

Original Paper

Effect of Oxygen Tension on the Amino Acid Utilisation of Human Embryonic Stem Cells

David R. Christensen^{a,b} Philip C. Calder^{b,c} Franchesca D. Houghton^{a,b}

^aCentre for Human Development, Stem Cells & Regeneration, ^bHuman Development and Health Academic Unit, Faculty of Medicine, University of Southampton, ^cNIHR Southampton Biomedical Research Centre, University of Southampton and University Hospital Southampton NHS Foundation Trust, Southampton, UK

Key Words

Embryonic stem cells • Amino acids • Hypoxia • Oxygen tension • Metabolism

Abstract

Background/Aims: Human embryonic stem cells (hESCs) are a potential source of cells for treatment of many degenerative diseases, but in culture have a propensity to spontaneously differentiate, possibly due to suboptimal conditions. Culture at low oxygen tensions improves hESC maintenance and regulates carbohydrate metabolism. Hence, a greater understanding of the nutrient requirements of hESCs will allow production of more appropriate culture media. This study aims to investigate the effect of environmental oxygen tension on the amino acid metabolism of hESCs. **Methods:** The production or depletion of amino acids by hESCs cultured at 5% or 20% oxygen in the presence or absence of FGF2 was measured by reverse-phase HPLC. **Results:** Atmospheric oxygen, or removal of FGF2 from hESCs cultured at 5% oxygen, perturbed the uptake or release of individual amino acids and the total amino acid turnover compared to hESCs cultured at 5% oxygen. In particular, serine uptake was reduced at 20% oxygen and by removal of FGF2. **Conclusions:** Highly pluripotent hESCs, cultured at 5% oxygen, demonstrate a greater amino acid turnover than hESCs cultured at 20% oxygen, or without FGF2. These data suggest that amino acid turnover could be used as a measure of the self-renewal capacity of hESCs.

Copyright © 2014 S. Karger AG, Basel

Dr. Franchesca Houghton

Centre for Human Development, Stem Cells and Regeneration
Faculty of Medicine, University of Southampton, Duthie Building (MP808)
Southampton General Hospital, Tremona Road, Southampton, SO16 6YD (UK)
Tel. +44(0) 23 8120 8731, Fax +44 (0)23 8120 4264, E-Mail F.D.Houghton@soton.ac.uk

Introduction

Pluripotent, human embryonic stem cells (hESCs) are derived from the inner cell mass of the blastocyst and have the capacity to differentiate into all cells of the body [1-4]. Thus, hESCs have the potential to provide an unlimited source of cells for transplantation to treat many degenerative diseases. To efficiently and safely generate cells for therapeutic use, it is necessary to ensure that hESCs are maintained in a highly pluripotent state before being directed down specific lineage pathways. However, hESCs have a tendency to spontaneously differentiate in culture. The conditions of culture may influence this.

It is now widely acknowledged that culture at a low oxygen tension is beneficial for hESC maintenance in terms of reduced spontaneous differentiation, increased expression of key pluripotency markers, enhanced proliferation, and decreased incidence of chromosomal abnormalities [5-9]. In addition, a reduced environmental oxygen tension has also been found to regulate the energy metabolism of hESCs [10]. Highly pluripotent hESCs cultured at a reduced oxygen tension displayed an increased expression of OCT4, SOX2, and NANOG, consumed less oxygen and pyruvate, and consumed more glucose, while producing higher levels of lactate than those maintained at 20% oxygen [10]. Interestingly, hESCs cultured at 5% oxygen in the absence of FGF2 for 16 hours displayed a metabolism similar to those maintained at atmospheric oxygen levels [10]. These observations are intriguing since the changes in energy metabolism occur prior to morphological differentiation and highlight the importance of understanding hESC nutrition and metabolism.

Amino acids represent a potential nutrient source, but the amino acid requirements of hESCs have received little attention. This is surprising since amino acids are required for many cellular processes, most notably protein synthesis, but also for pH regulation, the synthesis of nucleotides, glutathione, nitric oxide, and other signalling molecules, as well as being a potential source of energy [11-21]. Amino acids are routinely included in most culture media and have been shown to be beneficial for blastocyst development *in vitro* [22]. Moreover, the non-invasive measurement of amino acid utilisation by individual preimplantation human embryos following *in vitro* fertilisation was able to predict developmental competency not only to the blastocyst stage, but also those that would implant and give rise to a live birth [23-25].

Although amino acid metabolism of stem cell populations has been little studied, human mesenchymal stem cells (hMSCs) have been shown to consume glutamine, arginine, aspartate, asparagine, serine, and tyrosine, while alanine, glycine and glutamate were produced [26]. Moreover, hMSCs were found to metabolise non-essential amino acids differently from other, non-stem cell lines [26]. More recently, the amino acid requirements of a hESC line were characterised and found to vary with differences in the composition of the media [27]. These data suggest that not only do stem cells have a specific amino acid requirement, but also that the content of different media impacts metabolism which will affect cellular phenotype and self-renewal. Although culture at low oxygen tension has been shown to be beneficial for hESC maintenance and is associated with a reduction in oxidative metabolism, the effect on amino acid metabolism is unknown. However, data from other cell types suggest an increase in amino acid uptake occurs under hypoxic conditions [28-31].

This study aims to investigate the impact of environmental oxygen tension on the depletion or production of amino acids by hESCs. In addition, how early hESC differentiation, through the short-term removal of FGF2, affects amino acid metabolism will also be investigated. The findings suggest that highly pluripotent hESCs cultured at 5% oxygen have an increased requirement for amino acids compared to those maintained at atmospheric oxygen tensions.

Materials and Methods

hESC culture

Shef3 (supplied by the UK Stem Cell Bank) [32] and Hues-7 (D. Melton, Howard Hughes Medical Institute/Harvard University) [33] hESCs were cultured at 20% oxygen in Knockout DMEM (Invitrogen) supplemented with 15% knockout serum replacement (Invitrogen), 100 µg/ml penicillin/streptomycin (Invitrogen), 1 mM L-glutamine (Invitrogen), 1 x 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% oxygen. hESCs were maintained on Matrigel at both oxygen tensions for a minimum of 3 passages prior to use.

Measurement of amino acid utilisation

hESCs were passaged onto 12-well Matrigel-coated plates and cultured in MEF-conditioned medium at either 5% or 20% oxygen. On day 3 post-passage hESCs were pre-incubated in a defined metabolic medium [23] containing 5 mM glucose, 5 mM lactate, 0.47 mM pyruvate, 0.5% human serum albumin and a known concentration of amino acids [34] for 30 minutes. The medium was then replaced with a pre-determined quantity (300-500 µl) of the defined medium for 1.5-3.5 h. Cell-free wells containing medium only, subjected to the same incubation procedures, were used as controls and hence took account of any non-specific amino acid degradation or appearance. At the end of the incubation period, all but 100 µl of the used medium was collected from each well and stored at -80°C prior to analysis for amino acid content. Using measurements of cell-free control samples as a baseline, the change in amino acid concentration was calculated. The number of cells in each well was determined using a haemocytometer. This allowed the rates of amino acid depletion/production to be calculated as fmol/cell/h. To investigate the effect of early hESC differentiation, hESC culture medium was prepared in the absence of FGF2 prior to MEF-conditioning. This MEF-conditioned medium without FGF2 was used to culture hESCs for 16 hours prior to incubation in a defined metabolic medium.

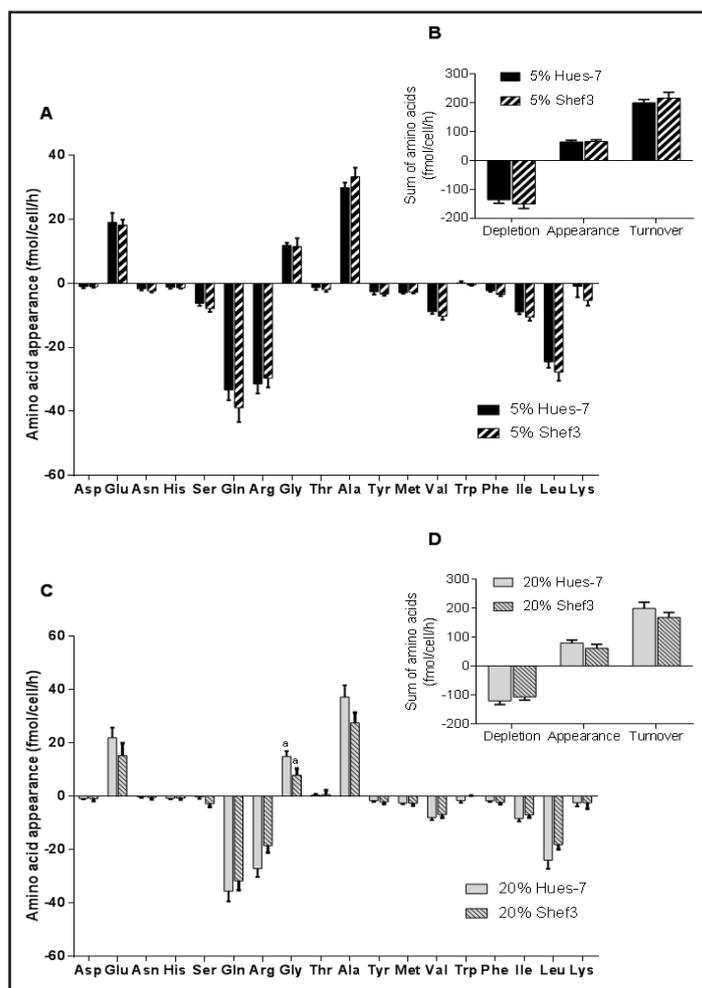
The concentration of amino acids in the media was measured by reverse-phase HPLC following the method of Houghton et al. [23], but using an Agilent 1100 series automated HPLC system fitted with a Gemini 50 x 4.6 mm column (Phenomenex). Media samples were diluted 2 in 25 with HPLC-grade water and pre-column derivatisation was achieved by the automated reaction of the diluted sample with an equal volume of the OPA (ortho-Phthalaldehyde)/ β -mercaptoethanol reagent (10 µl β -mercaptoethanol diluted in 5 ml OPA). The elution gradient operated at a flow rate of 2.5 ml/min. Solvent A consisted of 800 ml sodium acetate (83 mM, pH 5.9), 15 ml tetrahydrofuran (Fisher Scientific), and 200 ml HPLC-grade methanol (Fisher Scientific). Solvent B was made up from 800 ml HPLC-grade methanol and 200 ml sodium acetate (83 mM, pH 5.9). Using this method, it was not possible to detect proline or cysteine.

Individual and total amino acid depletion and production by hESCs were determined. Total amino acid depletion, and production, was calculated by summing the amino acids depleted from, and produced into, the medium, respectively. The total turnover was calculated as the sum of the amino acids depleted from and produced into the medium.

Statistical analysis

All data were tested to determine whether they were normally distributed using the D'Agostino-Pearson omnibus normality test. Any differences in the utilisation of amino acids with oxygen tension or the presence or absence of FGF2 were analysed using an unpaired t-test. Differences in the total amino acid depletion, production and turnover between hESCs maintained at 5%, 20%, 5% in the absence of FGF2, and 20% oxygen in the absence of FGF2 were determined using a two-way analysis of variance followed by a Fisher's test. All data represent a minimum of three independent experiments.

Fig. 1. Shef3 and Hues-7 hESCs show highly similar metabolic profiles. The utilisation of amino acids was compared between Hues-7 and Shef3 hESCs when cultured at 5% (A; n = 27 and n = 35 respectively) or 20% oxygen (C; n = 24 and n = 18 respectively). Positive values represent production of amino acids into the medium, whereas negative values represent amino acids that were depleted from the medium. The two hESC lines were also compared in terms of their total amino acid depletion, production and turnover at 5% (B) and at 20% oxygen (D). Bars with the same superscript are significantly different; ^ap = 0.035. Bars represent mean ± SEM.



Results

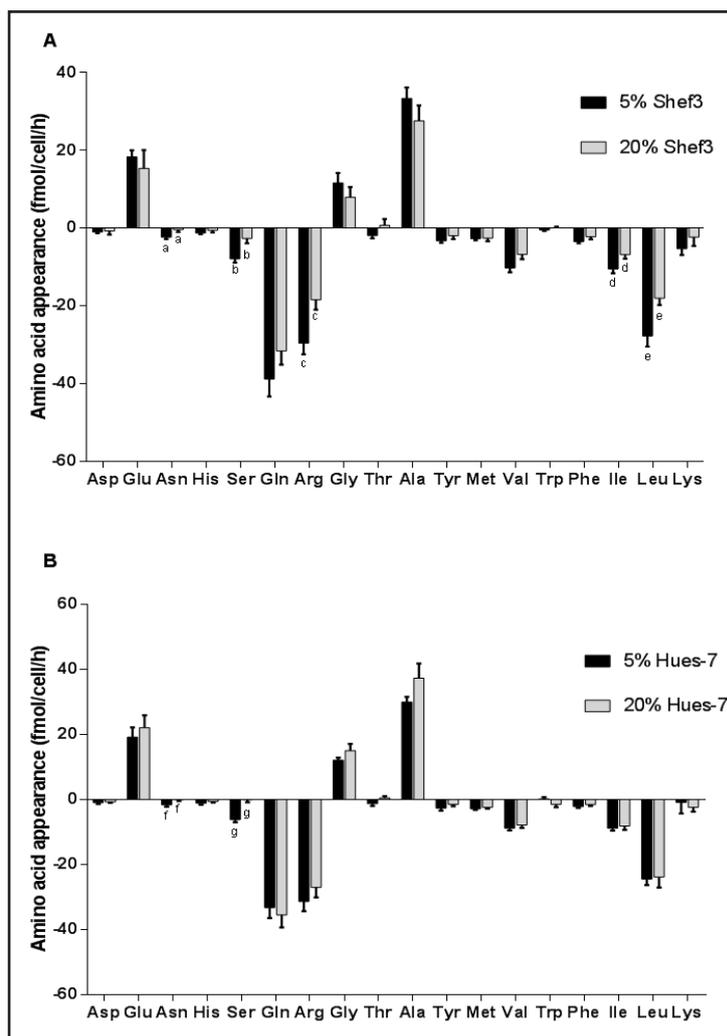
Two human embryonic stem cell lines show highly similar amino acid metabolic profiles at 5% and 20% oxygen

The amino acid depletion and production by two hESC lines, Shef3 and Hues-7, cultured at either 5% or 20% oxygen, were measured using HPLC following a two hour incubation in a defined medium. Negative values represent amino acids that were depleted from the medium and positive values represent production of amino acids into the medium. Total amino acid depletion, production, and turnover, by hESCs, were also calculated from the individual values.

No significant differences were found between the amino acid profiles for Shef3 and Hues-7 hESCs cultured at 5% oxygen (Fig. 1A). Only the utilisation of glycine was found to be significantly different in a comparison of the amino acid metabolic profiles of Shef3 and Hues-7 hESCs cultured at 20% ($p = 0.035$; Fig. 1C). The total amino acid depletion, production, and turnover for both Shef3 and Hues-7 hESCs cultured at 5% or 20% were also not significantly different (Fig. 1B, D). These data demonstrate a high degree of similarity in amino acid metabolism between these two hESC lines.

Shef3 and Hues-7 hESCs were found to consume glutamine, arginine, tyrosine, methionine, valine, phenylalanine, isoleucine, and lysine when cultured at 5% or 20% oxygen (Fig. 1). Asparagine and histidine were found to be consumed by Shef3 and Hues-7 hESCs cultured at 5%, but not at 20% oxygen (Fig. 1). Glutamine was consistently the most-consumed amino acid. Glutamate, glycine, and alanine were produced by both hESC lines cultured at both oxygen tensions (Fig. 1).

Fig. 2. Culture at 20% oxygen alters the amino acid metabolism of hESCs. The amino acid utilisation of hESCs. The amino acid utilisation of Shef3 (A) and Hues-7 (B) hESCs cultured at 5% oxygen (n = 35 and n = 27 respectively) was compared with that of hESCs cultured at 20% oxygen (n = 18 and n = 24 respectively). Bars with the same superscript are significantly different; ^ap = 0.013; ^bp = 0.003; ^cp = 0.016; ^dp = 0.04; ^ep = 0.021; ^fp = 0.042; and ^gp < 0.001. Bars represent mean ± SEM.



Culture at 20% oxygen alters the amino acid metabolism of hESCs

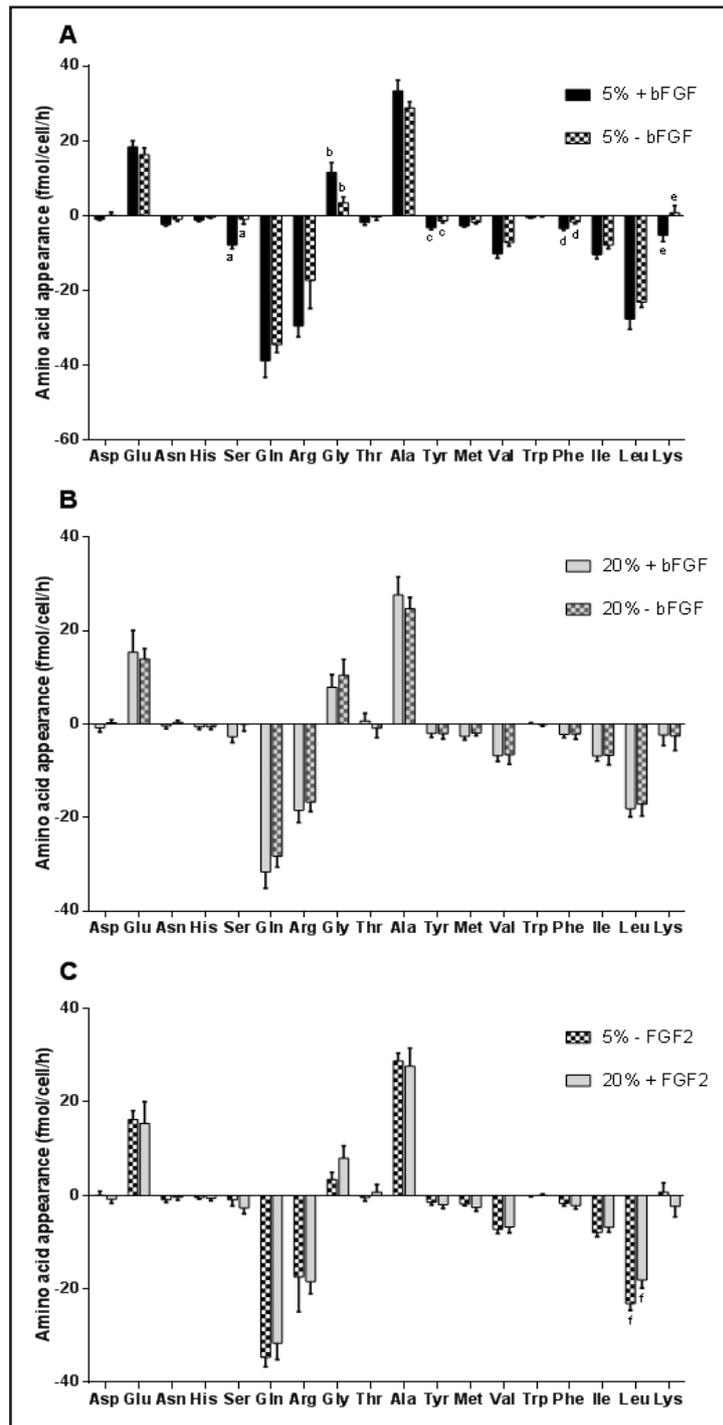
To investigate whether environmental oxygen tension affects the amino acid metabolism of hESCs, the amino acid profiles of hESCs cultured at 5% oxygen were compared to those maintained at 20% oxygen (Fig. 2). Both Shef3 and Hues-7 hESCs maintained at 20% oxygen were found to consume less asparagine (p = 0.013 and p = 0.042, respectively) and less serine (p = 0.003 and p < 0.001, respectively) than those cultured at 5% oxygen (Fig. 2). Shef3 hESCs cultured at 5% oxygen were also found to consume more arginine (p = 0.016), isoleucine (p = 0.04), and leucine (p = 0.021) than those maintained at 20% oxygen (Fig. 2A).

Short-term removal of FGF2 alters the amino acid metabolism of hESCs

Previously, we have shown that the short-term removal of FGF2 leads to a reduced rate of glucose uptake and lactate production in hESCs [10]. In addition, we demonstrated that either culture at 20% oxygen or the short-term removal of FGF2 results in a reduction of SOX2, OCT4 and NANOG expression [10]. The current study aimed to determine whether the short-term removal of FGF2 also affects amino acid metabolism.

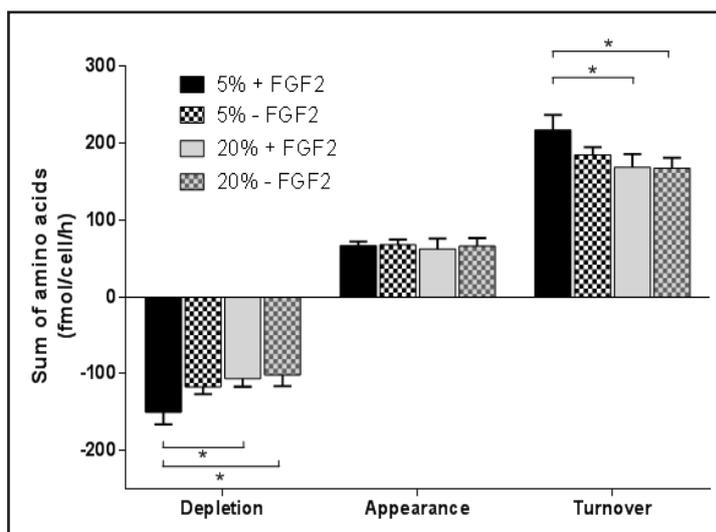
Removal of FGF2, for 16 hours, from the culture medium of Shef3 hESCs at 5% oxygen resulted in a reduced rate of serine (p < 0.001), tyrosine (p = 0.024), phenylalanine (p = 0.023), and lysine (p = 0.025) consumption and a decreased production of glycine (p = 0.022; Fig. 3A), compared to culture at 5% oxygen in the presence of FGF2. In contrast, the short-term removal of FGF2 from culture of Shef3 hESCs at 20% produced no differences in the uptake or production of any of the amino acids measured (Fig. 3B).

Fig. 3. Short-term removal of FGF2 alters the amino acid metabolism of hESCs maintained at 5% oxygen. The effect of removing FGF2 for 16 hours from the culture medium on the amino acid utilisation of Shef3 hESCs cultured at 5% (A; n = 35 with FGF2 and n = 23 without FGF2) and at 20% oxygen (B; n = 18 with FGF2 and n = 20 without FGF2) was analysed. Subsequently, the amino acid profile of Shef3 hESCs cultured at 5% oxygen without FGF2 was compared with that of hESCs maintained at 20% oxygen in the presence of FGF2 (C). Bars with the same superscript are significantly different; ^ap < 0.001; ^bp = 0.022; ^cp = 0.024; ^dp = 0.023; ^ep = 0.025; and ^fp = 0.030. Bars represent mean ± SEM.



As removal of FGF2 from 5% oxygen culture of hESCs had previously been shown to reduce rates of glucose utilisation and lactate production in a similar way to the effect of culture at 20% oxygen, the effect of removal of FGF2 from 5% oxygen culture on amino acid metabolism has been compared with culture at 20% oxygen (Fig. 3C). The amino acid metabolic profiles of Shef3 hESCs cultured at 5% oxygen in the absence of FGF2 for 16 hours was similar to that of Shef3 hESCs cultured at 20% oxygen. Only one significant difference was measured; a reduced rate of consumption of leucine was measured in Shef3 hESCs cultured at 20% oxygen compared with those cultured at 5% oxygen after the short-term removal of FGF2 ($p = 0.03$; Fig. 3C).

Fig. 4. Environmental oxygen tension and short-term removal of FGF2 alter the total amino acid depletion and turnover of hESCs. Shef3 hESCs cultured at 5% and 20% oxygen, with or without short-term removal of FGF2 were compared in terms of their total amino acid depletion, production and turnover (n = 35 for 5% with FGF2, n = 23 for 5% without FGF2, n = 18 for 20% with FGF2, and n = 20 for 20% without FGF2). *p < 0.05. Bars represent mean ± SEM.



Environmental oxygen and short-term removal of FGF2 alter the total turnover of amino acids in hESCs

Shef3 hESCs cultured at 5% oxygen were found to significantly increase the total amino acid depletion and turnover ($p = 0.028$ and $p = 0.016$, respectively; Fig. 4), compared to those maintained at 20% oxygen. The short-term removal of FGF2 from Shef3 hESCs cultured at 5% oxygen showed a trend towards a reduction in the total amino acid depletion and turnover to rates similar to those measured for culture at 20% oxygen in the presence or absence of FGF2 (Fig. 4). Removal of FGF2 from culture at 20% oxygen had no effect on total amino acid depletion, production or turnover when compared with culture at 20% oxygen in the presence of FGF2 (Fig. 4). However, there was a significant reduction in the rates of amino acid depletion and turnover between hESCs cultured at 20% oxygen where FGF2 was removed for 16 hours and those cultured at 5% oxygen in the presence of FGF2 ($p = 0.012$ and $p = 0.011$, respectively; Fig. 4).

Discussion

Until recently, hESC metabolism had received surprisingly little attention [10, 27], despite its importance for cell function. Previously, we have shown that environmental oxygen regulates energy metabolism in hESCs, by demonstrating an effect of oxygen tension on the rates of glucose uptake and lactate production, as well as oxygen consumption [10]. This study aimed to investigate whether oxygen tension similarly regulates amino acid metabolism in hESCs.

A number of studies have demonstrated the advantages of hESC culture at reduced oxygen tensions, including reduced spontaneous differentiation, increased expression of pluripotency markers, fewer chromosomal aberrations and increased rates of proliferation [5-9, 35] and low oxygen culture is thought to be more physiological [36-38].

The amino acid requirement of hESCs has received little attention, with only a single report being published to date [27]. This study found glutamine was consistently consumed and, perhaps unsurprisingly, that amino acid metabolism was altered by the addition of fetal calf serum to the medium [27]. The current study is the first to investigate the effect of environmental oxygen on the amino acid metabolism of hESCs. The high degree of similarity for the two hESC lines investigated here (Fig. 1) and their broad similarity with a previously described hESC line suggests that the findings of the current study may be characteristic of hESCs in general and therefore might not be cell-line specific. Glutamine, arginine, tyrosine, methionine, valine, phenylalanine, isoleucine, and lysine were all found to be consumed by both hESC lines investigated when cultured at either 5% or 20% oxygen and glutamine was

consistently the most-consumed amino acid (Fig. 1). Glutamate, glycine, and alanine were produced by hESCs of both lines in 5% and 20% oxygen culture (Fig. 1). Optimisation of culture media to improve maintenance of highly pluripotent hESCs requires an understanding of the nutrient requirements of hESCs.

Several differences were found between the amino acid metabolic profiles of Shef3 hESCs cultured at 5% and 20% oxygen (Fig. 2A), but only two differences were found in both Shef3 and Hues-7 hESCs (Fig. 2); uptake of asparagine and serine was greater in hESCs cultured under hypoxia compared with those maintained at atmospheric oxygen tensions. As maintenance of hESCs under hypoxia is associated with increased expression of pluripotency markers [5, 6, 8, 9], it may be that serine and asparagine are consumed at faster rates in hESCs at 5% oxygen because of the increased pluripotency or simply because of the hypoxic environment. Interestingly, glycine/serine metabolism has been receiving increasing levels of interest in cancer research recently because of roles that have been identified in tumorigenesis, survival of tumour-initiating cells (also known as cancer stem cells) and support of the Warburg effect, which is the characteristic of cancer cells to generate energy through glycolysis, even when oxygen is available [39-44]. Because of the general similarities between hESCs and cancer cells, including in their energy metabolism, it is possible that serine is required for the same metabolic pathways in hESCs as it is in breast and lung cancers [40-44]. Serine has also previously been shown to stimulate development of 1-cell hamster embryos into blastocysts *in vivo* [45, 46]. As hESCs are initially derived from the inner cell mass of blastocysts, it may be that serine is performing a similar role for hESCs as it does for cells of the developing embryo. Interestingly asparagine metabolism has also been implicated in cancer biology and for the adaptation of cancer cells to hypoxia [47-50].

To investigate further whether altered amino acid metabolism in atmospheric oxygen tension is related to pluripotency, FGF2, a factor that is required to maintain self-renewal and undifferentiated hESCs [10, 51, 52], was removed from the culture media for 16 hours. Previously, we had investigated the effect of removal of FGF2 for 16 hours in hESCs cultured at 5% oxygen and found that it induced a reduction in glucose utilisation, lactate production and expression of SOX2; similar effects to those cultured at 20% oxygen in the presence of FGF2 [10]. Interestingly, this current study found that the removal of FGF2 from hESCs cultured at 5% oxygen resulted in changes in the rate of uptake or production for five amino acids, including a reduced rate of uptake of serine, which was similar to the effect seen for culture at 20% oxygen (Fig. 3). In fact, a comparison of the amino acid profiles of hESCs cultured at 5% oxygen in the absence of FGF2 with those maintained at 20% oxygen in the presence of FGF2 found only a single difference (Fig. 3C), suggesting that removal of FGF2 has a similar effect on hESCs maintained at 5% to culture of hESCs at atmospheric oxygen. Finally, comparisons of total amino acid depletion, production and turnover in Shef3 hESCs maintained at 5% or 20% oxygen, with or without FGF2 added to the culture media, showed significantly reduced depletion and turnover in hESCs cultured at 20% oxygen and a similar trend in hESCs cultured at 5% oxygen after short-term removal of FGF2.

The current study has demonstrated that highly pluripotent hESCs cultured at 5% oxygen show a higher rate of amino acid depletion than those maintained at 20% oxygen. Initiating differentiation by removing FGF2 for 16 hours from hESCs cultured at 5% oxygen also leads to a reduction in the rate of amino acid depletion. These data suggest that amino acid turnover might also be able to be used as a measure of the self-renewal potential of hESC cultures. This investigation further highlights the importance of metabolism for hESC maintenance.

Acknowledgements

We are grateful to Kate Parry for technical support. This work was funded by the UK Medical Research Council G1000406 and G0701153.

References

- 1 Evans MJ, Kaufman MH: Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981;292:154-156.
- 2 Martin GR: Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 1981;78:7634-638.
- 3 Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM: Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282:1145-1147.
- 4 Thomson JA, Marshall VS: Primate embryonic stem cells. *Curr Top Dev Biol* 1998;38:133-165.
- 5 Ezashi T, Das P, Roberts RM: Low O₂ tensions and the prevention of differentiation of hES cells. *Proc Natl Acad Sci U S A* 2005;102:4783-4788.
- 6 Westfall SD, Sachdev S, Das P, Hearne LB, Hannink M, Roberts RM, Ezashi T: Identification of oxygen-sensitive transcriptional programs in human embryonic stem cells. *Stem Cells Dev* 2008;17:869-881.
- 7 Prasad SM, Czepiel M, Cetinkaya C, Smigielska K, Weli SC, Lysdahl H, Gabrielsen A, Petersen K, Ehlers N, Fink T, Minger SL, Zachar V: Continuous hypoxic culturing maintains activation of Notch and allows long-term propagation of human embryonic stem cells without spontaneous differentiation. *Cell Prolif* 2009;42:63-74.
- 8 Chen H-F, Kuo H-C, Lin S-P, Chien C-L, Chiang M-S, Ho H-N: Hypoxic culture maintains self-renewal and enhances embryoid body formation of human embryonic stem cells. *Tissue Eng Part A* 2010;16:2901-2913.
- 9 Forristal CE, Wright KL, Hanley NA, Oreffo RO, Houghton FD: Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. *Reproduction* 2010;139:85-97.
- 10 Forristal CE, Christensen DR, Chinnery FE, Petruzzelli R, Parry KL, Sanchez-Elsner T, Houghton FD: Environmental oxygen tension regulates the energy metabolism and self-renewal of human embryonic stem cells. *PLoS One* 2013;8:e62507.
- 11 Meijer AJ, Baquet A, Gustafson L, van Woerkom GM, Hue L: Mechanism of activation of liver glycogen synthase by swelling. *J Biol Chem* 1992;267:5823-5828.
- 12 Bredt DS, Snyder SH: Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem* 1994;63:175-195.
- 13 Häussinger D: The role of cellular hydration in the regulation of cell function. *Biochem J* 1996;313:697-710.
- 14 Dawson KM, Collins JL, Baltz JM: Osmolarity-dependent glycine accumulation indicates a role for glycine as an organic osmolyte in early preimplantation mouse embryos. *Biol Reprod* 1998;59:225-232.
- 15 Lane M, Gardner DK: Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 1998;13:991-997.
- 16 Wu G, Morris SM: Arginine metabolism: nitric oxide and beyond. *Biochem J* 1998;336:1-17.
- 17 Wu G, Meininger CJ: Regulation of nitric oxide synthesis by dietary factors. *Annu Rev Nutr* 2002;22:61-86.
- 18 Meijer AJ, Dubbelhuis PF: Amino acid signalling and the integration of metabolism. *Biochem Biophys Res Commun* 2004;313:397-403.
- 19 Morris SM: Arginine: beyond protein. *Am J Clin Nutr* 2006;83:508S-512S.
- 20 Chang L, Geng B, Yu F, Zhao J, Jiang H, Du J, Tang C: Hydrogen sulfide inhibits myocardial injury induced by homocysteine in rats. *Amino Acids* 2008;34:573-585.
- 21 Wu G: Amino acids: metabolism, functions, and nutrition. *Amino Acids* 2009;37:1-17.
- 22 Devreker F, Hardy K, Van den Bergh M, Vannin AS, Emiliani S, Englert Y: Amino acids promote human blastocyst development in vitro. *Hum Reprod* 2001;16:749-756.
- 23 Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, Leese HJ: Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod* 2002;17:999-1005.
- 24 Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, Lieberman BA, Leese HJ: Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod* 2004;19:2319-2324.
- 25 Stokes PJ, Hawkhead JA, Fawthrop RK, Picton HM, Sharma V, Leese HJ, Houghton FD: Metabolism of human embryos following cryopreservation: Implications for the safety and selection of embryos for transfer in clinical IVF. *Hum Reprod* 2007;22:829-835.
- 26 Higuera GA, Schop D, Spitters TWGM, van Dijkhuizen-Radersma R, Bracke M, de Bruijn JD, Martens D, Karperien M, van Boxtel A, van Blitterswijk CA: Patterns of amino acid metabolism by proliferating human mesenchymal stem cells. *Tissue Eng Part A* 2012;18:654-664.
- 27 Rathjen J, Yeo C, Yap C, Tan BSN, Rathjen PD, Gardner DK: Culture environment regulates amino acid turnover and glucose utilisation in human ES cells. *Reprod Fertil Dev* 2013; DOI: 10.1071/rd12276.
- 28 Soh H, Wasa M, Fukuzawa M: Hypoxia upregulates amino acid transport in a human neuroblastoma cell line. *J Pediatr Surg* 2007;42:608-612.
- 29 Gleason JE, Corrigan DJ, Cox JE, Reddi AR, McGinnis LA, Culotta VC: Analysis of hypoxia and hypoxia-like states through metabolite profiling. *PLoS One* 2011;6:e24741.

- 30 King N, Lin H, Suleiman MS: Oxidative stress increases SNAT1 expression and stimulates cysteine uptake in freshly isolated rat cardiomyocytes. *Amino Acids* 2011;40:517-526.
- 31 Fike CD, Sidoryk-Wegrzynowicz M, Aschner M, Summar M, Prince LS, Cunningham G, Kaplowitz M, Zhang Y, Aschner JL: Prolonged hypoxia augments L-citrulline transport by system A in the newborn piglet pulmonary circulation. *Cardiovasc Res* 2012;95:375-384.
- 32 Aflatoonian B, Ruban L, Shamsuddin S, Baker D, Andrews P, Moore H: Generation of Sheffield (Shef) human embryonic stem cell lines using a microdrop culture system. *In Vitro Cell Dev Biol Anim* 2010;46:236-241.
- 33 Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, Wang S, Morton CC, McMahon AP, Powers D, Melton DA: Derivation of embryonic stem-cell lines from human blastocysts. *New Engl J Med* 2004;350:1353-1356.
- 34 Tay JI, Rutherford AJ, Killick SR, Maguiness SD, Partridge RJ, Leese HJ: Human tubal fluid: production, nutrient composition and response to adrenergic agents. *Hum Reprod* 1997;12:2451-2456.
- 35 Forsyth NR, Musio A, Vezzoni P, Simpson AHRW, Noble BS, McWhir J: Physiologic oxygen enhances human embryonic stem cell clonal recovery and reduces chromosomal abnormalities. *Cloning Stem Cells* 2006;8:16-23.
- 36 Mitchell JA, Yochim JM: Measurement of intrauterine oxygen tension in the rat and its regulation by ovarian steroid hormones. *Endocrinology* 1968;83:691-700.
- 37 Rodesch F, Simon P, Donner C, Jauniaux E: Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol* 1992;80:283-285.
- 38 Fischer B, Bavister BD: Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil* 1993;99:673-679.
- 39 Vander Heiden MG, Cantley LC, Thompson CB: Understanding the Warburg effect: The metabolic requirements of cell proliferation. *Science* 2009;324:1029-1033.
- 40 Locasale JW, Grassian AR, Melman T, Lyssiotis CA, Mattaini KR, Bass AJ, Heffron G, Metallo CM, Muranen T, Sharfi H, Sasaki AT, Anastasiou D, Mullarky E, Vokes NI, Sasaki M, Beroukhi R, Stephanopoulos G, Ligon AH, Meyerson M, Richardson AL, Chin L, Wagner G, Asara JM, Brugge JS, Cantley LC, Vander Heiden MG: Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. *Nat Genet* 2011;43:869-874.
- 41 Possemato R, Marks KM, Shaul YD, Pacold ME, Kim D, Birsoy K, Sethumadhavan S, Woo H-K, Jang HG, Jha AK, Chen WW, Barrett FG, Stransky N, Tsun Z-Y, Cowley GS, Barretina J, Kalaany NY, Hsu PP, Ottina K, Chan AM, Yuan B, Garraway LA, Root DE, Mino-Kenudson M, Brachtel EF, Driggers EM, Sabatini DM: Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. *Nature* 2011;476:346-350.
- 42 Zhang WC, Shyh-Chang N, Yang H, Rai A, Umashankar S, Ma S, Soh BS, Sun LL, Tai BC, Nga ME, Bhakoo KK, Jayapal SR, Nichane M, Yu Q, Ahmed DA, Tan C, Sing WP, Tam J, Thirugananam A, Noghabi MS, Pang YH, Ang HS, Mitchell W, Robson P, Kaldis P, Soo RA, Swarup S, Lim EH, Lim B: Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. *Cell* 2012;148:259-272.
- 43 Maddocks ODK, Berkers CR, Mason SM, Zheng L, Blyth K, Gottlieb E, Vousden KH: Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. *Nature* 2013;493:542-546.
- 44 Vacanti NM, Metallo CM: Exploring metabolic pathways that contribute to the stem cell phenotype. *Biochim Biophys Acta* 2013;1830:2361-2369.
- 45 Gwatkin RBL, Haidri AA: Requirements for the maturation of hamster oocytes in vitro. *Exp Cell Res* 1973;76:1-7.
- 46 Bavister BD, McKiernan SH: Regulation of Hamster Embryo Development In Vitro by Amino Acids; in Bavister BD, (ed): *Preimplantation Embryo Development*. New York, Springer, 1993, pp 57-72.
- 47 Broome JD: Studies on the mechanism of tumor inhibition by L-asparaginase. Effects of the enzyme on asparagine levels in the blood, normal tissues, and 6C3HED lymphomas of mice: differences in asparagine formation and utilization in asparaginase-sensitive and -resistant lymphoma cells. *J Exp Med* 1968;127:1055-1072.
- 48 Aslanian AM, Fletcher BS, Kilberg MS: Asparagine synthetase expression alone is sufficient to induce L-asparaginase resistance in MOLT-4 human leukaemia cells. *Biochem J* 2001;357:321-328.
- 49 Ameri K, Luong R, Zhang H, Powell AA, Montgomery KD, Espinosa I, Bouley DM, Harris AL, Jeffrey SS: Circulating tumour cells demonstrate an altered response to hypoxia and an aggressive phenotype. *Br J Cancer* 2010;102:561-569.
- 50 Balasubramanian M, Butterworth E, Kilberg M: Asparagine synthetase: regulation by cell stress and involvement in tumor biology. *Am J Physiol - Endocrinol Metab* 2013;304:E789-E799.
- 51 Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK: Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 2001;19:971-974.
- 52 Amit M, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, Itskovitz-Eldor J: Human feeder layers for human embryonic stem cells. *Biol Reprod* 2003;68:2150-2156.