGATGTGAGTTGC; GLUT1 rev: CCATCACGGTCCTTCTTCATG; GLUT1 probe: AGGCTGAGCGTGAAA). qPCR was performed using an ABI 7900 HT Fast Real Time System (Applied Biosystems) in a 384 well plate.

### Statistical Analysis

All data were tested to determine whether they were normally distributed using the Anderson Darling normality test. Any differences in the utilisation of carbohydrates or glycolytic rate with O<sub>2</sub> tension were analysed using a Student's t-test. Differences in mRNA expression were normalised to the endogenous control, UBC and then to 1 for cells cultured at 5% O<sub>2</sub>, or to Allstars transfection controls when genes were silenced. Differences in protein expression were normalised to  $\beta$ -actin and then to 1 for cells cultured at 5% O<sub>2</sub>. In both cases a one sample t-test was used to determine significance from either 5% O<sub>2</sub> or transfection controls. Differences in O<sub>2</sub> consumption between hESCs maintained at 5%, 20% and 5% O<sub>2</sub> in the absence of FGF2 was determined using a one-way analysis of variance followed by a Fisher's test. Differences in binding of HIF-2 $\alpha$  to the GLUT1 promoter with O<sub>2</sub> tension was expressed as a percentage of input (non-immunoprecipitated chromatin) calculated using  $100 \times 2^{[Ct(\text{Input}) - Ct(\text{IP})]}$  for each sample and expressed as box and whisker plots. All data represent at least 3 independent experiments and are presented as mean  $\pm$  SEM.

## Results

### Environmental O<sub>2</sub> Tension Regulates Carbohydrate Utilisation

hESCs maintained at 5% O<sub>2</sub> display an increased proliferation and expression of pluripotency markers compared to those cultured at 20% O<sub>2</sub> [5]. The current study aimed to determine the impact of environmental O<sub>2</sub> tension on the energy metabolism of hESCs. Hues7 hESCs were cultured at either 5% or 20% O<sub>2</sub> and the consumption of glucose and pyruvate and the production of lactate were analysed on days two to four post-passage. Similarly, the effect of environmental O<sub>2</sub> tension on the depletion of glucose and production of lactate by Shef3 hESCs on day 3 post-passage was also determined. Under each O<sub>2</sub> tension, glucose was found to be the predominant energy substrate utilised. Interestingly, both cell lines consumed almost twice as much glucose ( $P < 0.001$ ) and produced approximately 2–3 times the amount of lactate ( $P < 0.01$ – $P < 0.001$ ) when cultured at 5% O<sub>2</sub> compared to 20% O<sub>2</sub> (Fig. 1A–D). A similar, low level ( $\sim 0.1$  pmol/cell/h) of pyruvate was consumed by Hues7 hESCs on each day post-passage (Fig. 1A–C).

### Environmental O<sub>2</sub> and Short-term Removal of FGF2 Alters hESC Energy Metabolism

To investigate whether the difference in energy substrate utilisation between hESCs cultured at 5% and 20% O<sub>2</sub> was a

reflection of the degree of cell pluripotency, FGF2 was removed from the medium used to culture cells at 5% O<sub>2</sub> for 16 hours. Morphologically, there was no overt differentiation observed in any of the treatment groups on day 3 post-passage (Fig. S1). This is in agreement with previous observations of hESCs cultured at 5% or 20% O<sub>2</sub> [5].

In terms of metabolism, removing FGF2 for 16 hours from hESCs cultured at 5% O<sub>2</sub> resulted in a significant reduction in the amount of glucose consumed in Hues7 cells and a near significant reduction in Shef3 cells compared to those maintained in the presence of FGF2 (Fig. 2A and B). However, in the absence of FGF2, both cell lines displayed a significant reduction in the amount of lactate produced. Interestingly, twice as much pyruvate was consumed when Hues7 cells were cultured at 5% O<sub>2</sub> in the absence of FGF2 for 16 hours compared to when FGF2 was present ( $P < 0.01$ ; Fig. 2A). This suggests that even the very early stages of differentiation are associated with an increased reliance on oxidative metabolism.

To determine the global ability of hESCs to produce energy, O<sub>2</sub> consumption was measured. Hues7 hESCs cultured at 20% O<sub>2</sub> were found to consume approximately 4  $\mu$ l O<sub>2</sub>/mg protein/h, which was significantly greater than hESCs cultured at both 5% O<sub>2</sub> ( $P < 0.001$ ) and 5% O<sub>2</sub> where FGF2 was removed for 16 hours ( $P < 0.001$ ; Fig. 2C). Interestingly, the removal of FGF2 for 16 hours from hESCs cultured at 5% O<sub>2</sub> significantly increased O<sub>2</sub> consumption compared to those cultured in the presence of FGF2 ( $P < 0.05$ ). This suggests that hESCs maintained at 20% O<sub>2</sub> have a greater energy requirement than those cultured at 5% O<sub>2</sub> in the absence of FGF2. hESCs cultured at 5% O<sub>2</sub> in the presence of FGF2 are the quietest metabolically having the lowest rate of O<sub>2</sub> consumption.

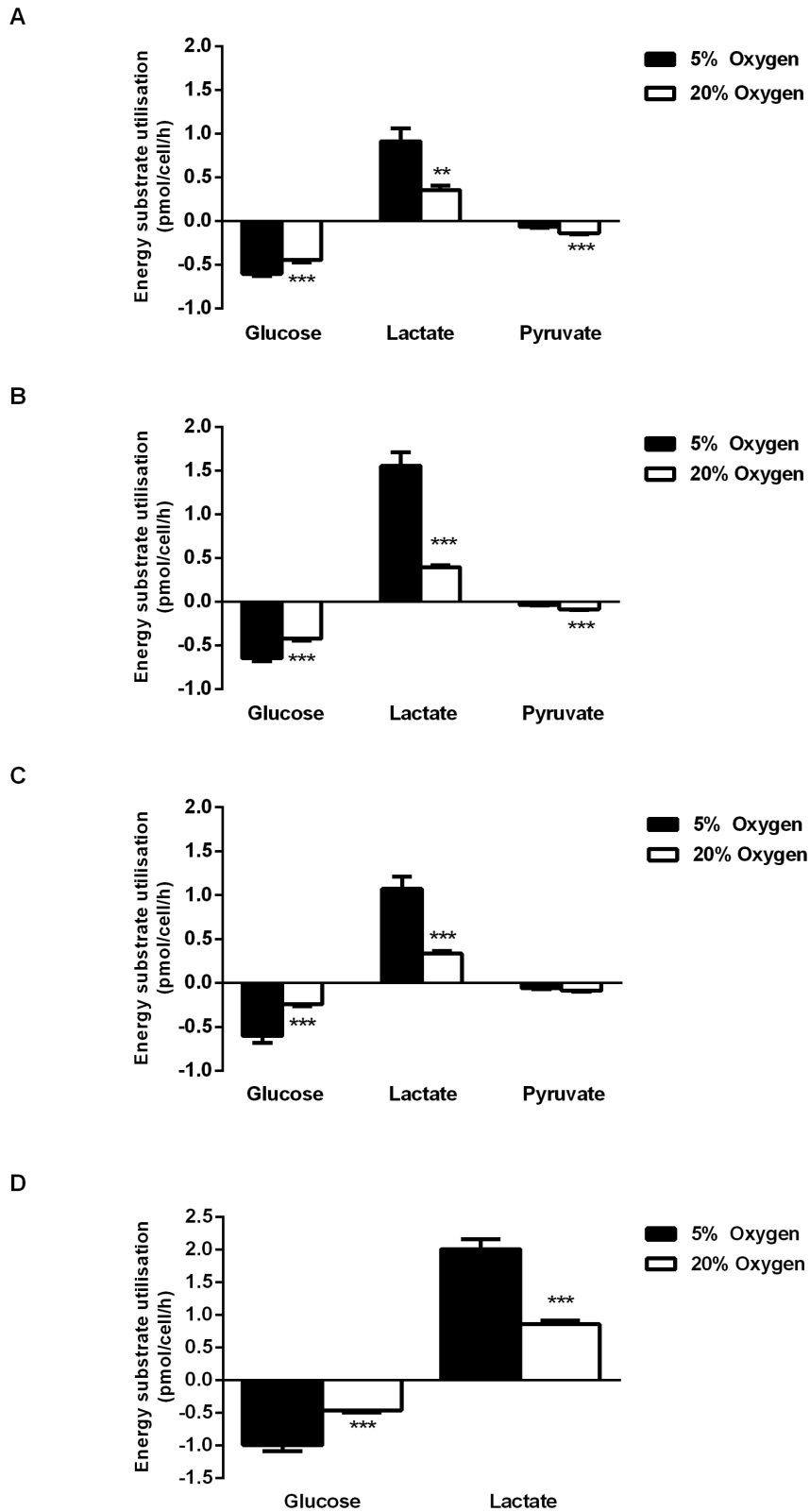
### Environmental O<sub>2</sub> and Short Term FGF2 Removal Regulates the Self-renewal of hESCs

In agreement with our previous report [5], OCT4 protein expression was significantly decreased in Hues7 hESCs maintained at 20% O<sub>2</sub> compared to those cultured at 5% O<sub>2</sub> ( $P < 0.05$ ; Fig. 3A, B). A similar reduction in SOX2 ( $P < 0.05$ ) and NANOG ( $P < 0.05$ ) expression was also observed at 20% O<sub>2</sub> (Fig. 3C–F). Interestingly, when FGF2 was removed for 16 hours from hESCs cultured at 5% O<sub>2</sub>, SOX2 protein expression decreased significantly while OCT4 and NANOG expression displayed a non-significant reduction to levels comparable to hESCs cultured under atmospheric O<sub>2</sub> tensions (Fig. 3A–F).

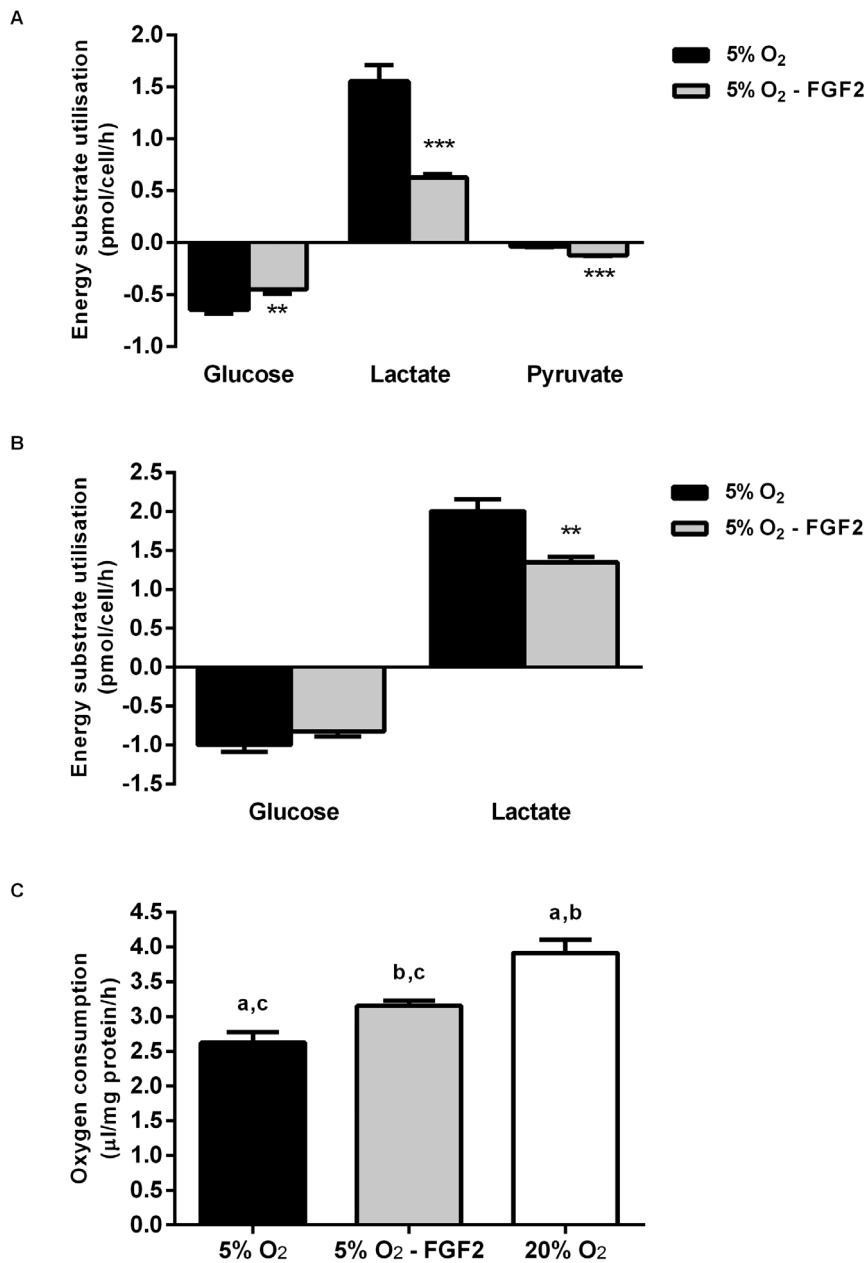
### GLUT1 mRNA is Differentially Expressed Under Hypoxic Conditions and Regulated by HIF-2 $\alpha$

To determine whether differences in glucose transport may be responsible for the increased consumption observed by Hues7 hESCs maintained at 5% O<sub>2</sub> compared to 20% O<sub>2</sub>, the expression of GLUT1 was investigated. GLUT1 mRNA expression was significantly decreased in cells maintained at 20% O<sub>2</sub> compared to those cultured at 5% O<sub>2</sub> (Fig. 4A). This suggests a correlation between the mRNA expression of GLUT1 and the uptake of glucose in hESCs.

Since HIFs are important regulators of the hypoxic response, siRNA was used to determine whether any of the HIF $\alpha$  subunits were responsible for the increased GLUT1 expression in hESCs cultured at 5% O<sub>2</sub>. GLUT1 mRNA expression was not affected when either HIF-1 $\alpha$  or HIF-3 $\alpha$  were silenced but was significantly reduced when HIF-2 $\alpha$  was knocked down ( $P < 0.001$ ; Fig. 4B). This suggests that HIF-2 $\alpha$  is an upstream regulator of GLUT1 in hESCs cultured at 5% O<sub>2</sub>.



**Figure 1. Hypoxic culture promotes glucose uptake and lactate production in hESCs.** Glucose, pyruvate and lactate utilisation were non-invasively measured in a defined hESC medium. More glucose was consumed and lactate produced by Hues7 hESCs cultured at 5% O<sub>2</sub> than at 20% O<sub>2</sub> on (A) day 2 (B) day 3 and (C) day 4 post-passage. In contrast, less pyruvate was consumed by hESCs at 5% O<sub>2</sub> compared to 20% O<sub>2</sub>. The rate of glucose consumption and lactate production was also greater on day 3 post-passage in Shef3 hESCs cultured at 5% O<sub>2</sub> compared to those maintained at 20% O<sub>2</sub> (D). \*\*P<0.01, \*\*\*P<0.001 significantly different to 5% O<sub>2</sub> (n=12–23). doi:10.1371/journal.pone.0062507.g001



**Figure 2. Short term removal of FGF2 at 5% O<sub>2</sub> alters hESC metabolism and promotes O<sub>2</sub> consumption.** Removal of FGF2 for 16 hours from Hues7 hESCs cultured at 5% O<sub>2</sub> (5% O<sub>2</sub>- FGF2) resulted in a reduction of glucose consumption and lactate production, whereas pyruvate consumption dramatically increased (A). Shef3 hESCs cultured at 5% O<sub>2</sub>- FGF2 displayed a significant reduction in lactate production (B). \*\*P<0.01, \*\*\*P<0.001 significantly different to 5% O<sub>2</sub>+FGF2 (n = 10–18). Hues7 hESCs cultured at 5% O<sub>2</sub> consumed less O<sub>2</sub> than when FGF2 was removed for 16 hours (C). hESCs maintained at 20% O<sub>2</sub> consumed the greatest amount of O<sub>2</sub>. Bars with the same superscript are significantly different; a, b, P<0.001, c, P<0.05 (n = 7–8).

doi:10.1371/journal.pone.0062507.g002

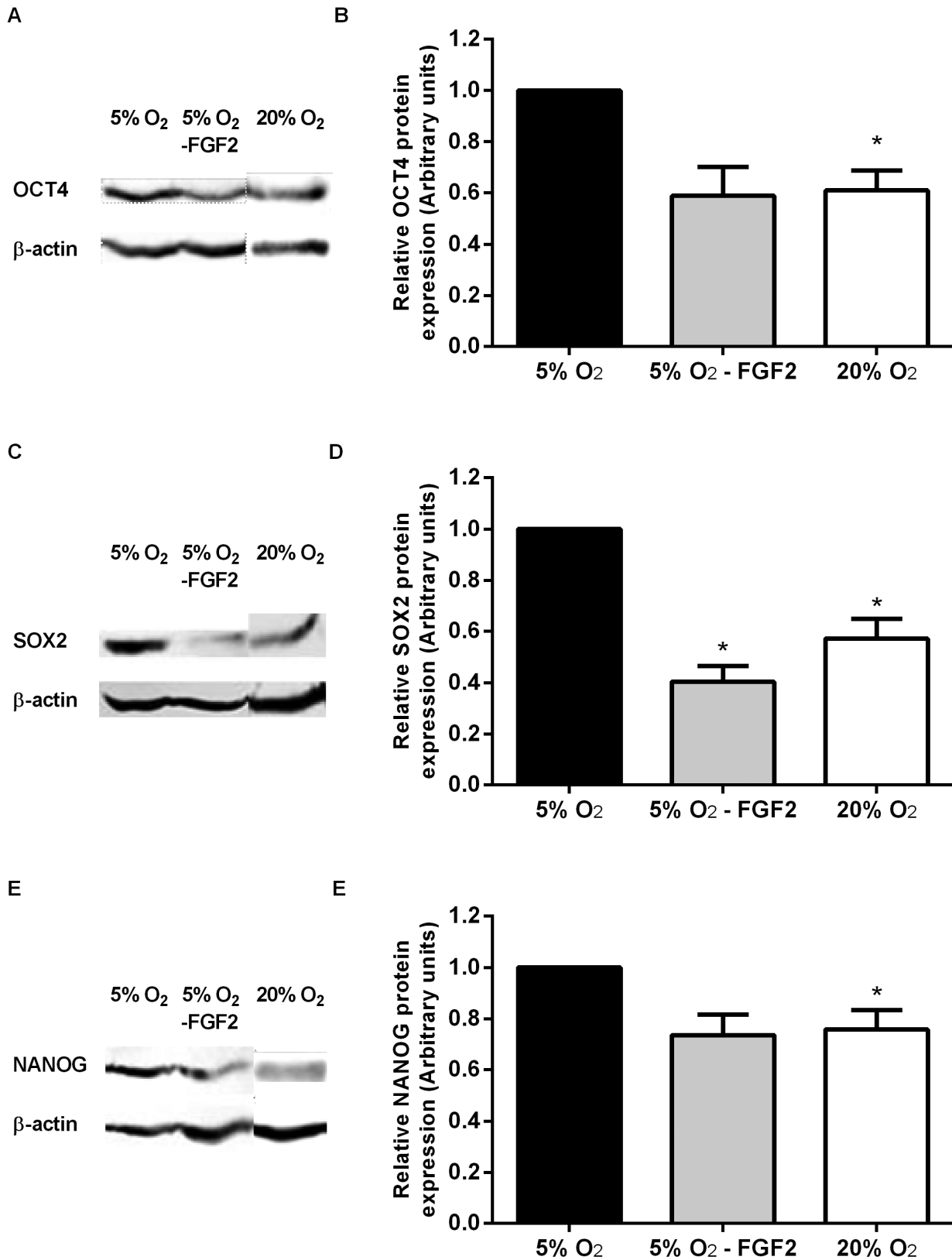
### HIF-2 $\alpha$ Binds Directly to the GLUT1 Promoter

To determine whether HIF-2 $\alpha$  binds directly to a potential HRE in the proximal promoter of GLUT1 ChIP assays were performed on Hues7 hESCs. We compared the enrichment, using qPCR, of the sequence corresponding to the GLUT1 proximal promoter when precipitating with an antibody specific for HIF-2 $\alpha$ , compared to the IgG isotype control. A 4-fold enrichment over the IgG control was observed in the chromatin isolated from hESCs maintained at 5% O<sub>2</sub> (Fig. 4C). In contrast, no significant binding of HIF-2 $\alpha$  was observed in chromatin isolated from hESCs

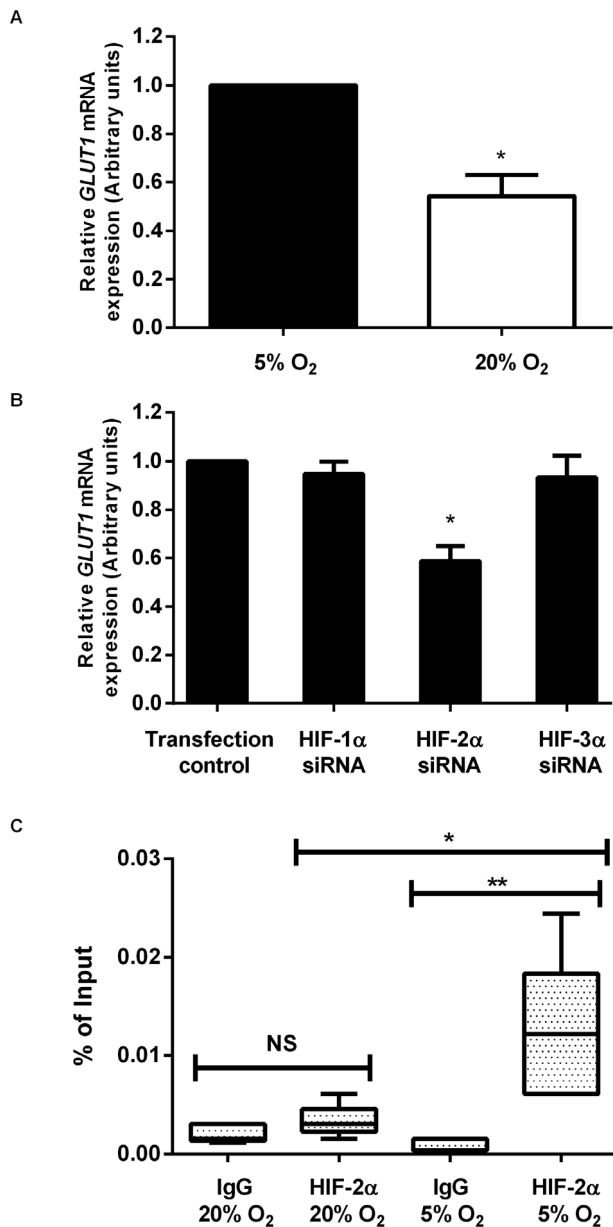
maintained at 20% O<sub>2</sub>. This data reveals a specific HIF-2 $\alpha$  interaction with the GLUT1 proximal promoter only in hESCs cultured under hypoxic conditions.

### Discussion

hESC metabolism has received little attention, despite being intrinsic to cellular function. Several studies have highlighted beneficial effects of culturing hESCs at low O<sub>2</sub> tensions including improved morphology, increased expression of pluripotency markers, a reduction in chromosomal abnormalities and a higher



**Figure 3. hESCs maintained at atmospheric O<sub>2</sub> levels express reduced levels of pluripotency markers compared to those cultured at 5% O<sub>2</sub>.** Hues7 hESCs were cultured at either 5% O<sub>2</sub>, 5% O<sub>2</sub> with FGF2 removed for 16 hours (5% O<sub>2</sub>- FGF2) or 20% O<sub>2</sub>. Protein was isolated and OCT4 (A and B), SOX2 (C and D) and NANOG (E and F) quantified using Western blotting. All data has been normalised to  $\beta$ -actin and to 1 for 5% O<sub>2</sub>. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 significantly different from 5% O<sub>2</sub> (n=3-4). doi:10.1371/journal.pone.0062507.g003



**Figure 4. GLUT1 expression parallels glucose utilisation and is directly regulated by HIF-2 $\alpha$  under hypoxic conditions.** RT-qPCR was used to quantify *GLUT1* mRNA expression in Hues7 hESCs cultured at either 5% O<sub>2</sub> or 20% O<sub>2</sub> on day three post-passage (A). All data has been normalised to *UBC* and to 1 for 5% O<sub>2</sub>. \*P<0.05 significantly different to 5% O<sub>2</sub> (n=3). Using siRNA to silence HIF- $\alpha$  subunits in Hues7 hESCs cultured at 5% O<sub>2</sub>, *GLUT1* mRNA was found to be regulated by HIF-2 $\alpha$  (B). All data has been normalised to *UBC* and to 1 for the transfection control. \*P<0.05 significantly different to transfection control (n=6). Using ChIP HIF-2 $\alpha$  was found to bind to the proximal promoter of *GLUT1* only in hESCs cultured at 5% O<sub>2</sub>. ChIP assays were performed with either a HIF-2 $\alpha$  or IgG control antibody on chromatin isolated from Hues7 hESCs cultured at either 20% O<sub>2</sub> or 5% O<sub>2</sub>. DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). \*P<0.05, \*\*P<0.01, NS indicates no significant difference (n=5). doi:10.1371/journal.pone.0062507.g004

rate of proliferation [3,4,5,22] but the impact on cellular metabolism is unknown. Thus, this study sought to investigate

the influence of environmental O<sub>2</sub> on the carbohydrate utilisation, energy metabolism and self-renewal of hESCs.

Independent of O<sub>2</sub> tension, glucose was found to be the predominant substrate utilised by hESCs. However, highly pluripotent hESCs cultured under hypoxic conditions were found to deplete significantly more glucose and produce higher levels of lactate than cells maintained at atmospheric O<sub>2</sub> tensions. This was intriguing and suggested a correlation between metabolism and self-renewal. To investigate this further, FGF2, a factor required to sustain self-renewal and support growth of undifferentiated hESCs [23,24], was removed for just 16 hours from highly pluripotent cells cultured at 5% O<sub>2</sub>. The removal of FGF2 resulted in a reduced utilisation of glucose and significant decrease in the amount of lactate produced. This was intriguing and highlights the ability of hESC metabolism to adapt to changes in environmental conditions. Moreover, the resultant rates of glucose utilisation and lactate production observed in hESCs cultured in the absence of FGF2 for 16 hours were similar to cells cultured at 20% O<sub>2</sub>. This was interesting since cells maintained at 20% O<sub>2</sub> expressed significantly less OCT4, SOX2 and NANOG than those cultured under hypoxic conditions. A similar trend was also mirrored by hESCs cultured at 5% O<sub>2</sub> in the absence of FGF2. These data suggest that energy metabolism may represent a novel parameter to quantify the self-renewal potential of hESC cultures.

The mechanism of how FGF2 regulates hESC energy metabolism under hypoxic conditions is unknown but data from adipocytes implicates the involvement of HIF-1 $\alpha$  [25]. These investigators found that the culture of adipocytes under hypoxic conditions in the presence of FGF2 caused an increase in both *GLUT1* expression and lactate production through the induction of HIF-1 $\alpha$ . In hESCs HIF-1 $\alpha$  is degraded after ~48 h of hypoxic culture after which HIF-2 $\alpha$  is stabilised [5]. Thus, it could be speculated that the reduced amount of glucose consumed and lactate produced by hESCs cultured at 5% O<sub>2</sub> in the absence of FGF2 may be due to the destabilisation/degradation of HIF-2 $\alpha$  and the resultant decrease in expression of hypoxia responsive genes.

Hues7 hESCs cultured at 5% O<sub>2</sub> consumed significantly lower levels of O<sub>2</sub> and pyruvate than those maintained at either 20% O<sub>2</sub> or 5% O<sub>2</sub> in the absence of FGF2. Since O<sub>2</sub> consumption provides the best global indication of the ability of a cell to produce energy, this suggests that hESCs cultured at 5% O<sub>2</sub> are more metabolically quiescent than those cultured at 5% O<sub>2</sub> in the absence of FGF2, or at 20% O<sub>2</sub>. As OCT4, SOX2 and NANOG expression were also significantly reduced in hESCs cultured at 20% O<sub>2</sub> compared to 5% O<sub>2</sub>, this suggests that as differentiation occurs a more active metabolism ensues. These results are comparable to that in the mouse blastocyst where the ICM was found to be metabolically relatively quiescent consuming low levels of O<sub>2</sub> compared to the differentiated trophectoderm [15].

Our data also suggest that hESCs display a glycolytic metabolism consuming glucose and producing lactate. This is in agreement with mouse ES cells and mesenchymal stem cells which utilise glycolysis as a primary source of ATP production in the undifferentiated state and switch to oxidative phosphorylation upon differentiation [26,27]. Similarly, nuclear reprogramming associated with induced pluripotent stem cells has been shown to be associated with a shift from an oxidative metabolism to one dependent on glycolysis [18,39]. Together with the current data, this highlights the importance of glycolysis for maintaining the pluripotent state.

hESCs cultured at 5% O<sub>2</sub> expressed significantly more *GLUT1* than those maintained at 20% O<sub>2</sub>. Glucose transporter expression is known to increase the amount of glucose taken up by cells and

GLUT1 is thought to be the predominant transporter in many cell types including mouse ESCs, brain, placenta, and retina [28,29,30]. Our finding of an increase in GLUT1 expression under hypoxic conditions is in agreement with that observed in mouse ESCs [31]. As a HRE is present in the promoter region of the GLUT1 gene [32,33] we were interested to determine whether any of the 3 regulated HIF- $\alpha$  subunits were responsible for this increased expression. Using siRNA, HIF- $\alpha$  subunits were silenced individually and the effect on GLUT1 expression determined. GLUT1 mRNA was down-regulated only when HIF-2 $\alpha$  was silenced, suggesting that HIF-2 $\alpha$  is an upstream regulator of GLUT1. The HIF family of transcription factors have also been found to mediate the expression of GLUT1 in mouse ESCs, MEFs and cardiomyocytes [34,35,36]. However, in these cell types, it was HIF-1 $\alpha$ , not HIF-2 $\alpha$  which regulated GLUT1 expression. This represents a fundamental difference in the regulation of GLUT1 between mouse and human ESCs.

Using ChIP, HIF-2 $\alpha$  was found to bind directly to the region containing a putative HRE in the proximal promoter of the GLUT1 gene only in hESCs cultured at 5% O<sub>2</sub>. This was an exciting finding since although GLUT1 has been extensively studied as a hypoxia inducible gene, to the best of our knowledge this is the first report of HIF-2 $\alpha$  binding directly to GLUT1. It is therefore possible that the increased expression of GLUT1 observed may be responsible for the greater uptake of glucose into hESCs cultured at 5% O<sub>2</sub>. However, since many of the genes involved in glucose metabolism including hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, enolase, pyruvate kinase and lactate dehydrogenase have also been shown to be regulated by environmental O<sub>2</sub>, alternative mechanisms of regulation remain a possibility [34,37,38].

## References

- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, et al. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145–1147.
- Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, et al. (2006) Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* 24: 185–187.
- Ezashi T, Das P, Roberts RM (2005) Low O<sub>2</sub> tensions and the prevention of differentiation of hES cells. *Proceedings of the National Academy of Sciences of the United States of America* 102: 4783–4788.
- Westfall SD, Sachdev S, Das P, Hearne LB, Hannink M, et al. (2008) Identification of oxygen-sensitive transcriptional programs in human embryonic stem cells. *Stem cells and development* 17: 869–881.
- Forristal CE, Wright KL, Hanley NA, Oreffo RO, Houghton FD (2010) Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. *Reproduction* 139: 85–97.
- Lofstedt T, Fredlund E, Holmquist-Mengelbier L, Pietras A, Ovenberger M, et al. (2007) Hypoxia inducible factor-2 $\alpha$  in cancer. *Cell Cycle* 6: 919–926.
- Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC (2003) Differential roles of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and HIF-2 $\alpha$  in hypoxic gene regulation. *Mol Cell Biol* 23: 9361–9374.
- Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, et al. (2002) Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod* 17: 999–1005.
- Brisson DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, et al. (2004) Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod* 19: 2319–2324.
- Sathananthan H, Pera M, Trounson A (2002) The fine structure of human embryonic stem cells. *Reproductive biomedicine online* 4: 56–61.
- Van Blerkom J, Davis P, Mathwig V, Alexander S (2002) Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos. *Hum Reprod* 17: 393–406.
- Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, et al. (2002) Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells* 20: 329–337.
- Draper JS, Fox V (2003) Human embryonic stem cells: multilineage differentiation and mechanisms of self-renewal. *Arch Med Res* 34: 558–564.
- Hewison LC, Leese HJ (1993) Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. *J Exp Zool* 267: 337–343.
- Houghton FD (2006) Energy metabolism of the inner cell mass and trophectoderm of the mouse blastocyst. *Differentiation* 74: 11–18.
- Varum S, Momcilovic O, Castro C, Ben-Yehudah A, Ramalho-Santos J, et al. (2009) Enhancement of human embryonic stem cell pluripotency through inhibition of the mitochondrial respiratory chain. *Stem cell research* 3: 142–156.
- Varum S, Rodrigues AS, Moura MB, Momcilovic O, Easley CA, et al. (2011) Energy metabolism in human pluripotent stem cells and their differentiated counterparts. *PLoS One* 6: e20914.
- Folmes CD, Nelson TJ, Martinez-Fernandez A, Arrell DK, Lindor JZ, et al. (2011) Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. *Cell Metab* 14: 264–271.
- Tay JL, Rutherford AJ, Killick SR, Maguiness SD, Partridge RJ, et al. (1997) Human tubal fluid: production, nutrient composition and response to adrenergic agents. *Hum Reprod* 12: 2451–2456.
- Petruzzelli R, Gaudino S, Amendola G, Sessa R, Puzone S, et al. (2010) Role of the cold shock domain protein A in the transcriptional regulation of HBG expression. *Br J Haematol* 150: 689–699.
- Weinmann AS, Farnham PJ (2002) Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. *Methods* 26: 37–47.
- Forsyth NR, Musio A, Vezzoni P, Simpson AH, Noble BS, et al. (2006) Physiologic oxygen enhances human embryonic stem cell clonal recovery and reduces chromosomal abnormalities. *Cloning Stem Cells* 8: 16–23.
- Amit M, Margulets V, Segev H, Shariki K, Laevsky I, et al. (2003) Human feeder layers for human embryonic stem cells. *Biology of reproduction* 68: 2150–2156.
- Xu C, Inokuma MS, Denham J, Golds K, Kundu P, et al. (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nature biotechnology* 19: 971–974.
- Kihira Y, Yamano N, Izawa-Ishizawa Y, Ishizawa K, Ikeda Y, et al. (2011) Basic fibroblast growth factor regulates glucose metabolism through glucose

## Summary

These studies demonstrate that hESCs utilise glucose as a predominant source of energy. Highly pluripotent hESCs cultured at 5% O<sub>2</sub> have a low level of O<sub>2</sub> consumption, consume high levels of glucose and produce large amounts of lactate. The onset of early differentiation, through the removal of FGF2 for 16 hours in hESCs cultured at 5% O<sub>2</sub>, or by maintaining cells at 20% O<sub>2</sub> leads to a more oxidative metabolism, demonstrated by an increased consumption of O<sub>2</sub> and a decreased uptake of glucose and production of lactate. The rise in glucose uptake observed under hypoxic conditions corresponds to an increased expression of GLUT1 which is directly regulated by HIF-2 $\alpha$ . This data provides further metabolic support for maintaining hESCs under hypoxic conditions, rather than culturing at atmospheric levels of O<sub>2</sub>. Finally, our data highlights the intrinsic importance of energy metabolism for hESC maintenance and may provide a novel method for the assessment of self-renewal.

## Supporting Information

**Figure S1 Typical morphology of Shef3 hESCs on day 3 post-passage cultured at 5% O<sub>2</sub> (A), 5% O<sub>2</sub> in the absence of FGF2 for 16 hours (5% O<sub>2</sub>- FGF2; B) and 20% O<sub>2</sub>. Scale bar = 100  $\mu$ m. (TIF)**

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## Author Contributions

Technical support: KLP. Conceived and designed the experiments: FDH CEF. Performed the experiments: CEF DRC FEC RP. Analyzed the data: CEF DRC FEC RP. Wrote the paper: FDH CEF TS RP DRC.



- transporter 1 induced by hypoxia-inducible factor-1alpha in adipocytes. *Int J Biochem Cell Biol* 43: 1602–1611.
26. Kondoh H, Leonart ME, Nakashima Y, Yokode M, Tanaka M, et al. (2007) A high glycolytic flux supports the proliferative potential of murine embryonic stem cells. *Antioxid Redox Signal* 9: 293–299.
  27. Chen CT, Shih YR, Kuo TK, Lee OK, Wei YH (2008) Coordinated changes of mitochondrial biogenesis and antioxidant enzymes during osteogenic differentiation of human mesenchymal stem cells. *Stem Cells* 26: 960–968.
  28. Flier JS, Mueckler M, McCall AL, Lodish HF (1987) Distribution of glucose transporter messenger RNA transcripts in tissues of rat and man. *J Clin Invest* 79: 657–661.
  29. Froehner SC, Davies A, Baldwin SA, Lienhard GE (1988) The blood-nerve barrier is rich in glucose transporter. *J Neurocytol* 17: 173–178.
  30. Kahn BB, Flier JS (1990) Regulation of glucose-transporter gene expression in vitro and in vivo. *Diabetes Care* 13: 548–564.
  31. Lee SH, Heo JS, Han HJ (2007) Effect of hypoxia on 2-deoxyglucose uptake and cell cycle regulatory protein expression of mouse embryonic stem cells: involvement of Ca<sup>2+</sup>/PKC, MAPKs and HIF-1alpha. *Cell Physiol Biochem* 19: 269–282.
  32. Hayashi M, Sakata M, Takeda T, Yamamoto T, Okamoto Y, et al. (2004) Induction of glucose transporter 1 expression through hypoxia-inducible factor 1alpha under hypoxic conditions in trophoblast-derived cells. *J Endocrinol* 183: 145–154.
  33. Zelzer E, Levy Y, Kahana C, Shilo BZ, Rubinstein M, et al. (1998) Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. *The EMBO journal* 17: 5085–5094.
  34. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, et al. (1998) Cellular and developmental control of O<sub>2</sub> homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev* 12: 149–162.
  35. Seagroves TN, Ryan HE, Lu H, Wouters BG, Knapp M, et al. (2001) Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. *Mol Cell Biol* 21: 3436–3444.
  36. Wright G, Higgin JJ, Raines RT, Steenbergen C, Murphy E (2003) Activation of the prolyl hydroxylase oxygen-sensor results in induction of GLUT1, heme oxygenase-1, and nitric-oxide synthase proteins and confers protection from metabolic inhibition to cardiomyocytes. *J Biol Chem* 278: 20235–20239.
  37. Ryan HE, Lo J, Johnson RS (1998) HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *EMBO J* 17: 3005–3015.
  38. Semenza GL, Jiang BH, Leung SW, Passantino R, Concorde JP, et al. (1996) Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* 271: 32529–32537.
  39. Panopoulos AD, Yanes O, Ruiz S, Kida YS, Diep D, et al. (2012) The metabolome of induced pluripotent stem cells reveals metabolic changes occurring in somatic cell reprogramming. *Cell Res* 22: 168–177.
  40. Prigione A, Fauler B, Lurz R, Lehrach H, Adjaye J (2010) The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. *Stem Cells* 28: 721–733.
  41. Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, et al. (2004) Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 350: 1353–1356.