The effect of physiological oxygen levels on GABAergic neuronal differentiation from mouse embryonic stem cells

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Abstract

Embryonic stem cells (ESCs) have the ability to generate any kind of cell in the body. They, therefore, have great potential for use in cell therapies for neurodegenerative disorders such as Huntington's disease. Establishing a culture environment to mimic components of physiological conditions may help to maintain and differentiate ESCs more successfully. One of the important conditions is the level of oxygen. Traditionally, 20% oxygen (O_2) has been used to culture cells, but this is much higher than physiological levels (2% O_2). In this study, we used the mouse ESC line 46C (Sox1-GFP knock-in) to investigate the effect of physiological oxygen on proliferation of mESCs, and their differentiation to neural progenitors (where Sox1 is expressed) and mature GABAergic neurons. mESCs were cultured in either high (20%, H) or low (2%, L) oxygen levels for four days before induction of differentiation, and subsequently differentiated under either high or low oxygen, in a 2×2 factorial design (H-H, H-L, L-H, L-L). mESCs placed in low oxygen levels during the differentiation phase showed less proliferation (a decreased proportion of Ki67$^+$ cells), complete loss of the self-renewing population (Oct4$^+$ cells), and a decrease in Sox-1$^+$ neural precursors. Consistent with this, neurons generated under low levels of oxygen showed a more mature morphology with an increased number of primary neurites and increased levels of GABA neurotransmitter. There was no significant difference in the percentage of neurons generated from either condition. We conclude that mESC culture in low oxygen conditions promotes maturation during neuronal differentiation and helps eliminate the residual Oct4$^+$ population. The adoption of low oxygen environments during neuronal differentiation may, therefore, decrease teratoma formation and increase the potential for ESC use in cell therapies for neurodegenerative disease.

Introduction

Embryonic stem cells (ESCs) have a great potential for use in cell therapies in neurodegenerative disorders such as Huntington's disease. Establishing a culture environment that is similar to physiological conditions may help ESC maintenance and differentiation. One important condition is the level of oxygen. The oxygen level of adult brain tissue ranges between 1 to 5%[1-3] and is also low in the uterine environment (2-8%).[4,5] Traditionally, 5% CO_2 and 95% of atmospheric air has been used for ESC culture, thus the oxygen level is 20%, which is hyperoxic to cells. Hyperoxia can increase the number of reactive oxygen species within cells, which then can damage proteins, lipids, and nucleic acids.[6,7] Previous studies have reported that ESCs in physiological oxygen showed enhanced ESC clonal recovery and decreased chromosomal abnormalities.[8,9] In addition, both human and mouse ESCs retained an undifferentiated phenotype when appropriate signals were given in low O_2 conditions, whereas, in the same culture, medium in high (20%) O_2, parts of ESC colonies began to lose expression of their pluripotency marker Oct4.[10,11] In other studies, under low O_2 conditions, cortical, ganglionic eminence (GE), and ventral mesencephalic (VM) precursor/stem cells showed increased proliferation when they were incubated with mitogens.[12-17] In addition, the culture of human and mouse VM precursors in low O_2 has been shown to lower cell death and delay senescence.[13,18] When differentiation was induced, more tyrosine hydroxylase (TH)$^+$ dopaminergic neurons were generated from rat VM cultures maintained in lowered O_2 conditions[12] and more TH$^+$ sympathoadrenal cells were generated from rat neural crest stem cells.[19] In the case of GE cultures, the neurotransmitter phenotype was changed from GABAergic to glutamatergic after culture in low O_2.[17] Recently, one study reported increased dopaminergic differentiation of mESCs using a 5-stage protocol in a 3.5% low O_2 level.[20] In this study, mESCs were differentiated in high O_2 until cells became neural progenitor. Then cells were exposed to low O_2 during the neural progenitors expansion (stage 4) and neuronal differentiation (stage 5). Recently, Mondragon-Teran *et al*. reported the effect of low O_2 on neuronal differentiation from mESCs.[21] They found enhanced cell proliferation and neuronal differentiation during monolayer differentiation in low O_2 although did not investigate which specific neurons were formed. There are no reports which describe how mESCs will respond to low O_2 conditions during GABAergic neuronal differentiation. Therefore, in the present study, we investigated the effect of low O_2 on GABAergic neuronal differentiation by exposing cells to a low O_2 tension from the mESC proliferation stage to the end of the neuronal differentiation stage, using the monolayer method.

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Materials and Methods

Unless stated otherwise, all reagents were from Sigma, UK (Sigma-Aldrich Corp., St. Louis, MO, USA).

mESC culture

The mouse ESC line 46C[22] was used in this study. This ESC line contains a green fluorescent protein (GFP) and puromycin resistance construct driven by the Sox-1 promoter which is switched on during the neurectoderm phase of stem cell differentiation. To see the effect of a physiological level of oxygen on mESCs (passage 29-31), cells were cultured in either high (20%) or low (2%) oxygen levels for four days in proliferating conditions with one passage

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(10^6 cells in a T25 flask with 10 mL 10% fetal calf serum and 100 U/mL LIF in GMEM; FCS/LIF) before being divided into two further groups and differentiated at either high or low oxygen levels (*i.e.* generating four experimental groups: H-H, H-L, L-H, L-L, Figure 1). mESCs were differentiated using the monolayer method which was first described by Ying *et al.*[23] and compared with other methods by Shin *et al.*[24] Briefly, mESCs were plated onto 0.1% gelatin-coated dishes at a density of 10^4 cells/cm^2 in N2B27 medium (1:1 mixture of DMEM/F12 (Invitrogen, Life Technologies Ltd., Paisley, UK) supplemented with modified N2; 25 μg/mL insulin, 100 μg/mL apo-transferrin, 6 ng/mL progesterone, 16 μg/mL putrescine, 30 nM sodium selenite, and 50 μg/mL bovine serum albumin fraction V; and neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen). Medium was renewed every other day. On Day 7, 3×10^4 dissociated cells in 30 µL N2B27 medium with 1 µM retinoic acid (RA) were seeded on poly-D-lysine and laminin coated 13 mm glass coverslips (Fisher Scientific, Loughborough, UK). After 4-6 h, 500 µL of the medium (N2B27+RA) was added, and this was replaced every other day for seven days. For some cultures, cells were cultured in N2B27 for ten days and then N2B27 and RA for a further seven days. In this condition, 0.5 µg/mL puromycin was added from Day 6 to Day 11. For high O_2 conditions, cells were incubated in a standard humidified incubator at 37ºC in 5% CO_2 and 95% air, whereas for low O_2 conditions, cells were incubated in a modular incubator (RS Biotech, Irvine, UK) at 37ºC in 5% CO_2, 2% O_2 and 93% N_2. The levels of the O_2 and CO_2 were monitored constantly. N_2 gas was supplied from a NG400A Nitrogen Generator (Peak Scientific, UK).

Immunocytochemistry and data analysis

All cultured cells were fixed by washing with phosphate buffered saline (PBS) followed by 20 min in 4% paraformaldehyde at 4°C. Fixed cells were permeabilized with 100% ethanol for 2 min at room temperature and rinsed 3 times with PBS. Non-specific binding was blocked in 5% normal goat serum (NGS, DAKO, Ely, UK) for 1 h at 4°C. Cells were incubated overnight at 4°C in 1% NGS containing primary antibodies (mouse anti- -III-tubulin, 1:400; Rabbit anti-GFP, 1:500, Chemicon, Millipore Corp., Billerica, MA, USA; mouse anti-Oct4, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit anti-GABA, 1:750; rabbit anti-Ki67, 1:500, AbCam, Cambridge, UK). The next day, cells were washed 3 times with PBS and incubated with secondary antibodies (Goat anti-rabbit 546 or 488 and Goat anti-mouse 546 or 488, 1:200 each,

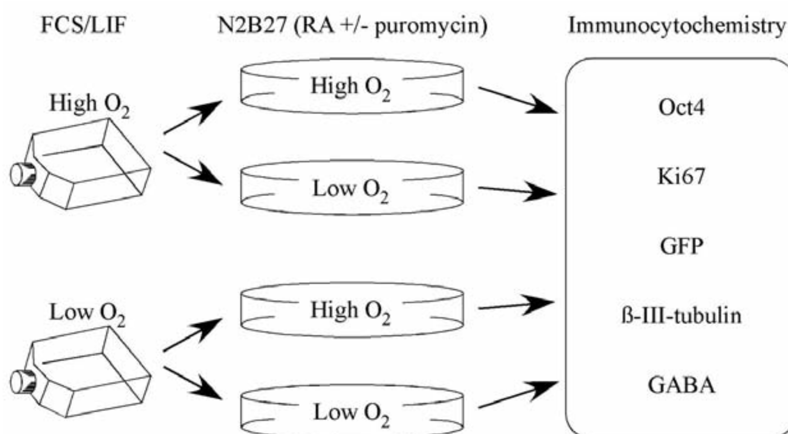

Figure 1. Experimental design. Mouse embryonic stem cells were expanded in either high or low O_2 conditions for four days in fetal calf serum/LIF medium before being split into two groups. After differentiation in the N2B27 medium with retinoic acid for 14 (without puromycin) to 17 (with puromycin) days, cells underwent immunocytochemistry using various antibodies.

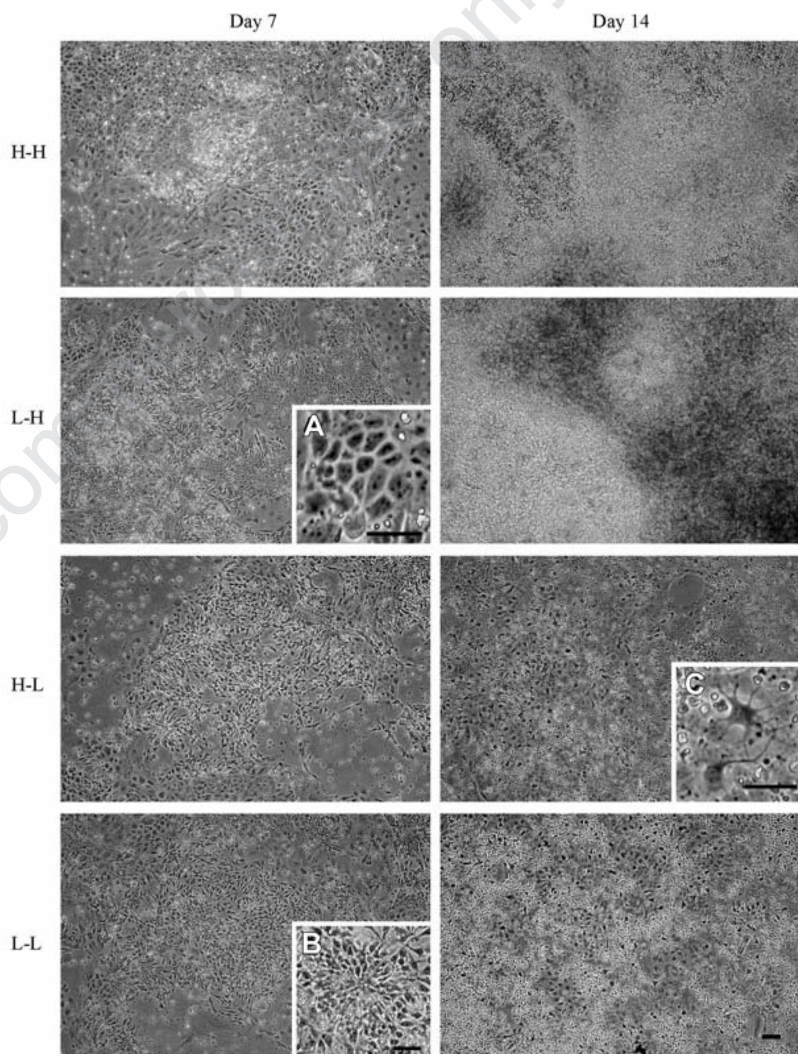

Figure 2. Live images of Day 7 and day 14 cultures in 4 conditions. Cells were cultured in N2B27 for seven days and replated on Day 7. Day 7 images were taken before trypsinisation. Cells were further cultured in N2B27 and RA for seven days. Day 14 images were taken before fixation on Day 14. Scale bar 100 µm applies to all low magnification pictures. Scale bars in inserts 40 µm.

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Invitrogen) in 1% NGS for 2 h at 4°C. After 3 PBS washes, coverslips were mounted using Vectashield hardset mounting medium containing DAPI (Vector Laboratories, Peterborough, UK).

After immunostaining, cells were viewed using a Nikon Eclipse 80i microscope and images were acquired with a Hamamatsu ORCA camera using NIS Elements imaging software (Nikon Corp., Tokyo, Japan).

Images were captured using equivalent intensity settings and the fluorescence intensity for GFP^+ and $GABA^+$ cells was measured using the NIS image software. Cells were classed as having strong expression of GFP at a quantitative fluorescence calibration intensity threshold of 200 or more. *Strong* expression was usually in the intensity range of 240-260. Weak GFP expression was most commonly seen at levels of 160-180.

Similarly, for GABA intensity, the threshold for classification as *strong* expression was set at a quantitative fluorescence calibration of 200 or more. *Strong* $GABA^+$ cells usully fell in the range of 220-260, and *weak* $GABA^+$ cells in the range 100-150. Three independent experiments were carried out and three coverslips were examined for each condition in each experiment to measure specific cell population and neurites. For all data analysis, one way ANOVA and Tukey's multiple comparison test were employed (GraphPad Prism version 4.00). P<0.05 was considered significant.

Results

The proliferating population was decreased in cells exposed to low O_2 conditions during the differentiation period

When cells were counted using trypan blue exclusion before replating on coverslips, at Day 7, there was no significant difference in the number of viable cells between groups (*data not shown*). However, mESCs cultured in high O_2 during the later half of the differentiation period (Days 8-14) produced more total cells than populations differentiated in low O_2 conditions (Figure 2). In addition, the colonies in high O_2 conditions appeared more 3-dimensional, with packed cells, often attributed to faster-dividing pluripotent cells.

Therefore, the cell division marker Ki67 was used to detect proliferating cells at Day 14. mESCs in high O_2 showed significantly higher numbers of proliferating cells compared to those in low O_2 although the proportion of proliferating cells in the cultures was fairly modest (Figure 3A-C, ANOVA: $F_{3,8}$=20.36, P<0.001).

$Oct4^+$ cells were removed completely in low O_2 conditions

Cells were cultured in N2B27 for seven days. On Day 7, cells were replated on coverslips and cultured for a further seven days before fixation. Figure 2 shows images from the 4 conditions (H-H, L-H, H-L, and L-L) on Day 7 and a further seven days after replating (Day 14). On Day 7, neural rosettes were observed throughout the cultures (Figure 2, insert B). However, L-L and H-L cultures contained more neural-like cells than H-H and L-H cultures. In H-H and L-H cultures, epithelial-like cells (Figure 2, insert A) were more abundant than in L-L and H-L cultures. On Day 14, H-H and L-H cultures were denser than L-L and H-L cultures and, though many neurons were found in both cultures, mature looking cells were more prevalent in L-L and H-L cultures (Figure 2, insert C). Cells were fixed on Day 14 and labeled with Oct4 antibodies to determine whether low O_2 reduced/removed the undifferentiated mESC population. The number of $Oct4^+$ colonies was counted on each coverslip (Figure 3A and B). There was no significant difference in the number of $Oct4^+$ colonies (ANOVA: $F_{3,8}$=4.00, n.s.). Even in H-H cultures, $Oct4^+$ colonies were very few. Some H-H coverslips did not have any $Oct4^+$ colonies at all, and others contained one colony (Figure 3A, insert A). There were no $Oct4^+$ colonies in L-H, H-L or L-L cultures in all coverslips examined.

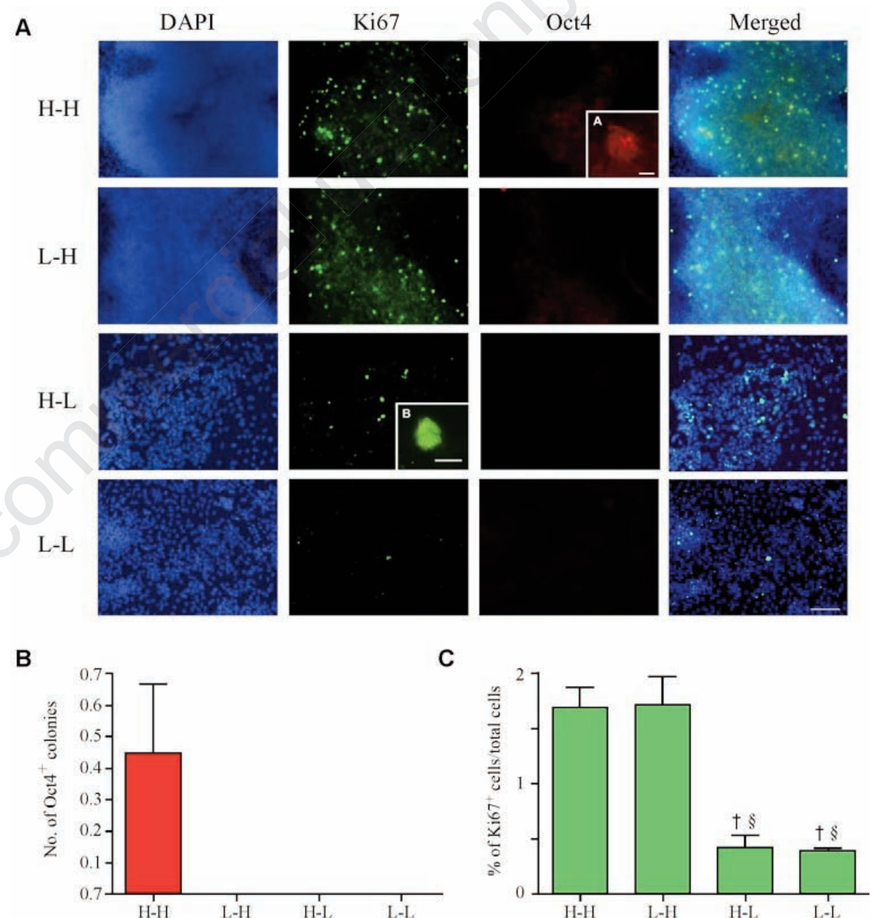

Figure 3. Effect of low O_2 on the pluripotent cell population and proliferative cell population during differentiation. Cells were cultured for 14 days and stained. (A) Immunocytochemistry of Oct4 and Ki67. An example of an $Oct4^+$ colony. (B) A $Ki67^+$ cell. Scale bar in the last merged picture is 100 μm and applies to all low power images. The scale bar in (A) is 20 μm and the scale bar in (B) 10 μm. (B) The number of $Oct4^+$ colonies per coverslip. Data was not significantly different. However, there were zero $Oct4^+$ cells in low O_2 conditions. (C) Percentage of $Ki67^+$ cells over total cells. The proliferating population was significantly reduced in cultures incubated in low O_2 during the neuronal differentiation period. †, P<0.01 compared to H-H; §, P<0.01 compared to L-H.

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The Sox1-GFP+ cell population was decreased in low O_2 conditions

Next, all non-neuronal cells were removed using puromycin and RA treatment to allow easier visual examination of the neural cells which had formed.[24] Cells were cultured in N2B27 for ten days and puromycin was applied in the medium from Day 6 to Day 10 (Figure 4). Compared to non-purified cultures (Figure 2, Day 7), non-neural looking cells were dramatically decreased and in all 4 conditions many neural rosettes were found (Figure 4, arrows). On Day 10, cells were replated on coverslips and cultured for seven more days. Compared to non-purified cultures (Figure 2, Day 14), the number of cells was substantially reduced in all 4 conditions and neuron-looking cells were more frequently and easily seen (Figure 5). In particular, cells in low O_2 during differentiation (*i.e.* H-L and L-L) showed mature and healthy looking morphologies (Figure 5, arrows) compared to those in high O_2 (H-H and L-H).

After seven days on coverslips, cells were labeled with Oct4 and GFP antibodies. There were no Oct4+ pluripotent cells present in any condition (n=3), whereas GFP was expressed by the majority of cells to varying degrees. Some cells expressed much higher levels of GFP (Figure 6A, white arrow) than others (Figure 6A, yellow arrow); therefore, expression was quantified both to determine how many cells were expressing GFP regardless of expression intensity and also how many expressed only high levels of GFP. It was hypothesized that the strong GFP expression would be true Sox1+ neural precursor cells, and weakly expressing cells would be young neurons and glial cells which contained remnant GFP protein even though their *GFP* gene had been switched off, since GFP protein (half-life up to 26 h) tends to remain longer in the cell than the gene expression.[25] There was no significant difference in percentage of total GFP expressing cells (disregarding expression intensity) between groups (ANOVA: $F_{3,8}$=0.4588, n.s.; Figure 6A and B). However, there were significantly fewer cells which were expressing high levels of GFP in the low O_2 cultures during differentiation (*i.e.* H-L and L-L) compared to cells differentiated in high O_2 conditions (*i.e.* H-H and L-H) (ANOVA: $F_{3,8}$=11.76, P<0.01; Figure 6A and C).

Neurons in low O_2 conditions showed a more mature morphology than in high O_2 conditions

After 17 days in differentiation culture, cells were stained with TUJ1 and GABA antibody. Since cells in low O_2 cultures showed a more mature morphology, we considered whether neuronal differentiation was accelerated, thus producing more neurons. On investigation, there was no significant difference in the percentage of TUJ1+ neurons between groups (ANOVA: $F_{3,8}$=0.018, n.s.; Figure 7A and B).

The number of primary neurites was counted on 30-60 neurons per coverslip in each condition. As seen previously in the live images, neurons cultured in low O_2 (*i.e.* H-L and L-L) possessed significantly more primary neurites than neurons cultured in high O_2 conditions (*i.e.* H-H and L-H) (ANOVA: $F_{3,8}$=11.62, P<0.01; Figure 7A and C). GABAergic neuronal differentiation in high and low O_2 conditions was then investigated. Like GFP immunoreactivity, GABA labeling showed two different intensities: strong fluorescence (Figure 7A, white arrow) or more weak fluorescence (Figure 7A, yellow arrow). Therefore, we first quantified GABA+ neurons over total neurons regardless of staining intensity. The majority of neurons were GABAergic (70-75%) and the percentage was similar between groups (ANOVA: $F_{3,8}$=0.995, n.s.; Figure 7A and D). However, when we compared strongly stained GABAergic neurons (considered to be mature GABAergic neurons since strong staining reflects more GABA content), the percentage of more mature GABAergic neurons was increased in cultures differentiated in low O_2 conditions (*i.e.* H-L and L-L) compared to high O_2 conditions (*i.e.* H-H and L-H) (ANOVA: $F_{3,8}$=156.1, P<0.0001; Figure 7A and E); this was consistent with the results of neurite number. In addition, compared to high O_2 (H-H), low O_2 cultures (L-H, H-L, and L-L) tended to contain more evenly dispersed neurons across the coverslips (Figure 7, 10x TUJ1).

Discussion

We investigated the effect of low oxygen on mESC differentiation to GABAergic neurons together with cell proliferation and pluripotency. Here, we used the monolayer method to differentiate mESCs, based on previous findings that these stem cells differentiate into functional GABAergic neurons, expressing appropriate markers, and possessing functional electrophysiology similar to medium spiny GABAergic projection neurons in the develop-

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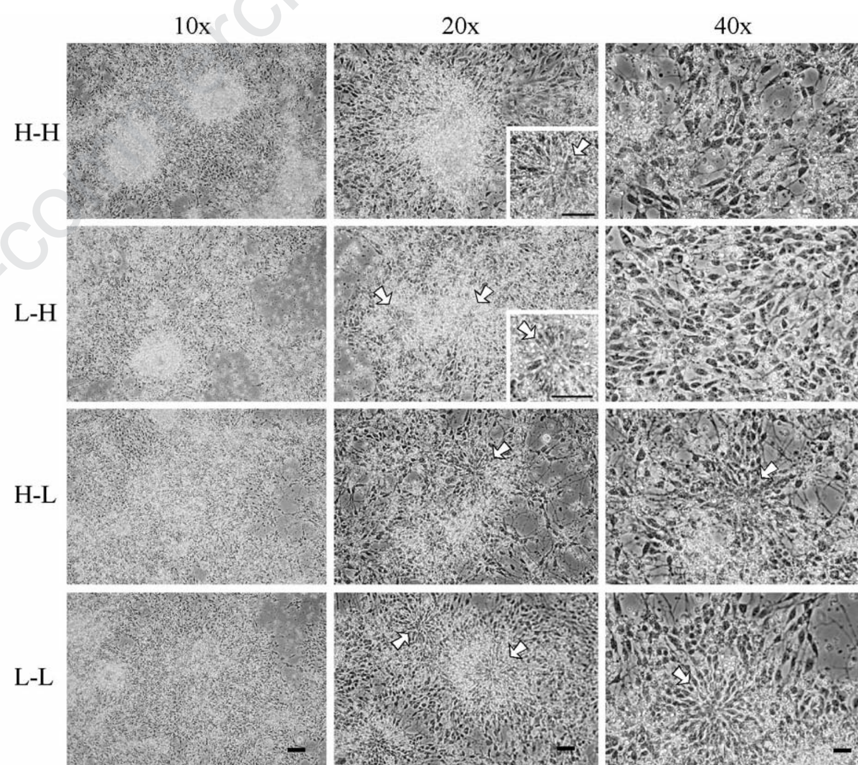

Figure 4. Day 10 cultures in 4 conditions. Cells were cultured in N2B27 for ten days. Puromycin was applied in the medium from Day 6 to Day 10. Many non-neural cells were removed and most of the cells appeared as neural rosettes (arrow) or bipolar cells. Scale bars in the first column (10x) 100 μm, in the second column including those in inserts (20x) 50 μm, and the last column (40x) 25 μm.

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ing striatum.[24] We exposed cells to low (2%) and high (20%) oxygen tension first during the proliferating/undifferentiated stage in the presence of FCS and LIF and then we further split cultures into low and high O_2 conditions when differentiation was induced. The exposure to low O_2 during the proliferating/undifferentiated stage had a positive effect on removal of Oct4+ pluripotent cells since H-H cultures very rarely showed Oct4+ colonies, but in L-H cultures there were no Oct4+ colonies in any of the three independent cultures analyzed in triplicate. However, the populations of Ki67+ mitotic cells, Sox1-GFP+ neural precursors, TUJ1+ neurons, and GABAergic neurons were not affected by low O_2 tension during the proliferating/undifferentiated stage. These populations were more affected by low O_2 exposure during the differentiation stage (*i.e.* H-H *vs* L-H, and H-L *vs* L-L did not show any significant differences; but H-H *vs* H-L and L-H *vs* L-L did show significant differences). This may reflect the short incubation period in low O_2 in the presence of FCS/LIF, since mESCs were cultured in FCS/LIF medium under low O_2 tension for only four days before starting differentiation. Therefore, four days might not be enough to see the full effect of low O_2 and mESCs might need to be cultured for a longer period in FCS/LIF conditions or even be derived in low O_2 conditions. Previous studies have reported that low oxygen tension favors proliferation and survival of stem/progenitor cells during their expansion period in embryonic rat and human VM progenitors,[12,13,18,26] human neonatal subventricular zone cells,[16] embryonic rat cortical cells,[15,27] embryonic rat GE cultures,[17] and embryonic rat neural crest stem cells.[19] Also in some studies using undifferentiated mESCs, low O_2 promoted proliferation of mESCs.[9,28]

Interestingly, previous research has shown that mESCs that were kept in low O_2 for 18 months but then once returned to higher O_2 conditions behaved very similarly to mESCs cultured in high O_2 throughout the study.[11] Forsyth *et al.* reported similar results from hESC cultures.[8] In low O_2, hESCs formed colonies more efficiently than in high O_2. When hESCs were re-oxygenated from 2 to 21% O_2 they did not form colonies as efficiently as those in low O_2 or, interestingly, with similar efficiency as those cultured in 21% throughout. We did not examine the number of Ki67+ cells of mESCs during the proliferation stage, therefore, we cannot conclude whether proliferation in low O_2 conditions leads to increased or lowered numbers of dividing stem cells. However, it appears from previous studies that culturing cells in low O_2 during the proliferation period might not affect differentiation to a significant extent, and lowering the oxygen tension only during the differentiation period may be more effective in influencing ESC fates. In this study, low oxygen tension during the differentiation period (*i.e.* H-L and L-L) lowered the number of Ki67+ proliferating cells. This agrees with a previous report in which the number of mESC-derived EBs was less in cultures placed in 5% O_2 compared with 20 and 40% O_2.[29] In addition, hESC-derived EBs were shown to have slower proliferation in 1 and 5% O_2 tension compared to 20% O_2 tension.[30] This is important when considering transplantation of ESC-derived neurons, as the introduction of cells that maintain the propensity to divide has been linked with teratoma formation by grafted cells. One recent study reported a similar experimental approach to the current study here.[21] Researchers differentiated E14Tg2a mESCs (the parental mESC line for 46C mESCs, which were used in the current study) for eight days using the monolayer method in low (2%) and high (20%) O_2 and then looked at cell proliferation by counting viable cells and the expression levels of nestin, TUJ1 and MAP2 by immunocytochemistry and flow cytometry. Authors found that there was increased live cell density at Day 8 in low O_2 conditions compared with high O_2. This is different to the current study, where cells at Day 7 showed no obvious difference in cell density (Figure 2, first column). The difference in findings might come from assessing the live cells at different time points during their differentiation. According to the same paper,[21] the viability of the cells goes down dramatically after Day 7. Also, differences in the seeding cell density might have affected the live cell density on Days 7 and 8.

Mondragon-Teran *et al.* found significantly more neural rosettes in low O_2 cultures (3- fold increase),[21] and the current study also found neural rosettes more easily in low O_2 condi-

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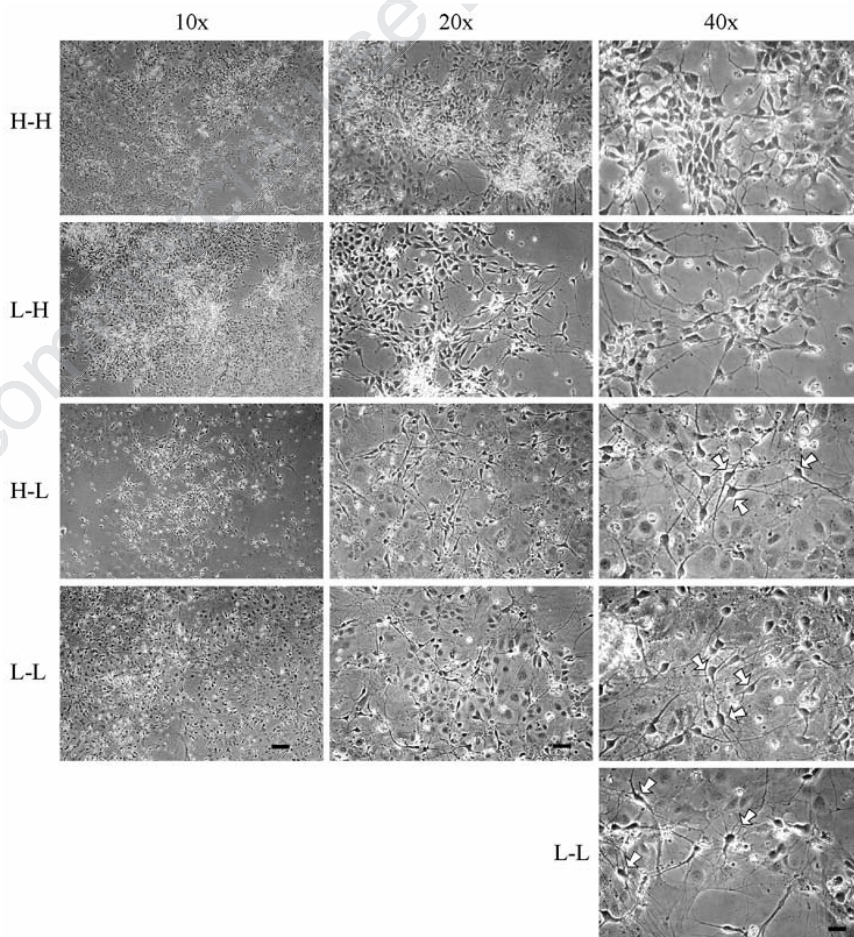

Figure 5. Day 17 cultures from the four conditions. Cells were cultured in N2B27 for ten days. Puromycin was applied in the medium from Day 6 to Day 10. On Day 10, cells were replated and further cultured in N2B27, puromycin, and RA for two days and without puromycin for five days. Cells developed into mature looking neurons (arrows) and glial cells. Scale bar in the first column (10x) 100 μm, second column (20x) 50 μm, and last column (40x) 25 μm.

tions. In the previous study, nestin+ neural precursors were slightly decreased in low O_2 conditions whereas TUJ1+ neurons and MAP2+ neurons were increased in low O_2 condition. In the current study, Sox1-GFP+ neural conditions were decreased when examined on Day 14 of the differentiation protocol. Even though the time and the culture medium were slightly different in two studies, incubating differentiating mESCs in low O_2 helped the progression of differentiation in both studies. However, unlike the study of Mondragon-Teran *et al.*, there was no significant difference in the percentage of TUJ1+ neurons in the current study in low and high O_2 cultures. One reason for this might be the different time point when the neuronal population was quantified. In the Mondragon-Teran *et al.* study, cells were cultured with N2B27 and neural cells were not selected from the total population. In the current study, mESCs were differentiated for 17 days with N2B27 and neural precursor cells were selected using puromycin and RA. Therefore, both in high and low O_2 cultures of the current study, non-neural cells were rarely present from Day 12 onwards. Accordingly, there were enriched neural populations already in both O_2 tensions, so the difference between low and high O_2 cultures was not as pronounced as that found by Mondragon-Teran *et al.* However, we did observe more rapid differentiation in the current study since there were more mature GABAergic neurons in low O_2 cultures. Consequently, our two studies agree that one of the effects of low O_2 in mESC neuronal differentiation using the monolayer method is in accelerating neuronal differentiation. One study comparing the effects of oxygen tension on mouse neural stem cells derived from embryonic GE reported an effect of different O_2 tensions on neuronal cell generation.[17] Neuronal differentiation (measured as TUJ1+ cells) was most efficient in 2% O_2 than other oxygen tensions (0, 1, 4, 10 and 20%). Similarly, hESCs proliferated under 3 and 5% O_2 tensions have been shown to have enhanced formation of EBs.[31,32] In addition to neuronal differentiation from primary cells, NSCs, and ESCs, other types of cell such as osteocytes,[33] platelets,[34] vascular tissues,[35] cartilage,[36] and cardiac cells[37] tend to develop better in low O_2 tensions. Therefore, this more rapid maturation effect seems a common phenomenon for stem cell differentiation. In support of this, in the current study, the percentage of the intense Sox1-GFP+ neural precursor population was decreased in low O_2 conditions. This does not indicate that low O_2 conditions inhibited neural differentiation, since the combined populations expressing both high and low levels of GFP were similar when comparing high and low O_2 cultures. Therefore, it seems likely that more neural precursor cells differentiated earlier into neurons and glial cells in low O_2 conditions than in high O_2, maturing and down-regulating GFP at a faster rate. In a rat VM culture study, nestin+ neural precursors were reduced in low O_2 and markers for neurons and glial cells appeared earlier in low O_2 cultures than in high O_2 cultures, although there was no significant difference in the percentage of TUJ1+ cells different between conditions.[12] The percentage of TUJ1+ neurons in our study was the same in both oxygen tensions which agrees with primary cell culture studies,[12,15,16] although some studies have reported increased neuronal differentiation[17,19] and some have reported decreased neuronal differentiation.[27] These discrepancies in neuronal number might be due to the different cell types used, different medium, different absolute oxygen levels, and different

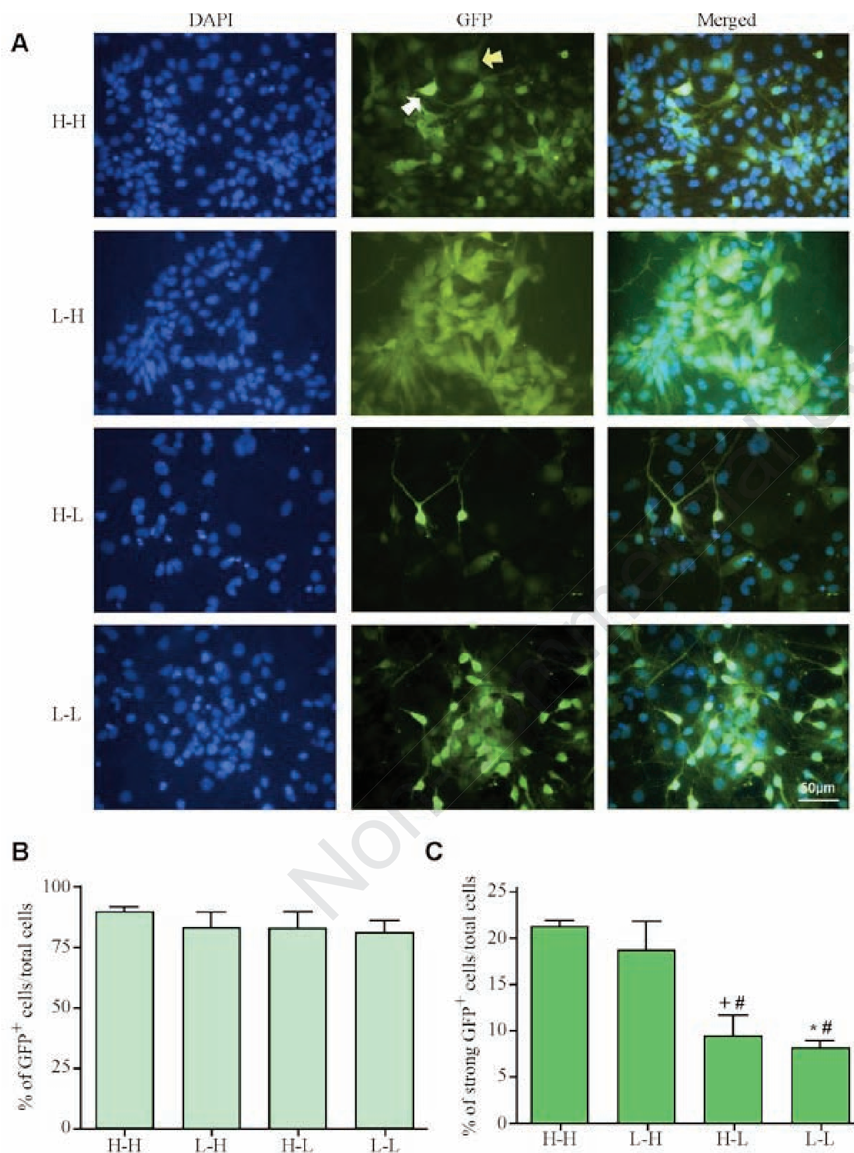

Figure 6. Effect of low O_2 on Sox1-GFP+ neural precursors. Cells were cultured for 17 days *in vitro* and stained for green fluorescent protein (GFP) immunoreactivity. (A) Immunocytochemistry labeling of GFP on Day 17. Some cells showed intense GFP expression (white arrow, range 240-260 quantitative fluorescence calibration) and some showed less intense GFP expression (yellow arrow, range 160-180 quantitative fluorescence expression). Scale bar 50 µm. (B) Percentage of cells expressing GFP over total cells. There was no significant difference between groups. (C) Percentage of cells expressing intense levels of GFP over total cells. The number of cells expressing intense GFP was decreased significantly in low O_2 conditions compared with high O_2 during differentiation. +, P<0.05 compared to H-H; *, P<0.01 compared to H-H; #, P<0.05 compared to L-H.

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incubation length. In this study, the neurons generated in low O_2 appeared to be more mature since they possessed more neurites, and more GABA neurotransmitter. Other studies using precursor/ adult stem cells also described better differentiation of different neurons under low O_2 conditions. Rat VM precursors produced more dopaminergic (DAergic) neurons able to release dopamine (DA).[12,13] Another study reported a change in the neurotransmitter phenotype from GABAergic to glutamatergic[17] and, in neural crest stem cells, the new appearance of a sympathoadrenal lineage.[19] In low O_2 cultures, expanded human SVZ cells and rat cortical progenitors produced oligodendrocytes, which were very rarely generated in high O_2 cultures.[15,16] One recent study generated DAergic neurons from mESCs using low O_2 (3.5%) tension and the 5-stage protocol.[20] There was no difference in the percentage of TUJ1+ neurons between high and low O_2 cultures, but there was an increase in absolute TH+ cell numbers and mRNA expression of TH, Nurr1, and engrailed-1 (En-1) in low O_2 conditions.

Taken together, low O_2 (2%) tension during the proliferating period and/or differentiation period removed the Oct4+ pluripotent stem cell population completely and lowered proliferation of mESCs. In addition, neurons differentiated in low O_2 tension showed better maturation with an increased number of primary neurites, and increased levels of GABA neurotransmitter. Therefore, it might be necessary to culture mESCs in low O_2 conditions to differentiate them to become GABAergic neurons to be used in cell transplantation, both to eliminate the Oct4+ dividing stem cells and for a more enhanced and specific differentiation.

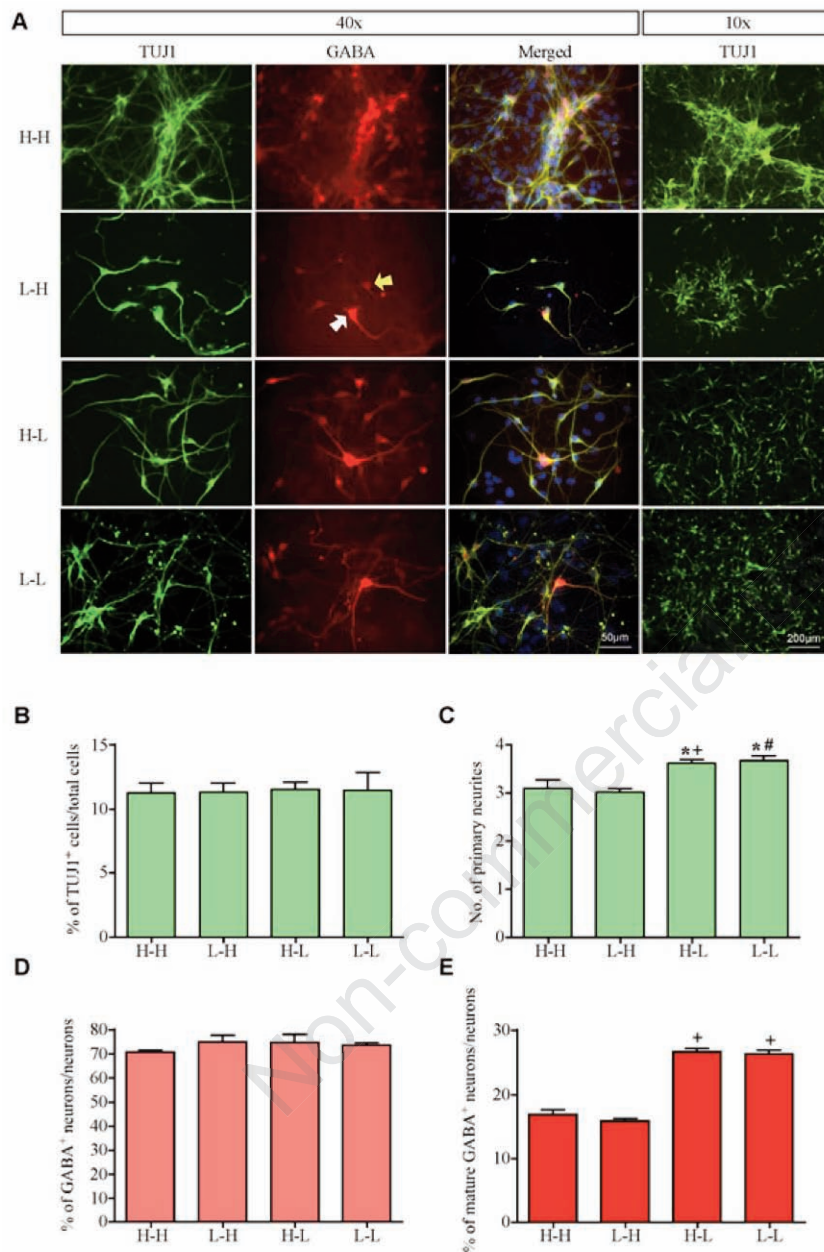

Figure 7. Effect of low O_2 on neuronal and GABAergic neuronal differentiation. Cells were cultured for 17 days *in vitro* and labeled with TUJ1 and GABA antibodies. (A) Immunocytochemistry of TUJ1 and GABA on Day 17. Some cells showed GABA expression (white arrow, range 220-260 quantitative fluorescence calibration) and some less strong GABA expression (yellow arrow, range 100-150 quantitative fluorescence calibration). Scale bar 50 μm in 40x and 200 μm in 10x images. (B) The percentage of TUJ1+ cells over total cells. There was no significant difference between groups. (C) The number of primary neurites per neuron. There were significantly more neurites in neurons cultured in low O_2 during differentiation (H-L and L-L). *, $P<0.05$ compared to H-H; +, $P<0.05$ compared to L-H; #, $P<0.01$ compared to L-H. (D) The percentage of all GABAergic neurons over total neurons. There was no significant difference between groups. (E) The percentage of neurons expressing intense GABA immunoreactivity over total neurons. Neurons expressed higher levels of GABA in cultures differentiated in low O_2 (H-L and L-L). +, $P<0.001$ compared to H-H and L-H.

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