Hypoxic regulation of preimplantation embryos: lessons from human embryonic stem cells

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Abstract

Development of the preimplantation embryo is reliant on nutrients present in the milieu of the reproductive tract. While carbohydrates, amino acids, lipids, and micronutrients are often considered when discussing preimplantation embryo nutrition, environmental oxygen is frequently overlooked. Although oxygen is not classically considered a nutrient, it is an important component of the *in vitro* culture environment and a critical regulator of cellular physiology. Oxygen is required to sustain an oxidative metabolism but when oxygen becomes limited, cells mount a physiological response driven by a family of transcription factors termed hypoxia inducible factors which promote expression of a multitude of oxygen sensitive genes. It is this hypoxic response that is responsible not only for the switch to a glycolytic metabolism but also for a plethora of other cellular responses. There has been much debate in recent years over which environmental oxygen tension is preferential for the culture of preimplantation embryos. The review will evaluate this question and highlight how research using human embryonic stem cells can inform our understanding of why culturing under physiological oxygen tensions may be beneficial for the development of embryos generated through clinical *in vitro* fertilisation.

Oxygen environment in the reproductive tract

Oxygen is essential for mammalian life and although the atmosphere contains 160mmHg oxygen at sea level, or approximately 20.9% oxygen, the concentration present in different tissues within the body varies widely but are much lower in the range of 5-100mmHg (Ast and Mootha, 2019). *In vivo*, the preimplantation embryo develops in an increasingly hypoxic environment (Figure 1); ~8.7% oxygen in the oviduct decreasing to 1.5-2% oxygen in the uterus of the rhesus monkey (Fischer and Bavister, 1993) while in the human, the intrauterine oxygen tension is ~2% oxygen (Ottosen et al., 2006). This dramatic reduction in oxygen tension as the embryo reaches the uterus is perhaps not surprising given that the implantation site in the rat uterus is devoid of blood vessels and hence thought to be anoxic (Rogers et al., 1983). Given

the hypoxic environment of the reproductive tract, exposure of embryos to supraphysiological oxygen tensions in incubators maintained at 5% CO_2 in air must be questioned.

There has been much debate over the nomenclature used to describe the oxygen tension in the female reproductive tract. This is because the low, 1.5-8.7% oxygen environment experienced by the preimplantation embryo *in vivo* is clearly physiological and hence would more accurately be described as normoxic, rather than hypoxic. Indeed, it would then follow that atmospheric oxygen should be classed as hyperoxic for the preimplantation embryo *in vitro*. However, convention in the field uses atmospheric, or 20% oxygen to describe normoxia, with a reduced oxygen tension with respect to atmospheric oxygen being termed hypoxia. Thus, for the purpose of this review, conventional terminology will be used but with particular care taken to avoid use of the term "normoxia". Instead, the oxygen tension experienced in the reproductive tract will be termed physiological, or hypoxic and compared to atmospheric, or 20% oxygen.

Effect of oxygen tension on the preimplantation embryo

The first reports demonstrating the beneficial effect of culturing mouse embryos at 5% oxygen were published by Whitten (Whitten, 1969, Whitten, 1971). In agreement with these studies, not only did more 2-cell mouse embryos develop to the blastocyst stage when cultured at 5% oxygen compared to 20% or 40% oxygen, but culture at atmospheric oxygen was also associated with a lower cell count in blastocysts compared to embryos cultured at 5% oxygen (Quinn and Harlow, 1978, Harlow and Quinn, 1979). It became apparent that not only the concentration of oxygen used, but also the stage at which the embryo is exposed to different oxygen levels is important. Exposure of pronucleate mouse embryos to atmospheric oxygen for just one hour before culturing to the blastocyst stage at 5% oxygen was found to inhibit development past the morula stage compared to continual culture at 5% oxygen (Pabon et al., 1989). Although continual culture of mouse zygotes at either 5% or 20% oxygen did not affect compaction or blastocyst rates, exposure to atmospheric oxygen caused a significant reduction in the number of inner cell mass (ICM) cells, and a reduction in fetal development per blastocyst transferred compared to culture under hypoxic conditions (Karagenc et al., 2004). This study highlights the importance of *in vivo* studies to fully elucidate the effect that perturbations during the preimplantation stages can have on subsequent embryo development.

Recently, the effect of culturing mouse embryos in the sequential oxygen environment observed *in vivo* was investigated (Nguyen et al., 2020). Embryos cultured at 7% oxygen from day 1 to day 3 followed by 2% from day 3 to day 5 displayed a significant increase in the rate of blastocyst formation and accelerated cell divisions at several stages of preimplantation development compared to those cultured at either 5% or 20% oxygen (Nguyen et al., 2020). This suggests that mimicking the oxygen levels found in the oviduct and

uterus may be beneficial, but blastocyst transfer studies are required to determine any impact on implantation and subsequent development.

It is also important to remember that the effect environmental oxygen tension exerts on the preimplantation embryo may be influenced by other variables in the culture system. For example, consideration should be given to whether embryos are cultured individually, or in groups. Compared to mouse embryos cultured at 5% oxygen in groups, there was a sequential reduction in the day 5 blastocyst cell number and hatching rate for embryos cultured individually at 5% oxygen, in groups at 20% oxygen or individually at 20% oxygen (Kelley and Gardner, 2016). Upon transfer of blastocysts to pseudopregnant recipients, although individual culture at 5% oxygen did not affect placental or fetal development, the placental labyrinth area was reduced compared to group culture at 5% oxygen. However, when culture at 20% was investigated, placental weight, fetal weight, crown-rump length and fetal weight to length ratio were all decreased when embryos were cultured individually compared to group culture (Kelley and Gardner, 2019). Similarly, medium composition can alter the effect of environmental oxygen on the embryo. In the bovine, embryo development to the blastocyst stage was improved by culturing at 5% oxygen compared to 20% oxygen (Fujitani et al., 1997). However, the addition of hypotaurine increased the rate of blastocyst formation at both 5% oxygen and at 20% oxygen (Fujitani et al., 1997). Together, these studies suggest that although culture at 5% oxygen was beneficial compared to 20% oxygen, the magnitude of the effect is dependent on other variables of the culture system and highlight the importance of optimising each individual component of the culture environment. Variation in media and culture components, together with heterogeneity of gamete sources and animal breeds may explain why some studies report no beneficial effects of culture at low oxygen tensions (Bahçeci et al., 2005), or even improved development at atmospheric oxygen tensions (Fischer-Brown et al., 2002, Mingoti et al., 2011).

Over the years, the benefit of culturing preimplantation embryos under hypoxic conditions has been shown in numerous species including the bovine (Thompson et al., 1990, Lim et al., 1999, Olson and Seidel, 2000), porcine (Berthelot and Terqui, 1996, Karja et al., 2004, Kitagawa et al., 2004), ovine (Thompson et al., 1990, Bernardi et al., 1996) and leporine (Li and Foote, 1993) but what about the human embryo?

It should be remembered that 5% oxygen has been used to culture embryos since the inception of human *in vitro* fertilisation (IVF) (Edwards et al., 1970, Steptoe et al., 1971, Steptoe and Edwards, 1978). Initial studies comparing the culture of human embryos at either 5% or 20% oxygen before transfer at 42-46 hours post-insemination found no difference in the rate of fertilisation, implantation or pregnancy (Dumoulin et al., 1995). Similar results were obtained in a prospective randomised study when embryos were cultured for 2 or 3 days in either 5% or 20% oxygen (Dumoulin et al., 1999). However, surplus embryos cultured at 5% oxygen were found to have an increased rate of blastocyst formation and cell number than those cultured at 20% oxygen (Dumoulin et al., 1999) suggesting that a reduced oxygen tension becomes increasingly important as the embryo develops. The fact that culturing embryos at 20%

oxygen until the cleavage stage, before transfer to the uterus had no deleterious effect on the rate of implantation was intriguing and it is tempting to speculate that the hypoxic environment present *in vivo* after transfer, provides a more favourable milieu for the embryo to complete compaction and undergo cavitation.

Two independent, prospective studies both using sibling human oocytes demonstrated that hypoxic culture did not affect fertilisation rates. However, the rate of blastocyst formation and the quality of blastocysts produced on day 5 of development was significantly improved when cultured at 5% compared to 20% oxygen throughout development (Kovacic and Vlaisavljevic, 2008, Ciray et al., 2009). These studies suggest that, in contrast to animal studies, the human early cleavage stage embryo may either be less sensitive to oxygen toxicity than the later stages of preimplantation development, or that perturbations acquired early in development are not manifest until the blastocyst stage. Investigating blastocyst formation is important but the ultimate clinical outcome is live birth rates. In a randomised controlled trial where gametes and embryos were cultured at either 5% or 20% oxygen and transferred on day 2-3 or day 5, live-birth implantation rate (number of live births divided by the number of embryos transferred), or the live birth rate (number of patients with at least one live born infant compared to the number of patients who had an oocyte retrieved) were measured as the primary endpoints (Meintjes et al., 2009). Oxygen tension had no effect on these parameters when embryos were transferred at the early cleavage stages. However, when embryos were cultured under hypoxic conditions and transferred at the blastocyst stage there was a dramatic increase in the live-birth implantation rate and the clinical pregnancy rate compared to those cultured at 20% oxygen. When the data for day 2-3 and day 5 transfers were combined, culture at 5% oxygen showed a significantly improved live-birth implantation rate and live birth rate (Meintjes et al., 2009). Similarly, more recent data has shown that culture at either 5% or 20% oxygen until day 2 or day 3 before transfer had no effect on the live birth rate per cycle but hypoxic culture did result in more good quality embryos for cryopreservation following transfer (Van Montfoort et al., 2020). Together, these data highlight the importance of hypoxic culture particularly when performing blastocyst transfers or cryopreserving human embryos.

The majority of studies have compared culture at 5% oxygen with 20% oxygen but the uterine environment has a much lower oxygen tension than the Fallopian tube. Hence, would culture at 5% oxygen for the first 3 days of development, followed by 2% oxygen be more physiological and beneficial for blastocyst formation? This precise question was investigated in two separate human studies (Kaser et al., 2018, De Munck et al., 2019). The first study using bi-pronucleate and tri-pronucleate embryos showed improved cleavage and blastocyst formation by the dual culture regime but reduced blastocyst cell number compared with continual 5% oxygen (Kaser et al., 2018). The second study found no difference in cavitation rate or the proportion of good quality blastocysts between the two (De Munck et al., 2019). Thus, it appears that 2% oxygen from day 3 to the blastocyst stage does not improve embryo quality above that already

observed by 5% oxygen. However, there are a couple of caveats; neither of these studies looked at development beyond the blastocyst stage and therefore implantation or live birth rates may still be affected. Also, moving embryos from 5% oxygen to 2% oxygen on day 3 may be developmentally early and perhaps day 4 might be preferential to ensure the embryo is at the morula stage, where *in vivo,* it would be located in the uterus.

There has now been a plethora of reports, including a Cochrane systematic review showing improved embryo development and clinical outcomes for human embryos cultured under hypoxic as opposed to atmospheric oxygen conditions (Bontekoe et al., 2012, Kasterstein et al., 2013, Kirkegaard et al., 2013, Guo et al., 2014, Ruiz et al., 2020).

Why is a low oxygen tension beneficial for preimplantation development?

Understanding how physiological oxygen tensions regulate embryo development is an area of active research and has been summarised in Figure 2. The functional significance of a hypoxic reproductive tract may be to protect the blastocyst from the detrimental effects of reactive oxygen species (ROS) since embryos cultured at 5% oxygen produce less ROS than those cultured at 20% oxygen (Goto et al., 1993, Leite et al., 2017). This is important since ROS causes DNA damage, induction of apoptosis and lipid peroxidation (Takahashi, 2012) and has been shown to be deleterious to development in the murine (Cebral et al., 2007, Ma et al., 2017), bovine (Takahashi et al., 2000) and human (Bedaiwy et al., 2004, Bedaiwy et al., 2010) embryo.

Environmental oxygen can also impact cellular senescence, a phenomenon where cells undergo persistent arrest but remain viable and able to secrete soluble factors which influence surrounding cells (Childs et al., 2014). Mouse blastocysts cultured at 5% oxygen displayed significantly lower levels of senescence-associated β-galactosidase (SA-β-galactosidase) and phosphorylated histone H2A.X, a protein associated with DNA damage than those cultured at atmospheric oxygen (Meuter et al., 2014). These results are intriguing and suggest that environmentally-induced stressors, such as 20% oxygen can induce senescence in embryos.

Oxygen tension is intrinsically linked to metabolism and the generation of ATP. It has long been known that mitochondria are immature, spherical in shape, contain few cristae with a proportion being vacuolated during early mouse embryo development (Hillman and Tasca, 1969). In contrast, by the blastocyst stage, mitochondria within the trophectoderm are slender and contain prominent cristae whereas in the ICM, they remain spherical and immature (Stern et al., 1971). This results in the trophectoderm being metabolically energetic, consuming more oxygen, producing more ATP and possessing more active mitochondria than the quiescent ICM (Houghton, 2006). The precise mechanisms which regulate mitochondrial development in the embryo remain to be discovered but environmental oxygen has been shown to be involved (Belli et al., 2019). Mouse IVF embryos cultured at 20% oxygen contained fewer

normal mitochondria and more vacuoles compared to those cultured at 5% oxygen, or *in vivo* produced embryos (Belli et al., 2019). Further research is required to understand the mechanisms regulating these results but the authors' suggestion that atmospheric oxygen may alter mitochondrial division, leading to an abnormal morphology and a reduction in total mitochondrial number warrants further investigation. This hypothesis would also be supported by work in astrocytes where exposure to hypoxia for 3 hours caused an increase in mitochondrial number compared to 20% oxygen (Quintana et al., 2019).

Metabolism is integral for embryo survival. The preimplantation embryo displays remarkable plasticity in terms of energy metabolism often being able to adapt and utilise whichever nutrients are present in the culture medium. For example, human embryos will develop to the blastocyst stage in a simple medium (Conaghan et al., 1998), but the addition of amino acids enhances development (Devreker et al., 2001). During the cleavage stages, mouse embryos consume pyruvate whereas at compaction glucose becomes the predominant energy substrate utilised (Martin and Leese, 1999). Environmental oxygen has also been shown to alter embryo metabolism. When mouse embryos were cultured at 5% oxygen during the cleavage stages, they consume less amino acids and pyruvate than those cultured at atmospheric oxygen. In contrast, when post-compaction embryos were cultured at 5% oxygen, amino acid and glucose consumption was increased compared to those cultured at 20% oxygen (Wale and Gardner, 2012). As hypoxic culture was associated with a significantly increased rate of blastocyst formation and cell number, it suggests that metabolism may be associated with developmental competency and be regulated by oxygen tension.

Ammonium is a waste product of amino acid metabolism and toxic to preimplantation embryos (Lane and Gardner, 1994). Thus, it is important that ammonium is sequestered to facilitate development. Mouse blastocysts are able to alleviate ammonium via transamination to glutamine and alanine but interestingly, only when cultured under hypoxic conditions and not at atmospheric oxygen tensions (Wale and Gardner, 2013). These findings were intriguing but provide further metabolic support for culturing embryos at a reduced oxygen tension.

Perhaps some of the most compelling data to support the culture of preimplantation embryos under hypoxic conditions looked at global gene (Rinaudo et al., 2006) and protein (Katz-Jaffe et al., 2005) expression of blastocysts. Mouse blastocysts cultured from the zygote stage at 5% oxygen displayed a similar global gene expression pattern to that of *in vivo* derived blastocysts. In contrast, embryos cultured at 20% oxygen displayed a decreased blastocyst cell number and a global gene expression pattern more disparate to that of *in vivo* blastocysts (Rinaudo et al., 2006). Similar results were obtained at the proteome level; mouse blastocysts cultured at 5% oxygen exhibited a protein expression profile more similar to *in vivo* derived blastocysts, while the proteome of blastocysts cultured at atmospheric oxygen was more divergent (Katz-Jaffe et al., 2005). These papers provide unequivocal evidence to support the culture of preimplantation embryos at 5% oxygen, at least in the mouse. It should be noted that environmental

oxygen is also able to modulate the epigenetic status of the embryo. In bovine embryos, atmospheric oxygen was found to increase global DNA methylation at both the 4-cell and blastocyst stage compared to 5% oxygen (Li et al., 2016). Similarly, the gene expression of lysine-specific histone demethylases (KDMs), specifically KDM1A, KDM4B and KDM4C were decreased in bovine blastocysts cultured at atmospheric oxygen compared to 5% oxygen (Skiles et al., 2018) suggesting that environmental oxygen also alters the chromatin landscape of the embryo.

Regulation of oxygen homeostasis

It is clear that atmospheric oxygen is deleterious for embryo development and while many physiological processes have been shown to be affected, the question remains; how is oxygen homeostasis in development regulated? Under conditions of low oxygen tension, cells mount a physiological response regulated by a family of transcription factors called hypoxic inducible factors (HIFs) which ensure oxygen homeostasis is maintained for critical oxygen-dependent processes. HIFs are known to regulate many hundreds of genes including those involved in energy metabolism, apoptosis, proliferation, self-renewal and vasculogenesis (Carmeliet et al., 1998, Ramírez-Bergeron et al., 2006, Goda and Kanai, 2012, Petruzzelli et al., 2014). HIFs form a heterodimeric complex consisting of an oxygen-dependent alpha subunit, either HIF-1α, HIF-2α or HIF-3α, and the constitutively expressed beta subunit, HIF-1β (also known as ARNT – aryl hydrocarbon receptor nuclear translocator). When oxygen is in a plentiful supply, HIF alpha subunits are hydroxylated by proline hydroxylases, recognised by the von Hippel-Lindau protein and ubiquitin ligase complex, and targeted for degradation by the proteasome. In contrast, when oxygen supply is limited the HIF alpha subunits can no longer be hydroxylated by proline hydroxylases, are stabilised and translocate to the nucleus where they bind to the HIF-1β subunit. HIFs bind to a conserved consensus sequence (A/G)CGTG termed a hypoxic response element (HRE) in the proximal promoter or enhancer of hypoxia regulated genes and increase transcription (Semenza and Wang, 1992). HIF-1α was the first discovered, is ubiquitously expressed and hence was considered to be the master regulator of the hypoxic response (Wang et al., 1995, Semenza, 1998). HIF-2α (also known as EPAS1) was first characterised by three independent groups (Ema et al., 1997, Tian et al., 1997, Flamme et al., 1998) and found to be structurally similar to HIF-1α having a 48% amino acid homology (Hu et al., 2003). However, in comparison to HIF-1α, HIF-2α has a more restricted pattern of expression including vascular endothelial cells, liver parenchymal cells and renal interstitial cells (Tian et al., 1997, Wiesener et al., 2003). Although HIF-1α and HIF-2α bind to the same HRE, HIF-1α is thought to be responsible for the initial, acute transcriptional response to hypoxia, while HIF-2α regulates the chronic hypoxic response (Holmquist-Mengelbier et al., 2006, Mole et al., 2009, Koh et al., 2011). HIF-3α, or IPAS has a limited pattern of expression including thymus, corneal epithelium and Purkinje cells of the cerebellum (Gu et al., 1998, Makino et al., 2001). It is the least well characterised of the HIF-α subunits and has multiple splice variants which have been shown to inhibit HIF-1α and HIF-2α (Gu et al., 1998, Hara et al., 2001, Heikkila et al., 2011).

HIFs are crucial for embryo development. Both HIF-1α and HIF-2α null mice are embryonic lethal. HIF-1α -/- mice die around day E11 with vascular defects, the neural tube fails to close due to mesenchymal cell death and there are also cardiovascular malformations. (Iyer et al., 1998, Kotch et al., 1999). HIF-2α -/- mice die around day E12.5 – E16.5 from bradycardia (Tian et al., 1998). In some cases, HIF-2α null mice die shortly after birth from respiratory distress syndrome (Compernolle et al., 2002). Interestingly, loss of HIF-2α was also found to dramatically decrease the number of primordial germ cells present in E8.5 embryos (Covello et al., 2006).

In the bovine blastocyst, the mRNA expression of both HIF-1α and HIF2α were unaffected by environmental oxygen. However, in embryos cultured at 7% oxygen before compaction and then transferred to either 2%, 7% or 20% oxygen, HIF-1α protein was not expressed in any of the blastocysts. In contrast, HIF-2α protein was expressed predominantly in the nuclei of blastocysts cultured in each treatment group but expression appeared increased in the ICM of blastocysts cultured at 2% oxygen post-compaction compared to 7% oxygen (Harvey et al., 2004). Interestingly, the number of cells in the ICM were significantly increased in the bovine blastocysts cultured at 2% oxygen compared to those cultured at 7% or 20% oxygen post-compaction despite there being no overall difference in total blastocyst cell number (Harvey et al., 2004). Intriguingly, culture at 2% oxygen post-compaction was also associated with an increase in GLUT1 (Harvey et al., 2004) and lactate dehydrogenase A (Harvey et al., 2007) mRNA. Both GLUT1 (Petruzzelli et al., 2014) and lactate dehydrogenase A (Cui et al., 2017) have been shown to be target genes for HIF-2α and hence may be responsible for their increased expression. More recently, HIF-2α was observed predominantly in the nuclei of mouse blastocysts cultured from the 2-cell stage at 3% oxygen, whereas those cultured at 20% oxygen displayed only very weak cytoplasmic staining (Ma et al., 2017). These investigators also found increased GLUT3 and VEGF transcript expression in blastocysts cultured under hypoxic as opposed to atmospheric oxygen. Again, both GLUT3 and VEGF are known to be HIF regulated (Maxwell et al., 1997). It is tempting to speculate that HIF-2α may be responsible for the increased glucose uptake (Wale and Gardner, 2012) observed under hypoxic conditions by enhancing the expression of GLUT1, GLUT3 and lactate dehydrogenase A. Similarly, the increased VEGF expression under hypoxia may stimulate angiogenesis required at implantation. However, caution must be exercised as the increase in these genes were observed at the mRNA level and further work is required to determine whether there is a concomitant increase in protein expression.

Importance of oxygen homeostasis – lessons from embryonic stem cells

Embryonic stem cells (ESCs) derived from the ICM of the blastocyst have the characteristics of self-renewal and pluripotency, the ability to give rise to cells of all 3 germ lineages. Being able to differentiate into all cells of the body, they hold great potential for regenerative medicine and the treatment of degenerative disorders. Human ESCs (hESCS) are notoriously difficult to maintain *in vitro* as they have a propensity to differentiate, likely due to a suboptimal culture environment. Like the preimplantation embryo, there are

numerous reports of the beneficial effects of culturing hESCs under hypoxic conditions. These include a reduction in chromosomal abnormalities and enhanced clonal efficiency (Forsyth et al., 2006), protection against spontaneous differentiation (Ezashi et al., 2005, Prasad et al., 2009), increased proliferation (Ludwig et al., 2006, Forristal et al., 2010), enhanced expression of key transcription factors which regulate self-renewal, namely OCT4, SOX2 and NANOG (Ludwig et al., 2006, Forristal et al., 2010, Forristal et al., 2013, Petruzzelli et al., 2014), reduced oxygen consumption, greater consumption of glucose and production of lactate and thus an increased rate of flux through glycolysis (Forristal et al., 2013, Harvey et al., 2016), and a greater turnover of amino acids (Christensen et al., 2014) compared to cells maintained at atmospheric oxygen tensions. In addition, the derivation and culture of hESCs under physiological oxygen (5% oxygen) has been shown to allow the retention of two active X chromosomes, representing the ground state of pluripotency whereas exposure to 20% oxygen induced X chromosome inactivation (Lengner et al., 2010). The efficiency of reprogramming somatic cells to induced pluripotent stem cells is also improved under hypoxic conditions (Yoshida et al., 2009). Together, these data suggest that maintenance of the pluripotent state is improved under hypoxia.

The mechanisms which regulate the beneficial effects of maintaining hESCs under hypoxia are beginning to be unravelled and HIFs have a central role. HIF-1α was found to be responsible for regulating the initial hypoxic response. Upon exposure to hypoxia, HIF-1α was located in the nucleus of hESCs and remained for ~48 hours but was absent following long-term culture under hypoxic conditions. In contrast, hESCs express HIF-2α in the cytoplasm under atmospheric oxygen and for the first 48 hours of hypoxia. However, following three passages under hypoxia, HIF-2α protein had translocated to the nucleus to regulate the long-term hypoxic response (Forristal et al., 2010). Thus, although HIF-1α is important for regulating the initial hypoxic response in hESCs, HIF-2α drives the chronic response to hypoxia.

Under conditions of hypoxia, HIF-2α promotes the self-renewal of hESCs by binding directly to an HRE in the proximal promoters of OCT4, SOX2 and NANOG to enhance their expression (Covello et al., 2006, Petruzzelli et al., 2014). hESCs possess bivalent chromatin comprising both active and silencing histone modifications. This ensures that although developmental genes are silenced, they are poised for activation (Bernstein et al., 2006). The hypoxic culture of hESCs was found to be associated with an altered histone modification profile around the HRE site in the proximal promoters of OCT4, SOX2 and NANOG. hESCs cultured at atmospheric oxygen contained high levels of H3K9me3, a marker of gene repression and significantly reduced H3K4me3 and H3K36me3, markers of gene activation around the HRE sites of NANOG and SOX2 compared to hESCs maintained at 5% oxygen. In contrast, the proportion of H3K36me3 was dramatically increased at the HREs of OCT4, SOX2 and NANOG in hESCs cultured at 5% compared to 20% oxygen (Petruzzelli et al., 2014). These data suggest that hESCs maintained under hypoxic conditions are more euchromatic, allowing transcription factors and chromatin remodelling factors to bind whereas culture at atmospheric oxygen is associated with a more heterochromatic state.

hESCs have a metabolism based on glycolysis. Compared to atmospheric oxygen, under hypoxic conditions, hESCs consume more glucose and produce more lactate leading to an increased rate of flux through glycolysis. In contrast, oxygen consumption, the best global indication of the ability of a cell to produce ATP is decreased. These metabolic changes under hypoxia correlate with an increased expression of OCT4, SOX2 and NANOG and suggest that environmental oxygen regulates energy metabolism which is intrinsically linked to the self-renewal of hESCs (Forristal et al., 2013). This finding was subsequently supported by the observation that naïve hESCs which more closely represent cells of the ICM, exhibit an increased glycolytic flux compared to primed hESCs (more akin to epiblast stem cells) and that a decrease in glycolysis reduces the self-renewal of naïve hESCs (Gu et al., 2016).

Glucose is transported into hESCs through GLUT3 which is located in cell membranes, rather than via GLUT1, which displays a largely cytoplasmic localisation. GLUT3 expression was upregulated in hESCs under hypoxia and silencing GLUT3 decreased both glucose uptake and lactate production but interestingly, also reduced the expression of OCT4. Further analysis revealed a significant positive correlation between GLUT3 and OCT4 expression suggesting that hESC self-renewal is regulated by the rate of glucose uptake (Christensen et al., 2015). Together, these findings were intriguing, but the question remained, how does glucose metabolism regulate hESC self-renewal under hypoxia? This question was addressed with the use of glycolytic inhibitors; 2-deoxyglucose which competes with glucose for binding hexokinase, and 3-bromopyruvate which inhibits hexokinase through alkylation. When hESCs were cultured under hypoxic conditions in the presence of either of these inhibitors, as expected, the rate of lactate production was decreased, the mRNA expression of a panel of differentiation markers were increased and OCT4, SOX2 and NANOG protein expression decreased. However, surprisingly HIF-2α expression was also significantly decreased (Arthur et al., 2019). This suggests that under hypoxia, glycolysis promotes hESC self-renewal through HIF-2α. HIF-2α can then bind the proximal promoters of OCT4, SOX2 and NANOG to enhance expression and thus the self-renewal of hESCs (Arthur et al., 2019). How glycolysis regulates HIF-2α expression in hESCs remains to be determined but the mechanism is likely to be complex since HIF-2α itself is also known to promote glycolysis through the increased expression of GLUT transporters (Forristal et al., 2013).

Conclusions and future perspectives

There is now overwhelming data to support the culture of preimplantation embryos under a reduced oxygen tension (Table 1). In the human, it appears that a hypoxic environment is particularly important for the later stages of preimplantation development, from compaction through to blastocyst formation. Originally, culturing embryos under low oxygen tensions was unwieldy, requiring pre-mixed gas cylinders containing 5%O_2, 5% CO_2 and 90% N_2 and desiccators, or sealed chambers. With the development of incubators capable of regulating oxygen tensions, cost became a barrier to implementing the culture of embryos at reduced oxygen tensions. However, with the accessibility of commercially available incubators

that accurately regulate reduced oxygen tension, prices have become more affordable. Thus, it must be questioned why some IVF clinics continue to culture embryos under atmospheric oxygen; particularly those performing blastocyst transfers.

Data from hESCs may provide critical insight into why hypoxic conditions are beneficial for preimplantation embryos and particularly from compaction onwards in clinical IVF. Compaction is the stage where differential cell division occurs generating inner cells destined to become the ICM and outer cells which will give rise to the trophectoderm of the blastocyst. A mathematical model found that human morula cultured in static drops at 5% oxygen would be mildly hypoxic (Byatt-Smith et al., 1991). The authors recommended stirring to increase oxygen availability and hence development. On the contrary, mechanisms regulating the hypoxic response of hESCs would suggest that the centre of the morula being hypoxic may actually be an essential developmental phenomenon. It is proposed that hypoxic inner cells in the core of the morula will stabilise HIFs which translocate to the nucleus and increase the transcription of hypoxia regulated genes. This may provide a critical mechanism to drive the expression of OCT4, SOX2 and NANOG while simultaneously promoting proliferation and a glycolytic metabolism to ensure the blastocyst contains a wholly pluripotent ICM and hence optimal embryo development upon implantation (Figure 3). Of course, further research will be required to verify this hypothesis. However, the proposition does highlight the importance of understanding the physiological role that environmental oxygen has on the earliest stages of embryo development as well as its implications for later life. It is proposed that recapitulating the environment of the reproductive tract in terms of nutrient availability and oxygen tension will allow *in vitro* embryo development to more closely replicate that *in vivo,* leading to improved clinical outcomes.

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Figure legends

Figure 1 Schematic representation of the oxygen tension in the reproductive tract during preimplantation development.

Figure 2 Schematic representation of the beneficial effects of culturing preimplantation embryos to the blastocyst stage at 5% oxygen compared to atmospheric oxygen.

Figure 3 Schematic representation of the impact of environmental oxygen on hESCs and a proposed mechanism to explain the beneficial effect of culturing preimplantation embryos in hypoxic conditions. It is proposed that culturing embryos at 5% oxygen leads to increasingly hypoxic inner cells in the morula, which stabilise HIFs and increase the transcription of hypoxia regulated genes resulting in blastocysts with increased cell numbers, highly pluripotent ICMs and improved developmental competency compared to those cultured at 20% oxygen.

Table Legend

Table 1 Effects of culturing preimplantation embryos in either a hypoxic or atmospheric oxygen tension.