Effect of Hypoxia On Proliferation and Neural Commitment of Embryonic Stem Cells at Different Stages of Pluripotency

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***Abstract* —** We have studied the effect of low oxygen levels (2% O_2), or hypoxia, in the expansion and neural commitment of mouse embryonic stem (ES) cells.

When ES cells were maintained in culture with leukemia inhibitory factor (LIF), cell proliferation was reduced at low oxygen levels and a simultaneous reduction in cell viability was also observed. Morphological changes and different cell cycle patterns also occurred, suggesting some early differentiation under hypoxic conditions. However, when cells were maintained in a ground state of pluripotency, by inhibition of autocrine FGF4/ERK and GSK3 signaling, hypoxia did not affect cell proliferation, and did not induce early differentiation.

Nevertheless, during neural commitment, low oxygen tension exerted a positive effect on early differentiation of ES cells, resulting in a faster commitment towards neural progenitors. Overall our results demonstrate the need to specifically regulate the oxygen content, especially hypoxia, along with other culture conditions, when developing new strategies for ES cell expansion and/or controlled differentiation.

Index Terms — Embryonic stem cells; expansion; neural commitment; hypoxia.

I. CONTEXT

Stem cells possess the ability for unlimited or prolonged self-renewal and to differentiate into highly distinct cell lineages. In particular, pluripotent embryonic stem (ES) cells can give rise to cells derived from the three embryonic germ layers (ectoderm, endoderm, and mesoderm) [1], which makes them attractive for a wide range of clinical and pharmacological applications. However, a better understanding of the mechanisms that control the expansion and differentiation of ES cells is of great importance for the systematic production of cells for therapeutic applications.

Mouse ES cells, in particular, can be maintained in culture in an undifferentiated state in the presence of leukemia inhibitory factor (LIF) and serum, or under serum-free conditions with LIF and bone morphogenetic protein (BMP-4) [2]. However, recent evidence has highlighted the existence of metastable pluripotent states, demonstrating that ES cells can assume distinct states of pluripotency *in vitro* [3]. Therefore, two stages of pluripotency can be defined: a naïve or ground state, and a primed or committed state of pluripotency. Culture conditions for the maintenance of ground-state ES cells were developed and involve the utilization of small-molecule inhibitors of fibroblast growth factor (FGF)/extracellular signal-regulated kinases (ERK) and glycogen synthase kinase-3 (GSK3) signaling [4]. Auto-inductive FGF4/ERK signaling poises ES cells susceptible for lineage commitment, and cells enter into a primed pluripotent state. Thus, inhibition of ERK signaling, augmented by inhibition of GSK3 to restore cellular growth and viability, allows the propagation of naïve pluripotent ES cells.

The influence of oxygen levels on this dynamic regulatory mechanism has not yet been studied, and may potentially provide new insights into ES cell proliferation, metabolism, and phenotype retention. Interestingly, the oxygen tensions to which cells are exposed during development are substantially lower than atmospheric levels, making O_2 content a potentially important parameter when designing new strategies for stem cell expansion and/or controlled differentiation. Therefore, we have focused our efforts in studying the effects of low oxygen levels (2% O_2), or hypoxia, in the expansion and neural commitment of mouse embryonic stem (ES) cells [5].

II. GOALS

The specific aim of this work was the evaluation of the effect of one important microenvironmental factor, oxygen tension, in embryonic stem cell fate. ES cells can be expanded in cultured with different culture media supplemented with a variety of soluble molecules to promote their self-renewal and pluripotency maintenance. These soluble signals can modulate important signaling pathways and can thus modulate the choice between stem cell self-renewal and differentiation. In this work we focused on the role of three of these signaling pathways,

LIF/STAT3, FGF4/ERK and Wnt/ β -Catenin, under different oxygen tensions and we studied how this important culture parameter can interfere with signaling networks modulating ES cell behavior.

III. Team and Institutions

This work is within the frame of the scientific activities currently being developed at the Department of Bioengineering (DBENG, IST) and Institute for Biotechnology and Bioengineering (IBB). In particular, it is being conducted at the Stem Cell Bioengineering Laboratory, which has been focusing for several years in the development of highly controlled culture systems for the *ex-vivo* expansion of stem cells. This project is being coordinated by Prof. Joaquim Sampaio Cabral, the IBB director and current head of the DBENG at IST.

IV. Implementation

We have studied the effect of low oxygen levels (2% O_2), or hypoxia, in the expansion and neural commitment of mouse embryonic stem (ES) cells.

A hypoxic chamber was used to maintain low oxygen tensions throughout time in culture. Cell supernatants were collected to evaluate the levels of relevant metabolites (*e.g.* glucose, glutamine, lactate and ammonia) and calculate consumption/production rates and metabolic yields for 20% and 2% O_2 conditions. Furthermore, the levels of specific proteins, such as the pluripotency markers SSEA-1, Oct-4 and Nanog, or the neuroectoderm marker Sox1, were analyzed by flow cytometry for both conditions.

This strategy allowed us to evaluate the influence of different oxygen levels not only at a cellular level, but also at a molecular level. Quantitative real-time PCR, coupled with cell cycle analysis and proliferation assays (*e.g.* PKH analysis and clonal assays) were used to evaluate the commitment stage of mouse ES cells maintained at different oxygen tensions. This also allowed us to study the neural specification of these cells in monolayer cultures.

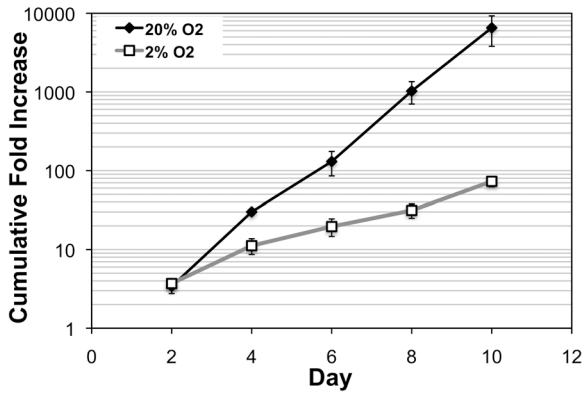
Overall, the implementation of this project was successful, and the strategy and procedures used were also very useful to reveal potential mechanisms of ES cell response to hypoxic conditions.

V. Results

Effect of Hypoxia on Mouse ES Cell Expansion and Metabolism in the Presence of LIF

In the presence of leukemia inhibitory factor (LIF), cell proliferation was reduced under hypoxia (Fig. 1a) and a simultaneous reduction in cell viability was also observed (77% vs. 91% for hypoxia and normoxia, respectively, after 10 days in culture). Specific growth rate was also reduced in hypoxia (0.43 ± 0.03 day^{-1}), as compared to 20% O_2 (0.84 ± 0.11 day^{-1}). Slight morphological changes were observed suggesting some early differentiation for hypoxic conditions (Fig. 1b), even though cells retained high levels of pluripotency marker expression. These results suggest that hypoxic conditions prompt mouse ES cells early commitment when LIF is used to maintain cell pluripotency. In fact, LIF/STAT3 signaling is suppressed by HIF-1α, which results in hypoxia-induced *in vitro* differentiation. In addition to affecting mouse ES cell proliferation and pluripotency, low oxygen levels also influenced cell metabolism. Overall, there was an increase in lactate specific production rate and a significant increase in the glucose consumption in hypoxic conditions.

A.



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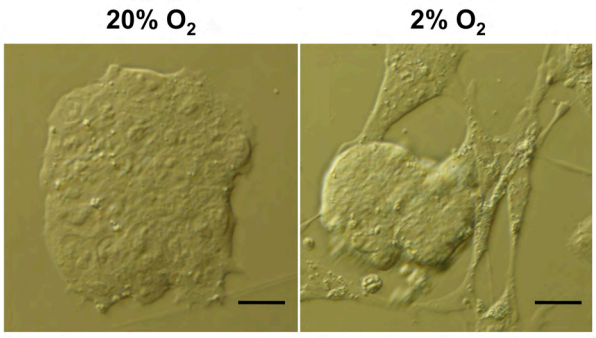

Figure 1. Hypoxia resulted in reduced mouse ES cell proliferation in the presence of LIF. (a) Cumulative fold increase in total cell number of five consecutive passages performed in triplicate. (b) Mouse ES cell morphology under 20 and 2% O_2 conditions. Scale bar: 50 μm.

Effect of Hypoxia on Expansion and Metabolism of Ground-State Pluripotent Mouse ES Cells

When cells were maintained in a ground-state of pluripotency [4], by inhibition of GSK3 and autocrine FGF4/ERK signaling, hypoxia did not affect cell proliferation, and did not induce early differentiation. The proliferation of mouse ES cells under these conditions was not affected and the overall cumulative fold increase in total cells was similar (FI= 3911 ± 1429 for 20% O_2 and FI= 2646 ± 488 for 2% O_2), independently of the oxygen level used. This resulted in identical growth rate values for both conditions tested (0.82 ± 0.04 day^{-1} for 20% O_2 and 0.79 ± 0.02 day^{-1} for 2% O_2). In addition, over 95% of cells were positive for Oct-4 and Nanog, either at low or at atmospheric oxygen tensions, as quantified by flow cytometry. In these conditions, the contribution of GSK3 inhibition appears to be of pivotal importance in the regulation of cell functions under hypoxia, possibly acting through activation of the canonical Wnt pathway. Under ground-state culture conditions, hypoxia seemed to increase to some extent glucose and lactate metabolism, similar to that obtained for mouse ES cell expansion in the presence of LIF.

Neural Commitment of Ground-State Mouse ES Cells under Hypoxic Conditions

During neural commitment, low oxygen tension exerted a positive effect on early differentiation of ground-state ES cells, resulting in a faster commitment towards neural progenitors. The use of a green fluorescent protein (GFP) knock-in reporter ES cell line (46C) allowed the examination of the process by which ES cells acquire neural identity since the open reading frame of the Sox1 gene was replaced with the coding sequence of GFP [4]. This revealed that the maximum expression of Sox1-GFP was reached faster at 2% O_2 than at 20% O_2, and by day 4 cells maintained under low oxygen levels were already over 80% Sox1-positive (83.8 ± 3.1%) whereas under 20% O_2 the percentage of Sox1-positive cells was only 65.6 ± 7.3% (Fig. 2). In addition, by day 8, flow cytometry quantification of neuronal class III β-Tubulin (Tuj1) also revealed a slightly higher percentage of neuronal cells at low oxygen tension. Cell metabolism during neural commitment was also influenced by hypoxic conditions. High levels of glucose consumption and lactate production were seen for 2 and 20% O_2 during the first days of commitment. Nevertheless, later in culture, hypoxia led to substantially higher glucose and lactate consumption /production rates due to an increase in anaerobic metabolism at low oxygen tensions.

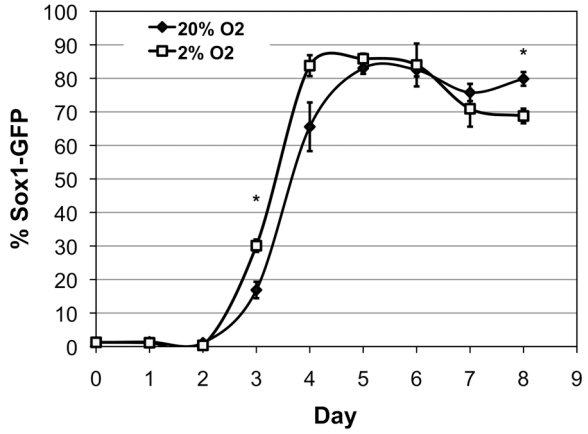

Figure 2. Percentage of Sox1-GFP-positive cells during neural commitment at different O_2 levels. Error bars represent the standard error of the mean (SEM) of three independent experiments. Asterisks denote statistical significance ($P<0.05$).

VI. Conclusions

The influence of low oxygen levels (2%) on the proliferation and neural differentiation of ground state embryonic stem cells was therefore evaluated in this work. A comprehensive analysis of ground state ES cell proliferation kinetics, cell viability, expression of pluripotency markers, cell cycle, and metabolic profile was performed for low oxygen tensions and compared with the same results obtained for atmospheric oxygen levels. In addition, the conversion of ground state ES cells into neural precursors under low oxygen tension was also studied.

Overall our results demonstrate the need to specifically regulate the oxygen content, along with other culture conditions, when developing new strategies for ES cell expansion and/or controlled differentiation. Consequently, this work is expected to contribute with valuable information for the improvement of current strategies for the expansion and neural differentiation of pluripotent stem cells.

VII. Planned Developments

Future developments will involve the use of rational and systematic approaches to study the influence of oxygen in stem cell fate. Work will be dedicated and more focused on the molecular mechanisms behind the pathways already mentioned. The main goal will be explaining some of the experimental evidences that were observed in the experiments shown here and try to investigate some of the mechanistic hypothesis that were raised during this project. On the other hand, future work will also be dedicated to a more detailed characterization of ES cell populations obtained under different culture conditions. For those purposes, several techniques like quantitative real time PCR, flow cytometry, western blotting and TUNEL assays will be used. The supplementation of culture medium with several signaling molecules will also be performed since this would help to reinforce some of the points discussed here and potentially find new avenues of research.

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