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Hypoxia Enhances Proliferation of Mouse Embryonic Stem Cell-Derived Neural Stem Cells

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ABSTRACT: Neural stem (NS) cells can provide a source of material with potential applications for neural drug testing, developmental studies, or novel treatments for neurodegenerative diseases. Herein, the ex vivo expansion of a model system of mouse embryonic stem (mES) cell-derived NS cells was characterized and optimized, cells being cultivated under adherent conditions. Culture was first optimized in terms of initial cell plating density and oxygen concentration, known to strongly influence brain-derived NS cells. To this end, the growth of cells cultured under hypoxic (2%, 5%, and 10% O_2) and normoxic (20% O_2) conditions was compared. The results showed that 2–5% oxygen, without affecting multipotency, led to fold increase values in total cell number about twice higher than observed under 20% oxygen (20-fold vs. 10-fold, respectively) this effect being more pronounced when cells were plated at low density. With an optimal cell density of 10^4 cells/cm^2, the maximum growth rates were 1.9 day^{-1} under hypoxia versus 1.7 day^{-1} under normoxia. Cell division kinetics analysis by flow cytometry based on PKH67 tracking showed that when cultured in hypoxia, cells are at least one divisional generation ahead compared to normoxia. In terms of cell cycle, a larger population in a quiescent G_0 phase was observed in normoxic conditions. The optimization of NS cell culture performed here represents an important step toward the generation of a large number of neural cells from a reduced initial population, envisaging the potential application of these cells in multiple settings.

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KEYWORDS: neural stem cells; hypoxia; expansion; monolayer

Introduction

Neural Stem Cells

The development of the central nervous system (CNS) supposes the existence of a population of cells capable of forming both neurons and glia, according to differentiation cues sent by their niche. These cells, named herein neural stem (NS) cells, can be expanded in vitro in the presence of fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF), via propagation of floating cell clusters called "neurospheres" (Reynolds and Weiss, 1992). Neurospheres contain NS cells and progenitors mixed with differentiated cells embedded in a complex extracellular matrix. Although this method has been widely used, it shows limitations for cell culture (Conti et al., 2005; Lederer and Santama, 2008). For instance, heterogeneity occurs within and between neurospheres, stem cells maintained within neurospheres have an uncertain relationship to CNS precursors in vivo (Suslov et al., 2002), there are oxygen and nutrient diffusion limitations and waste accumulation in the cluster center, and neurospheres differentiate much more readily into astrocytes than neurons in vivo (Mukhida et al., 2008; Winkler et al., 1998). Nevertheless, diffusion limitations, in neurosphere culture, can be prevented by using stirred suspension systems (Kallos et al., 1999).

The expansion of NS cells under adherent conditions would circumvent most of these limitations. In some reports, though, neural progenitor cells propagated in adherent cultures, without genetic transformation, lose neural differentiation potential after long-term passaging (Qian et al., 2000; Temple, 1989). However, the derivation and expansion in vitro of cultures of NS cells under adherent conditions, capable of generating high numbers of neuronal cells, with no significant decline in potential over time, was recently demonstrated (Conti et al., 2005; Pollard et al., 2006). The derivation of these lines was performed from several sources, such as mouse embryonic stem (mES) cells. The procedure consists firstly in a differentiation protocol of ES cells into neural progenitors (Diogo et al., 2008; Ying et al., 2003), followed by the expansion of a subset of progenitors in medium with FGF-2 and EGF. These cells show long-term self-renewal capacity and maintain the capacity to generate neurons, astrocytes, and oligodendrocytes (Glaser et al., 2007). In the present work, the cell line used

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was derived from CGR8 ES cells, being thus called CGR8-NS cells. Due to the inherent limited availability of brain-derived NS cells, the generation of populations of NS cells from ES cells and their further differentiation allows the potential use of these cells for the cure of neurodegenerative diseases and for neural drug testing.

Hypoxia

Oxygen is an important energy source for cell metabolism and its concentration is tightly regulated in the CNS, where the pO_2 values are similar among different mammalian species and range from 2.53% to 5.33% (19–40 mmHg) in the cortex (Chen et al., 2007; Erecinska and Silver, 2001). Cellular responses to changes in oxygen availability are well studied in different cell types (Hermitte et al., 2006; Jeong et al., 2007; Ma et al., 2009; Mondragon-Teran et al., 2009; Powers et al., 2008; Schipani et al., 2001) but most in vitro studies of NS cells are performed under atmospheric oxygen tension, or normoxia (95% air/5% CO_2, O_2 tension around 20%), which is not the CNS physiological range. There have been some studies investigating the effect of lower concentrations of oxygen on the proliferation and differentiation of NS cells from different origins, both in terms of species or tissue. Studies have demonstrated a positive effect of low oxygen tensions on the proliferation of these cells and influenced their fate, when induced to differentiate (Chen et al., 2007; Horie et al., 2008; Milosevic et al., 2005; Morrison et al., 2000; Storch et al., 2001; Studer et al., 2000).

ES cell-derived NS cell lines were only relatively recently derived (Conti et al., 2005) and thus little is known about the effect of oxygen on the proliferation of these cells. In fact, even under normoxia, the culture of these cells has not been fully characterized, for instance in terms of growth rate or optimal plating density. In this work, together with these aspects, the effect of hypoxia on the expansion of the ES cell-derived NS cell line CGR8-NS was evaluated. Our results clearly demonstrate that low oxygen environment enhances the proliferation of CGR8-NS, while fully retaining their multipotency.

Materials and Methods

Cell Line

The NS cell line CGR8-NS, derived from the mouse ES cell line CGR8 and provided by the laboratory of Professor Austin Smith, Wellcome Trust Centre for Stem Cell Research, Cambridge, United Kingdom, was used as the cell model.

CGR8-NS cells used in this work were always at passages P20–P25. In all the experiments, cells were thawed from the frozen stock and passaged once under 20% O_2 before the procedures described.

Expansion of NS Cells

Except for the initial cell density (ICD) studies, CGR8-NS cells were plated at an ICD of 10^4 cells/cm^2. Cells were expanded under static conditions in uncoated tissue culture (TC) plates or T-flasks (Falcon, BD Biosciences, San Jose, CA) using RHB-A medium (Stem Cell Sciences, Cambridge, UK) supplemented with 15 ng/mL FGF-2 and EGF (Peprotech, London, UK), 1% penicillin, and 1% streptomycin (Gibco, Invitrogen Corporation, Carlsbad, CA). Cells were passaged, after 3 days of culture, using Accutase (Sigma, St. Louis, MO). In each passage, viable and dead cells were determined by counting in a hemocytometer under an optical microscope (Olympus, Hamburg, Germany) using the trypan blue dye exclusion test (GibcoBRL). Viabilities were always above 90%. Fold increase (FI) in cell number was calculated as the ratio between the cell number determined at the end of the culture and the number of cells initially plated.

Gas Phase pO_2 Control

Cells were cultured at 37°C in a 5% CO_2 humidified atmosphere. For culture under hypoxic conditions (10%, 5%, and 2% O_2), cells were incubated inside a chamber with a customized computer-controlled system (Biospherix Ltd., Lacona, NY). The oxygen level inside the incubator (as well as CO_2) was controlled automatically by the injection of nitrogen (and CO_2). Cells were cultured in 12-well TC plates (Falcon), which have a surface area of 3.6 cm^2 and a working volume of 1 mL, or T25 plates (Falcon), with a working volume of 5 mL. These volumes correspond to a depth of medium of 2.7 and 2.0 mm, respectively. According to the studies by Powers et al. (2008), under similar conditions, the magnitude of the pO_2 drop across the culture medium is expected to be negligible.

Neuronal and Astrocyte Differentiation of NS Cells Under Normoxic Conditions

For astrocyte differentiation, after expansion for 3 days, CGR8-NS cells were plated (10^4 cells/cm^2) and exposed to RHB-A medium supplemented with 1% fetal bovine serum (FBS, GibcoBRL) and 1% penicillin and 1% streptomycin. Half of the volume of medium was exchanged by fresh medium at day 2. At day 3, cells were harvested with Accutase and astrocyte differentiation efficiency was quantified by flow cytometry with the antibody against glial fibrillary acidic protein (GFAP), PE-conjugated (dilution 1:20, Santa Cruz Biotechnology, Santa Cruz, CA). The negative control was mouse IgG_1-PE (dilution 1:20, Santa Cruz Biotechnology).

For neuronal differentiation, cells were plated (1.0×10^5–1.5×10^5 cells/cm^2) on uncoated T25 flasks using RHB-A medium supplemented with 1% B27 (Invitrogen, Carlsbad, CA), 0.5% NDiff Neuro-2 Supplement (Milipore, Billerica,

MA), and 10 ng/mL FGF-2. After 3 days at 37°C, cells were gently dissociated with Accutase. Cells were then plated at a density of 5×10^4–7.5×10^4 cells/cm^2 on laminin (Sigma)-coated (3 μg/mL for 3 h at 37°C) plates in medium composed of a 1:1 mixture of RHB-A and neurobasal media (Invitrogen) containing 0.5% NDiff Neuro-2, 1% B27, and FGF-2 (10 ng/mL). All media for neuronal differentiation were supplemented with 1% penicillin and 1% streptomycin. After 4 days under these conditions, cells were harvested using Accutase and neuronal differentiation efficiency was also assessed by flow cytometry with Alexa Fluor® 488 labeled Neuronal Class III β-Tubulin (TUJ1) (Covance, Princeton, NJ, dilution 1:200). As negative control, Alexa Fluor® 488 labeled Rat IgG_{2b} (Biolegend, San Diego, CA) was used (dilution 1:150).

Intracellular Staining for Flow Cytometry

Cells were analyzed for the expression of intracellular proteins by flow cytometry after intracellular staining. Briefly, 0.5–1×10^6 cells were washed with phosphate-buffered saline (PBS, GibcoBRL) and fixed in 2% paraformaldehyde (PFA, Sigma) for 20 min. Cells were then washed with 1% normal goat serum (NGS, Sigma), resuspended in 3% NGS and permeabilized with a 0.5% saponin solution (Sigma). Cells were incubated with blocking solution (3% NGS) for 15 min and then with the antibody for 2 h at room temperature. Finally, cells were washed with 1% NGS and resuspended in PBS, prior to analysis. All flow cytometry analyses in these studies were performed in a FACScalibur cytometer (Becton Dickinson, San Jose, CA) and a minimum of 10,000 events was collected for each sample. The CellQuest software (Becton Dickinson) was used for all acquisition/analysis unless other is specified.

Clonal Growth Studies

Clonal growth studies were performed by plating 50 cells into each well of 4-well culture plates (Nunc, Roskilde, Denmark) and scoring colonies after 6 days. Medium was exchanged 3 days after plating.

Lactate Production Determination

Lactate concentrations were determined in the supernatant samples collected throughout the experiments by using an automatic analyzer (YSI 7100MBS, Yellow Spring Instruments, Yellow Springs, OH). The specific lactate production rates (LPR) were calculated for every time interval using Equation (1):

$$\mathrm{LPR} = \frac{\Delta L}{\overline{X_v}\Delta t} \tag{1}$$

where ΔL is the variation in lactate concentration during the time period Δt and $\overline{X_v}$ the average viable cell number during the same time period.

Proliferation, Cell Cycle, and Apoptosis Studies

Cell proliferation was studied using the PKH67 Fluorescent Cell Linker Kit (Sigma). Since the PKH67 dye is stably incorporated in the cell membrane, the molecules are equally distributed between daughter cells during cell division. Each generation of cells is therefore half as fluorescent as the previous one, allowing the precise quantification of the number of divisions the cells have undergone (da Silva et al., 2009; Wallace and Muirhead, 2007; Wallace et al., 2008). The kit was used according to manufacturer's standard instructions. After staining, a sample from the cell suspension (5×10^5 cells) was analyzed by flow cytometry and the remaining cells were plated. After 3 days in culture under normoxia or hypoxia, proliferation was analyzed by flow cytometry using the software MODFIT-LT (Verify Software House, Topsham, ME) to determine the proportion of proliferating cells at each generation and to calculate the proliferation index (PI) (Wallace and Muirhead, 2007). PI is the ratio between the number of cells in all generations at the time of harvest and the back calculated number of parent cells theoretically present at the start of the experiment. Mathematic details on the calculation of PI are available elsewhere (Wallace and Muirhead, 2007).

To quantify and distinguish apoptotic and necrotic cells, the Annexin V:FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA) was used. The kit was used according to manufacturer's standard instructions.

Cell-cycle analysis was performed by culturing CGR8-NS cells for 3 days and performing a procedure similar to the one described for intracellular staining. Cells were incubated with a staining solution consisting of 5 μg/mL propidium iodide (BD Biosciences), 25 μg/mL RNAse A (Promega Corporation, Madison, WI), and 3% NGS (Sigma) in PBS (GibcoBRL) for 30 min at 37°C. To distinguish cells in cell cycle from G_0 cells, the anti-Ki-67-FITC antibody (dilution 1:38, Invitrogen) was used. Mouse IgG_1 antibody, FITC-conjugated (dilution 1:30, BD Biosciences), was used as the negative control.

Statistical Analysis of Data

Results are expressed as mean ± standard error of the mean (SEM, SEM = standard deviation/$\sqrt{n}$, where n is the number of experiments performed.). The two-sided Wilcoxon rank sum test was used to perform statistical analyses for hypoxia and normoxia comparison, considering a P-value < 0.05 to be significant.

Cell Growth Kinetics Modeling

The Weibull model (Tabatabai et al., 2005) was used to describe the growth of CGR8-NS cells. This model is described by Equation (2)

$$P(t) = M - (M - P_0)\,\mathrm{Exp}(-\beta t^{\gamma}) \tag{2}$$

where $P(t)$ is the number of viable cells after "t" days, M is the maximum number of cells, which is achieved in the stationary phase, P_0 is the initial number of cells, which corresponds to the population in the lag phase plateau, β is a scale factor, and γ is a shape factor. The fitting was performed by minimizing the squared errors using the Solver function in a Microsoft Excel spreadsheet (Microsoft Office 2007). The equations obtained with the model allow the calculation of instantaneous specific growth rates throughout all growth phases without numerical differentiation, which may introduce errors. Specific growth rates were calculated by Equation (3)

$$\mu(t) = \frac{1}{p}\frac{dp}{dt} \tag{3}$$

where P is the cell number, given by the model and dp/dt is the instantaneous derivative of the growth curve, given by Equation (4)

$$\frac{dp(t)}{dt} = (M - P(t))(\beta\gamma t^{\gamma-1}) \tag{4}$$

Doubling time was calculated according to Equation (5)

$$\text{doubling time} = \frac{\text{Ln}\,2}{\text{average } \mu \text{ (exponential phase)}} \tag{5}$$

The time required to achieve half the maximum population of cells, $t_{1/2}$, for both conditions was calculated from the Weibull model.

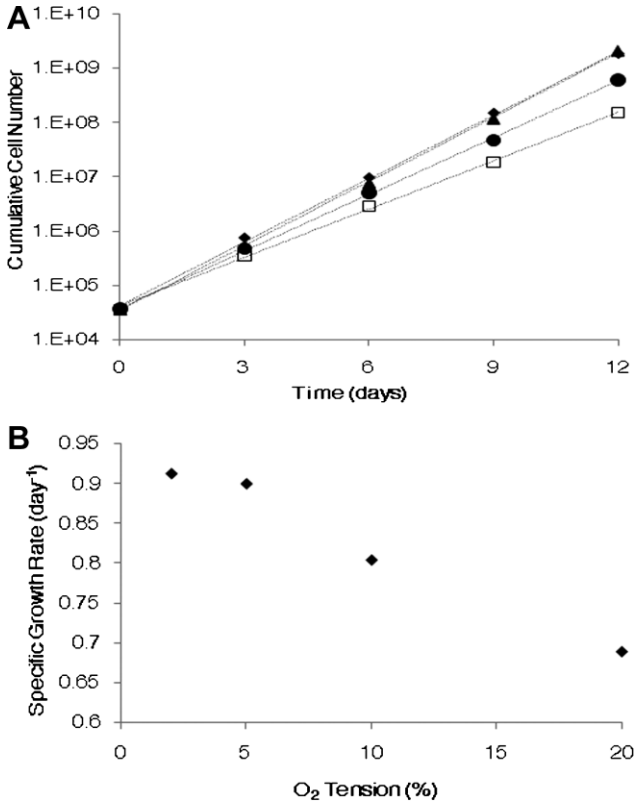

Figure 1. Effect of O_2 on CGR8-NS cell expansion. **A**: Cumulative number of cells during successive passaging under 20% (□), 10% (●), 5% (◆), and 2% (▲) O_2. Cells were passaged every 3 days during a maximum of 12 days. **B**: Specific growth rate determined from the slopes of the cumulative cell number curves plotted in (A).

Results

mES Cell-Derived NS Cell Growth Under Different Oxygen Tensions

The influence of oxygen on the optimization of mouse ES cell-derived NS cell culture was evaluated by testing different O_2 tensions: 20%, 10%, 5%, and 2%. Cells were plated with an ICD of 10^4 cells/cm^2 and passaged successively every 3 days, during a maximum of 12 days under an atmosphere with the different O_2 concentrations mentioned above (Fig. 1). After 3 days, at each cell passage, cells were found to be in late exponential phase of growth. It is clear from Figure 1A that, when compared to the standard 20% atmospheric O_2 tension, low oxygen tension strongly enhances the expansion of CGR8-NS cells. This positive effect was observed after the first passage and was consistently maintained throughout the following passages. These differences in cell proliferation thus led to different growth rates as determined from the slope of the cumulative cell number curves (Fig. 1B). Overall, after four passages (12 days), we observed a cumulative FI in cell number of $\approx 6 \times 10^4$ in hypoxia versus $\approx 5 \times 10^3$ under normoxia. These results indicate that to obtain the best performance in terms of cell proliferation, CGR8-NS cells must be cultured under an optimum O_2 tension range between 2% and 5%, approximately the physiological concentration of oxygen in the brain. Further studies were then performed under 2% O_2.

NS Cells Maintain Nestin Expression and Multipotency When Expanded Under Hypoxic and Normoxic Conditions

Before selecting 2% as the optimum oxygen tension value for the expansion of CGR8-NS cells, it was essential to verify if the expression of Nestin, a typical marker of NS/progenitor cells was impaired by this condition. Indeed, 95% of CGR8-NS cells (Conti et al., 2005) are expected to express this marker. The percentage of Nestin-positive cells was quantified by flow cytometry throughout six successive passages (18 days) and was found to be comparable under both 2% and 20% O_2 and always above 95% (result not shown). Thus, even after a relatively long period of time under hypoxia, most cells maintained the expression of this typical marker. The differentiative potential of NS cells

expanded for 3 days under 2% O_2 was also studied in order to assess the multipotency of these cells. In the case of astrocyte differentiation, after 3 days, the typical ''star-like'' cell morphology was observed and cells were harvested and analyzed by flow cytometry for GFAP expression. The results obtained show that under 2% and 20% O_2, almost 90% of the cells express GFAP (Table I), demonstrating that glial cell differentiation potential was not affected upon cell expansion under hypoxia. In terms of neuronal differentiation, cells were harvested after 6 days, when expression of βIII-tubulin is expected (Spiliotopoulos et al., 2009), and analyzed by flow cytometry. The results indicate an average expression of βIII-tubulin of 42% and 41% for cells previously expanded under hypoxia and normoxia, respectively (Table I), which suggests similar efficiency to undergo neuronal differentiation for the cells previously expanded under both conditions.

Influence of ICD on CGR8-NS Cell Growth Under Hypoxic or Normoxic Conditions

The influence of ICD on expansion of CGR8-NS cells under 2% and 20% O_2 was also evaluated. Cells were plated at different ICDs under both O_2 tensions and, after 3 days, the number of cells was determined and compared (Fig. 2A), as well as the respective FI in total cell number (Fig. 2B). According to Figure 2B, under normoxia, the FI values obtained were similar and slightly above 10-fold for 5–20×10^3 cells/cm^2 ICD values. For both O_2 conditions, an ICD of 10^3 cells/cm^2 led to a lower FI. When comparing hypoxia and normoxia, at the same ICD, the results show an average FI at least ≈1.5-fold higher under hypoxia versus normoxia (Fig. 2C), for all ICD, except for 2×10^4 cells/cm^2. In this case, in both conditions cells were too close to 100% confluency and therefore the FI values obtained under both conditions were not significantly different (Fig. 2B). Indeed, by the analysis of Figure 2A, we can conclude that a cell density of 2.6×10^5 cells/cm^2 is probably close to the maximum value that can be achieved with these cells.

It was also observed that, for low densities (10^3 cells/cm^2), the FI values are lower. This is probably due to the lack of autocrine signaling factors (e.g., mitogens) beyond a certain threshold concentration. To better evaluate the effect of low ICD, we performed expansion of CGR8-NS cells at clonal density (Fig. 2D–F). Under 2% O_2, not only a higher percentage of cells were able to adhere to the plate after seeding and generate colonies, but the formation of larger colonies was also observed. Indeed, colonies with more than 100 cells were hardly present in normoxic culture, while under hypoxia these were often present. This result may explain why when low ICDs were used, like 10^3 cells/cm^2, the difference between normoxic and hypoxic conditions is more evident (Fig. 2B and C).

Table I. Neuronal and astroglial differentiation of CGR8-NS cells after expansion under 2% and 20% O_2.

	Differentiated cells (%)	
	2%	20%
Neurons (TUJ1)	42 ± 19	41 ± 7
Astrocytes (GFAP)	83 ± 5	85 ± 10

Data presented are means ± standard error of the mean for two independent experiments.

Growth Kinetics of CGR8-NS Cells

To better characterize culture under normoxic and hypoxic conditions, the growth kinetics of the cells was evaluated during the usual time between passages (≈3 days). As observed in Figure 3A, expanding CGR8-NS cells under 2% O_2 produced two distinct effects: a shorter lag phase and a higher value for the maximum number of cells attained. Under normoxia, a longer lag phase was observed, leading to a higher $t_{1/2}$ (Table II). Furthermore, in terms of specific growth rate (Fig. 3B and Table II), the higher maximum specific growth rate was obtained for cells cultured under 2% O_2. Additionally, the maximum value is obtained 12 h earlier in time under hypoxia than under normoxia.

Importantly, in all experiments performed after 3 days of expansion described below, the differences in terms of FI in cell number between 2% and 20% O_2 were similar to those presented in Figure 3A.

Lactate Production Reflects Differences in Cell Number But Not in Oxygen Tension

Lactate production by CGR8-NS cells was determined after 3 days in culture, for the different ICD studied before (Fig. 4A). In terms of lactate accumulation, the maximum final concentrations reached both under hypoxia and normoxia were 2.3 ± 0.6 and 1.1 ± 0.2 mM, respectively. It was observed that higher ICDs led to higher final concentrations of lactate and, for each density, culture in hypoxia led to higher lactate production. This is a direct consequence of the existence of a higher number of cells; indeed, calculating the specific LPR (Fig. 4B), no significant differences were observed between the values for the three higher ICD either under hypoxia or normoxia. The results concerning LPR during one passage time frame (3 days, Fig. 4C and D) confirm these observations. In fact, in Figure 4C, lactate concentration increases throughout time, following cell growth, but in terms of specific production (Fig. 4D) there were no significant differences. In the case of the lowest ICD tested (10^3 cells/cm^2), probably the number of cells determined was too small, and consequently the final lactate concentration, being thus more affected by experimental errors, leading to higher LPR values. Finally, LPR was compared among cells cultured under 2%, 10%, and 20% O_2, without significant differences being observed (not shown).

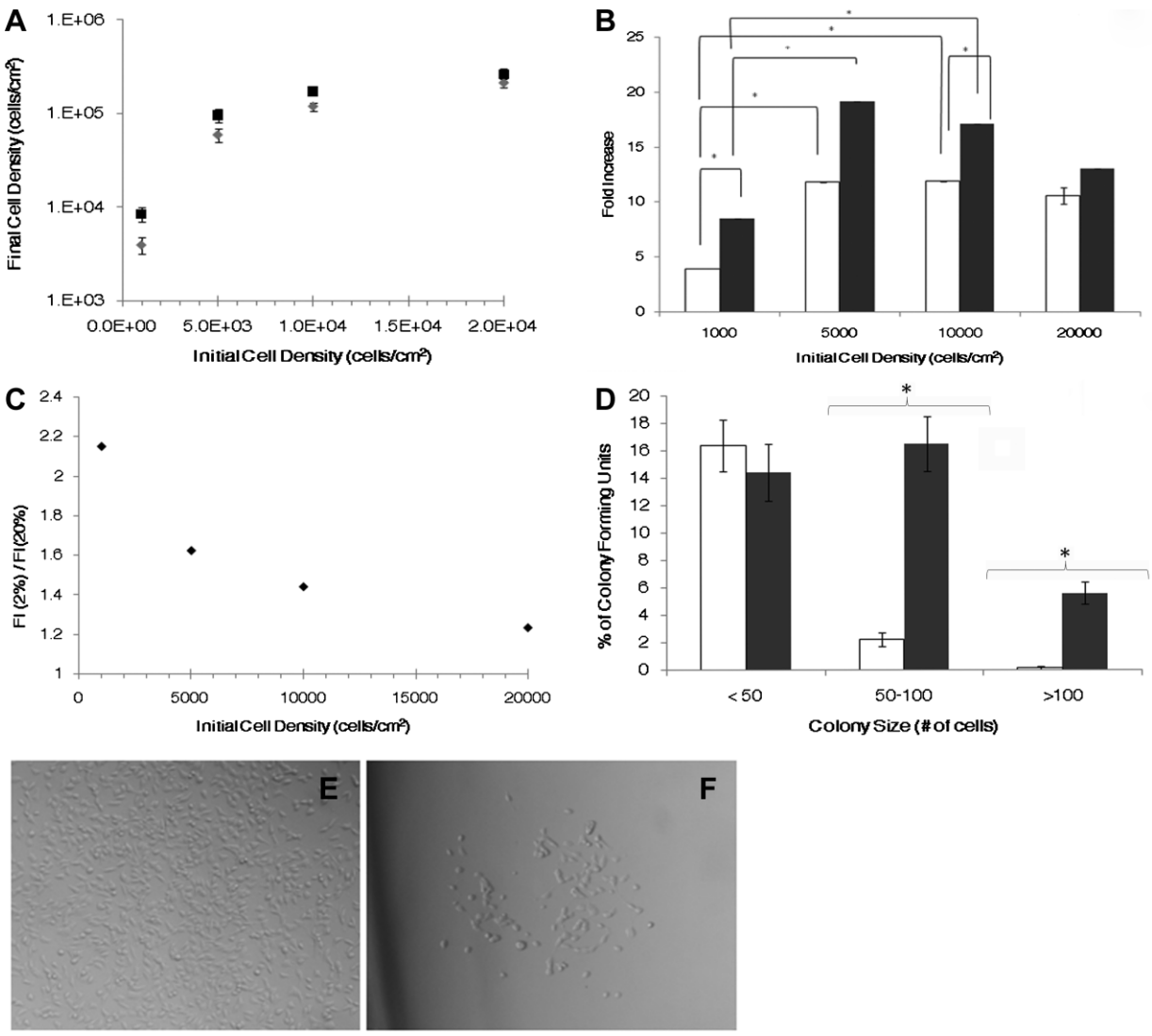

Figure 2. Influence of ICD on CGR8-NS cell expansion under 2% and 20% O_2 tension. **A**: Viable cell densities obtained 3 days after plating CGR8-NS cells at different ICD in 12-well culture plates, under hypoxic (■) and normoxic (◆) conditions, and respective fold increase values (**B**; white bars: normoxia, black bars: hypoxia). **C**: Fold increase ratio (hypoxia/normoxia) for the different ICD. Data presented are means ± standard error of the mean for two determinations from at least four independent experiments. **D**: Percentage of colony forming units and number of cells per colony, under hypoxic (black bars) or normoxic (white bars) conditions. **E**: Typical aspect of colonies obtained under hypoxic and normoxic (**F**) conditions 6 days after plating. Data presented are means ± standard error of the mean for four determinations from four independent experiments. $^*P < 0.05$.

Divisional Generation and Cell-Cycle Phase Distribution Versus Apoptosis/Necrosis After Expansion Under Hypoxic and Normoxic Conditions

The positive effect of low oxygen in the expansion of CGR8-NS cells may be the result of: (i) enhanced proliferation of the cells under 2% O_2; (ii) lower levels of cell death/apoptosis under low oxygen; or (iii) a combined effect of both factors. To test these hypotheses, cell divisional history, cell cycle, and apoptosis/necrosis studies were performed for both culture conditions.

Cell proliferation was studied by staining the cells with the PKH67 membrane dye, by quantifying the number of cell divisions in both conditions (Fig. 5A). We observed that, after 3 days, cells cultured under hypoxia were mostly in generations 4 and 5 (34 ± 3% and 38 ± 4%, respectively), while under normoxia, cells were mostly in generations 3 and 4 (28 ± 2% and 45 ± 4%, respectively). Furthermore, under hypoxia, a significantly higher number of cells were in generation 6 and a significantly lower number belonged to generation 3 ($P < 0.05$). Also, PI is higher (9.5 ± 0.7 vs. 6.5 ± 0.5) when cells are cultured under 2% O_2. The divisional history study suggests that cells cultured under hypoxia are at least one divisional generation ahead compared to normoxia. This is in agreement with the results obtained from the kinetic studies. Indeed, if we assume that the doubling times determined for hypoxia and normoxia cultures (11.1 and 13.9 h, respectively) are average

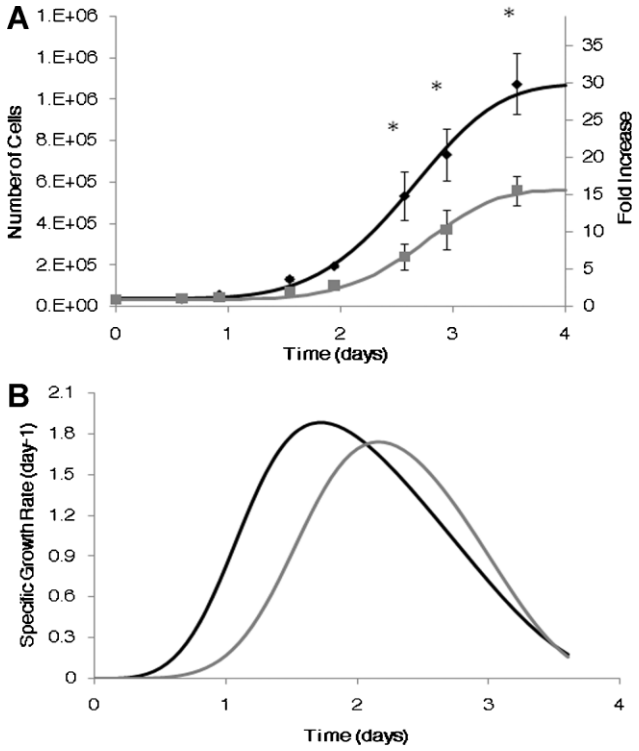

Figure 3. Growth kinetics of CGR8-NS cell expansion. **A**: Number of viable cells experimentally determined under hypoxic (◆) and normoxic (■) conditions and determined from the Weibull model for hypoxia (black line) and normoxia (gray line). **B**: Specific growth rate values (day^{-1}) during expansion of CGR8-NS cells under hypoxic (black line) and normoxic conditions (gray line) as calculated from Equation (3). Data presented are means ± standard error of the mean for two determinations from four independent experiments. $^{*}P < 0.05$.

values, with some cells dividing faster than others, and if we consider the culture period of 3 days (72 h), the conclusions are in agreement with PKH67 tracking results: after 3 days, up to six cell divisions are possible under hypoxia while under normoxic conditions only five divisions are likely to occur. Thus, cells cultured under 2% O_2 can divide at least one more time than cells cultured under 20%. The faster proliferation under hypoxia is also confirmed by the higher PI, a measure of the increase in cell number over the course of the experiment.

To better understand and characterize the proliferative behavior of cells under hypoxia, the cell-cycle phase distribution was determined. Thus, after 3 days of culture under 2% or 20% O_2, the DNA content was analyzed by flow cytometry (Fig. 5B). The results show that, without significant differences between hypoxia and normoxia, roughly around 50% of the cells were in G_0/G_1 phases, 40% were in S phase and 10% in G_2/M. Based on previous studies on the effect of hypoxia on G_0/cell-cycle transitions in stem cells (Hermitte et al., 2006), further analysis using the nuclear antigen Ki-67 was performed to distinguish the cells on G_0 phase from the other cell-cycle phases. Indeed, Ki-67 (Scholzen and Gerdes, 2000) is present during all active phases of the cell cycle, but not in G_0. Figure 5C shows the percentage of CGR8-NS cells in G_0 or cycling, after 3 days of culture, under 2% or 20% O_2, with a higher percentage of quiescent cells being observed under 20% O_2. Together with the proliferation study, this observation allows to conclude that not only cells underwent more cellular divisions under hypoxia, but also there was a higher number of cells able to exit G_0, being actively dividing. This result may explain the higher FI values obtained when cells are cultured under these conditions.

Besides proliferation, the effect of hypoxia on cell death/apoptosis was also studied. Thus, cells expanded for 3 days under 2% or 20% O_2 were stained with Annexin V and propidium iodide (Fig. 5D) with no significant differences being observed in terms of apoptotic/necrotic cells. Consequently, atmospheric oxygen tension does not induce CGR8-NS cell death, for instance by oxidative stress mediated by reactive oxygen species, as previously reported (Milosevic et al., 2005). In this case, exposure of CGR8-NS cells to 20% O_2 (vs. 2% O_2) seems to result not in a higher cell death, but either in a decrease of the cell fraction able to enter cell cycle, as shown in Figure 5C, or in longer cycling times.

Discussion

Low Oxygen Exerts Different Effects in Different NS Cell Populations

The effect of hypoxia on cell expansion appears to vary depending on different NS cell populations, not only in terms of species but also concerning the region from where cells were isolated. For E12 rat mesencephalic precursor cells it was reported (Studer et al., 2000) that hypoxic culture increases the proliferation rate, as determined by BrdU incorporation and lowers the levels of apoptosis, determined by the TUNEL assay. For E13.5 mouse fetal cortical precursors (Chen et al., 2007), cultured under 2–5% O_2, no differences in relation to 20% O_2 were found in

Table II. Maximal specific growth rates, time to achieve the maximum specific growth rate, doubling time, and time to achieve half the maximum population of cells for hypoxic and normoxic expansion cultures of CGR8-NS cells.

	Maximum specific growth rate (day^{-1})	Time to achieve the maximum specific growth rate (h)	Average specific growth rate during exponential phase (day^{-1})	Doubling time (h)	$t_{1/2}$ (h)
2%	1.9 ± 0.1	41 ± 0.8	1.5 ± 0.09	11.1 ± 0.9	61.9 ± 1.0
20%	1.7 ± 0.1	53 ± 3.2	1.2 ± 0.02	13.9 ± 0.3	64.1 ± 1.1

Data presented are means ± standard error of the mean for four independent experiments.

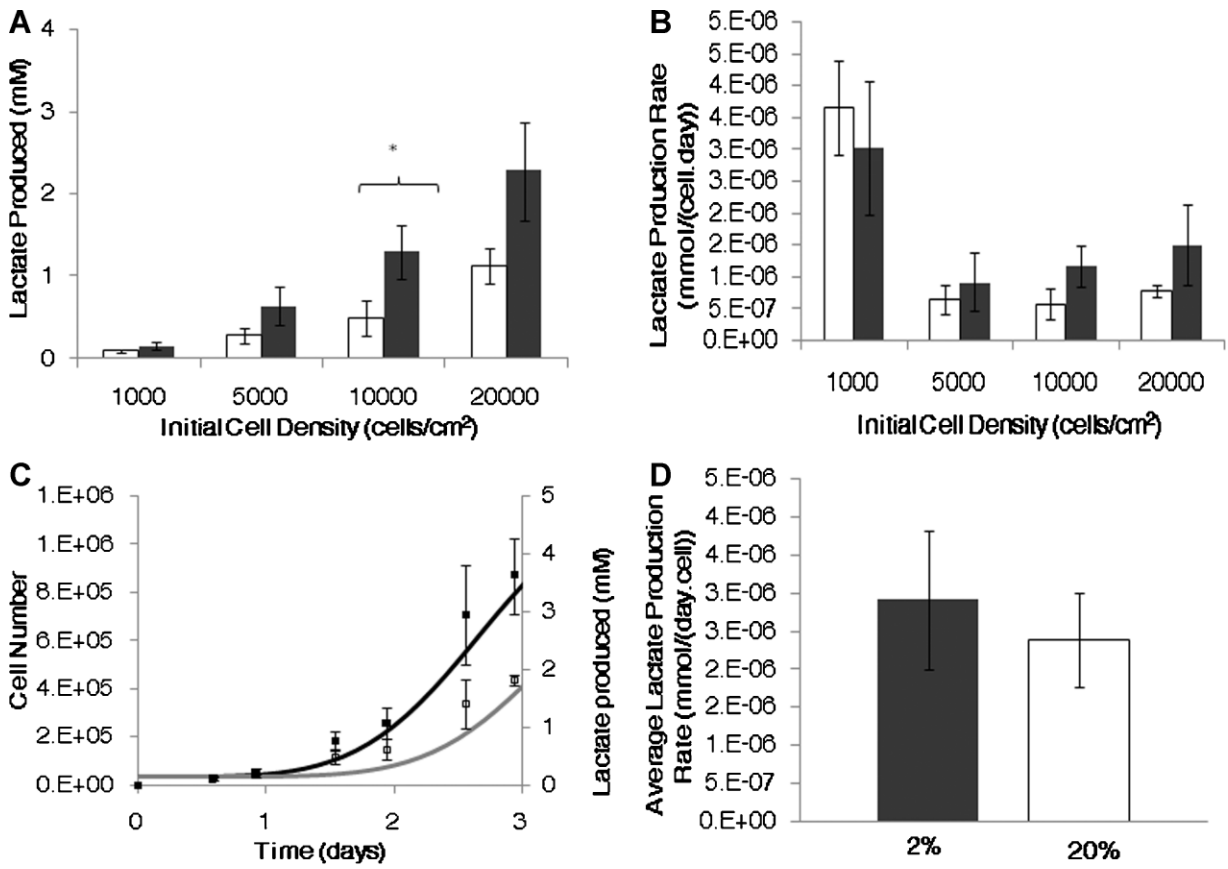

Figure 4. Effect of hypoxia on lactate production during CGR8-NS cell expansion. **A**: Concentration of lactate in cell culture medium after plating cells at different ICD under hypoxia (black bars) and normoxia (white bars) and respective lactate production rates (**B**; hypoxia: black bars, normoxia: white bars; four independent experiments). **C**: Lactate accumulated (squares) in culture medium (mM) and cell number (lines) during time in culture for the time period of one passage under hypoxic (■ and black line) and normoxic (□ and gray line) conditions and respective average lactate production rates (**D**). Data from two independent experiments. Data presented are means ± standard error of the mean. $^{*}P < 0.05$.

proliferation, also determined by BrdU incorporation, and higher levels of apoptosis were detected by activated proteolytically cleaved caspase 3 analysis. Human SVZ precursors (Pistollato et al., 2007) cultured under 5% O_2, on the other hand, showed no differences in terms of apoptosis, determined by the same method, whereas proliferation is increased, as determined by Ki-67 expression. These are some examples which illustrate the different effects that low oxygen tension may have. It is important, however, to note that the abundance of different assays used to assess proliferation or apoptosis may bring additional concerns to the comparison of these results (Elmore, 2007; Ross et al., 2008). In terms of the effect of expansion under hypoxia over the differentiation potential, Pistollato et al. (2007) report, for neurospheres expanded during one passage and differentiated under 20% O_2, a percentage of ≈45% TUJ1-positive neurons and ≈55% astrocytes. On the other hand, expansion during one passage under 5% and differentiation under 20% O_2 generated ≈50% neurons and ≈50% astrocytes. These results, obtained in similar O_2 conditions to those in the study here described, show slightly higher neuronal differentiation efficiency for cells expanded under hypoxia. This difference may be related to the different origin and culture conditions of the cells in both studies. Other authors described higher percentages of neuronal differentiation. For instance, Studer et al. (2000) report differentiation of 73% of rat NS cells cultured under hypoxia into neurons; Chen et al. (2007) report 80% of neuronal differentiation for neurospheres cultured under normoxia and other authors (Baghbaderani et al., 2008) obtained, with neurospheres cultured in bioreactors and differentiated under normoxia, ≈65% of TUJ1+ cells. These higher percentages of neuronal differentiation suggest that further optimization of the differentiation protocol used herein may be possible.

Hypoxia Does Not Increase Lactate Production Rate in CGR8-NS Cells

Lactate is a well-known metabolic end-product, resulting from both glucose and glutamine consumption and its

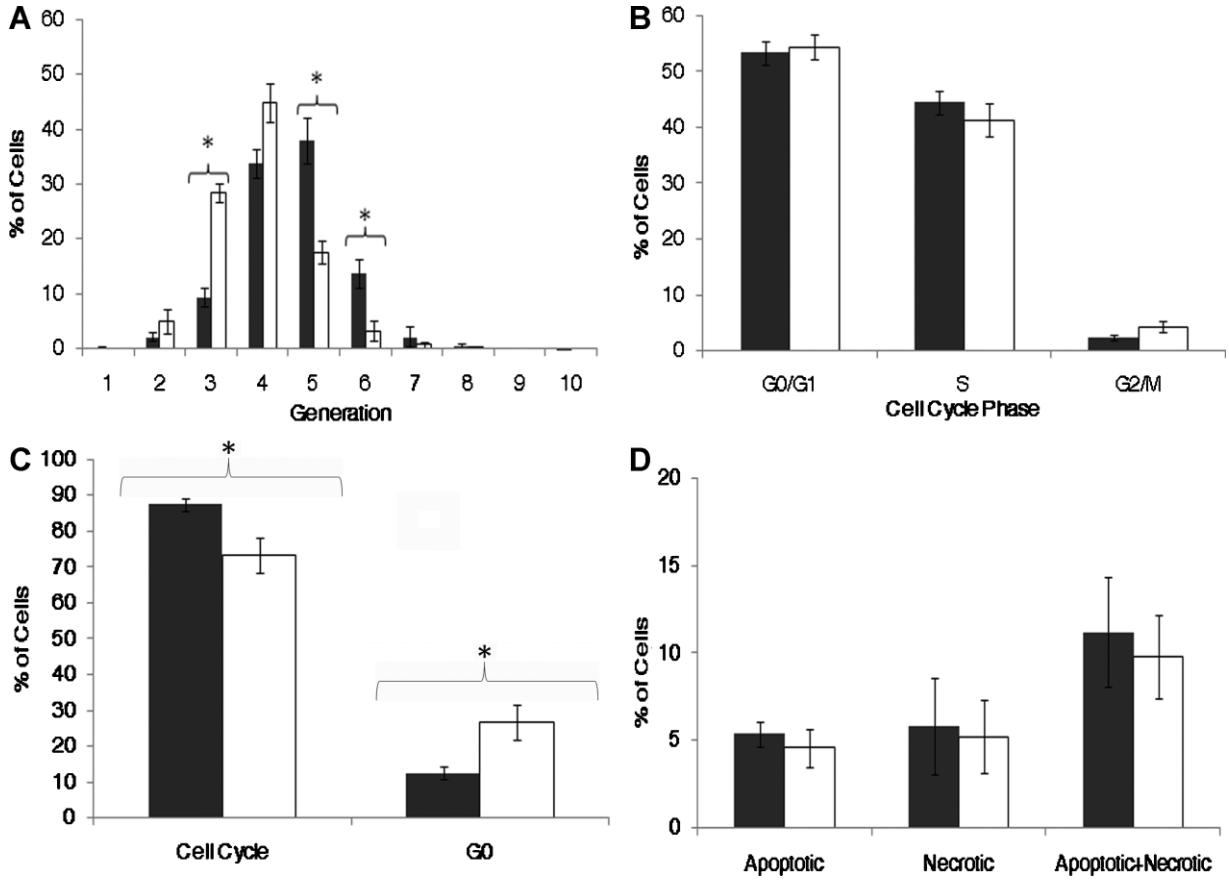

Figure 5. Effect of hypoxia on CGR8-NS cell expansion, cell cycle, and apoptosis. **A**: Distribution of CGR8-NS cells through different divisional generations after expansion during 3 days under hypoxic (■) and normoxic (□) conditions (three independent experiments). **B**: Cell-cycle phase distribution of CGR8-NS cells after 3 days of expansion under normoxic (□) and hypoxic (■) conditions after propidium iodide staining (at least five independent experiments). **C**: Distribution of CGR8-NS cells between G_0 and cell cycle (at least three independent experiments). **D**: Percentage of apoptotic and necrotic cells and total non-viable cells for CGR8-NS cells expanded during 3 days under hypoxic (■) and normoxic conditions (□) determined by flow cytometry after Annexin V/propidium iodide staining (four independent experiments). Data presented are means ± standard error of the mean. $^*P < 0.05$.

accumulation in the culture medium can be harmful by increasing medium osmolarity (Gódia and Cairó, 2005) or by decreasing pH (Kallos and Behie, 1999). In addition, lactate production may also be seen as an indicator of anaerobic metabolism (Powers et al., 2008). The lactate concentrations achieved in this work are lower than the inhibitory concentrations reported for other types of stem cells (16 mM for rat MSC, Schop et al., 2009), so its toxic effects should not be a concern. It was previously reported for murine neurospheres cultured in bioreactors, an increase in lactate production under low oxygen (Kallos and Behie, 1999), interpreted as being caused by a metabolic change triggered by hypoxia. In the work here reported there is no significant difference between both conditions in terms of LPR per cell. Thus, no evident shifting toward an anaerobic metabolism was demonstrated, probably because of the different origin of the cells and the culture medium used.

HIF-1 May Be Responsible for Higher Proliferation Under Hypoxia

Low oxygen concentrations induce a response in cells, with an important outcome that is the activation of the transcription factor hypoxia-inducible factor (HIF-1). HIF-1, in normoxic conditions, is efficiently degraded by the cells but hypoxia immediately stabilizes the protein through oxygen-sensing mechanisms and increases its activity (Semenza, 2000). Some of the genes induced by HIF-1 are known to regulate the proliferation and differentiation of neural precursors or to play a neuroprotective role (Chu et al., 2008). These facts give some biological explanation to the observations here presented, however, it remains to be studied which molecular mechanisms are actually behind the effect of hypoxia on CGR8-NS cells. Low oxygen is also reported to have other positive effects, for instance in terms of chromosomal stability, as chromosomal

instability is related directly to oxidant stress (Csete, 2005; Woo and Poon, 2004) and, in fact, fewer spontaneous chromosomal aberrations have been observed when hESCs are cultured at 2% versus 20% oxygen (Forsyth et al., 2006).

Conclusions

In this work, the influence of different O_2 concentrations on the ex vivo expansion of mouse ES cell-derived NS cells was studied. The results here presented demonstrated that hypoxia is optimal for achieving higher cell numbers, leading to faster cell proliferation but not to significant differences in the percentage of non-viable cells. Although throughout this text this condition was referred as hypoxia, like it has been traditionally designated, probably it would be more accurate to describe these low oxygen conditions as ''in situ normoxia'' and 20% O_2 as ''hyperoxia,'' as suggested elsewhere (Ivanovic, 2009).

We thus conclude that 2–5% O_2 is the most appropriate O_2 tension range for efficient large-scale expansion of CGR8-NS cells in order to provide the number of cells required for drug testing, developmental studies, and cellular therapy.

Nomenclature

CNS	central nervous system
EGF	epidermal growth factor
FGF-2	fibroblast growth factor-2
FI	fold increase
HIF-1	hypoxia inducible factor-1
ICD	initial cell density
LPR	lactate production rate
NGS	normal goat serum
NS cells	neural stem cells
PBS	phosphate-buffered saline
PI	proliferation index
SEM	standard error of the mean
$t_{1/2}$	time required to achieve half the maximum population of cells
TC plates	tissue culture plates

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