

**Effects of oxygen concentration on the proliferation and differentiation
of mouse neural stem cells *in vitro***

Short running title: Effects of oxygen concentration on neural stem cells

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Abstract

Background and purpose- Cerebral ischemia is known to elicit the activation of neural stem cells (NSCs), however its mechanism is not fully determined. Although oxygen concentration is known to mediate many ischemic actions, there has been little attention given to the role of pathological oxygen changes under cerebral ischemia on the activation of NSCs. We investigated the effects of various oxygen concentrations on mouse neural stem cells *in vitro*.

Methods- NSCs were cultured from the ganglionic eminence of fetal ICR mice on embryonic day 15.5 using a neurosphere method. The effects of oxygen concentrations on proliferation, differentiation and cell death of NSCs were evaluated by bromodeoxyuridine (BrdU) incorporation, immunocytochemistry and TUNEL assay, respectively.

Results- The highest proliferation and the neuronal differentiation of the NSCs were observed in 2 % oxygen, which yielded significantly higher proportions of both BrdU-labeled cells and Tuj1-positive cells when compared with 20 % and 4 % oxygen. On the other hand, the differentiation to the astrocytes was not affected by oxygen concentrations, except in the case of anoxia (0 % oxygen). The cell death of the NSCs increased in lower oxygen conditions and peaked at anoxia. Furthermore, the switching of the neuronal subtype differentiation from GABA-positive to glutamate-positive neurons was observed in lower oxygen conditions.

Conclusions- These findings raise the possibility that reduced oxygen levels occurring with cerebral ischemia enhances NSC proliferation and neural differentiation, and that mild hypoxia (2 % oxygen), which is known to occur in the ischemic penumbra, is suitable for abundant neuronal differentiation.

Key Words: neural stem cell, hypoxia, proliferation, differentiation, cerebral ischemia

Introduction

Cerebral ischemia is known to cause a complex cascade of cellular events, leading to both acute and delayed neural death and severe brain dysfunction in animal models as well as in human beings (Siesjo 1992). Interestingly, a recent line of evidence demonstrates that global and focal ischemia enhances the proliferation and the neural differentiation of neural stem cells (NSCs) in the subgranular zone of the dentate gyrus, the anterior subventricular zone and the posterior periventricular zone adjacent to the hippocampus. These conditions also increase the migration of the newborn neurons to the dentate granule cell layer, the olfactory bulb, the striatum, and the damaged hippocampal CA1 region (Arvidsson et al. 2002; Kee et al. 2001; Liu et al. 1998; Nakatomi et al. 2002; Sato et al. 2001; Takagi et al. 1999; Yagita et al. 2001). However, the mechanisms underlying the activation of the NSCs (enhanced proliferation, neural differentiation and migration) triggered by ischemia are not fully understood.

Among various incidents during ischemia, reductions in the supply of oxygen (hypoxia) and glucose (hypoglycemia) in the brain seem to be the main factors mediating ischemic actions in the brains. Recently, we have reported that when the NSCs were cultured at lowered concentrations of glucose, which is reported to occur in the brain under physiological and pathophysiological conditions, their proliferation and differentiation into neurons and astrocytes is facilitated *in vitro* (Horie et al. 2004). With regard to oxygen concentration, it has been reported that physiological oxygen concentrations in the brain (3-5%) enhances the proliferation of cultured NSCs and modulates their differentiation into neurons. This is in contrast conditions of 20% oxygen, which is non-physiologically high *in vivo*, but often used in the standard

method of cell culture (Jin et al. 2002; Morrison et al. 2000; Shingo et al. 2001; Studer et al. 2000). However, there have not been any studies investigating the effect of lower concentrations of oxygen seen in pathologically conditions on the proliferation and the differentiation of the NSCs. It is well known that acute ischemic stroke lowers the oxygen concentration to various levels depending on the brain area (Astrup et al. 1981; Fisher 1997; Harris et al. 1987; Liu et al. 2004; Studer et al. 2000). In the severely damaged ischemic core regions, blood supply is interrupted and oxygen concentration decreases to anoxia level (near 0 %). In the penumbra, which surrounds the core region and is initially viable tissue, there is a reduced but constant blood supply, leading to lowered tissue oxygen concentration of approximately 2 %, which is approximately half the oxygen concentration in the normal brain tissue (3 to 5 %) (Harris et al. 1987; Liu et al. 2004; Studer et al. 2000).

Considering the various concentrations of oxygen under physiological and pathophysiological conditions, we examine the effects of oxygen concentrations (0, 1, 2, 3, 4, 10 and 20 %) on the proliferation and differentiation, as well as, the cell death of the NSCs derived from embryonic mouse ganglion eminence by means of an *in vitro* NSC culture system. An *in vitro* system allows us to control oxygen concentrations and delineate the actions of the extrinsic factors.

Materials and Methods

Primary NSC culture

The NSCs were isolated and propagated using the neurosphere method developed by Reynolds, Weiss and colleagues (Reynolds et al. 1992; Reynolds and Weiss 1992).

Fetal ICR mice on embryonic day 15.5 (midnight of overnight mating is designated as embryonic day 0) were isolated from their mother under deep anesthesia with ether and placed into ice-cold 1 : 1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient (DMEM/F-12: Gibco-BRL, Gaithersburg, Md., USA). The ganglionic eminence tissue was carefully micro-dissected under a stereomicroscope and triturated with a commercial 1 ml plastic pipette to obtain a single cell suspension in N-2 medium (DMEM/F-12 supplemented with 36.4 mM glucose, 23 μ g/ml insulin, 92 μ g/ml transferrin, 55 μ M putrescine, 27.5 nM sodium selenite, 20 nM progesterone and 50 U/ml penicillin–streptomycin) (Mitome et al. 2001). The viable dissociated cells at a density of 25×10^4 cells/5 ml in N-2 medium containing 20 ng/ml EGF (Invitrogen Corp., Carlsbad, CA, USA) were seeded into uncoated T25 culture flasks (Becton-Dickinson, Franklin Lakes, NJ) and were maintained in a humidified incubator at 37 °C with 95% atmospheric air/5% CO₂. Cultures were incubated for 5 days to form enough primary neurospheres. The cell viability for all the primary cultures examined was over 90% at the beginning of the experiment (data not shown).

BrdU immunocytochemistry

The effect of oxygen concentrations on the proliferative activity of NSCs was evaluated by bromodeoxyuridine (BrdU) immunocytochemistry (Studer et al. 2000). The single cell suspensions from primary neurospheres were prepared by centrifugation (1,200 rpm, 5 min) followed by mechanical dissociation. They were seeded at a cell density of 2.2×10^4 cells/350 μ l in N-2 medium containing EGF (20 ng/ml), which had been equilibrated under each oxygen concentration (0, 1, 2, 3, 4, 10 and 20 %), in Lab-Tek[®]

Chamber SlideTM (Nunc, Inc., Naperville, IL, USA). The NSC cultures were placed in humidified portable isolation chambers (CO₂ multi-gas incubator, APMW-36, ASTEC, and AMP-30D, ASTEC, Japan), flushed continuously with a N₂ gas to maintain established O₂ concentrations at a constant temperature of 37 °C. The oxygen concentration was monitored with IRMA blood analysis system M3623A (Agilent Technologies, Tokyo, Japan) before the experiment. The analyzed data was calculated taking into consideration the appropriate atmospheric pressure of 1009 hPa (756.8mmHg) and the saturation stem pressure of 62.75 hPa (47.0 mmHg) at 37 °C. These measured values were compatible with the established oxygen concentrations in the chamber (data not shown). After 24 hr incubation at various concentrations of oxygen, cells were labeled with a 10µM of BrdU and incubated for an additional 6 hr. After the labeling medium was removed by aspiration, cells were fixed in PBS containing 4 % paraformaldehyde for 20 min at room temperature. To denature nuclear DNA into single-strand term, cells were incubated in 0.02 % pepsin in 0.01 N HCl for 20 min, 2 N HCl for 45 min, and 0.15 M Sodium Borate for 10 min. Cells were washed by PBS three times and incubated overnight with anti-BrdU antibody (1:50; rat monoclonal antibody, Oxford Biotechnology Ltd, Oxford, UK) in PBS containing 0.1 % Triton X-100 and 3% normal goat serum at 4 °C.

After removal of the primary antibody solution, cells were washed with PBS three times and were incubated with secondary antibodies (Alexa Fluoro[®] 568 goat anti-rat IgG, 1:200, Molecular Probes Inc., Eugene, OR) and 1.25 µg/ml of Hoechst 33258 for a nuclear counter staining for 2 hr at room temperature under light-shading conditions. After sequential washing with PBS and water, the slides were mounted with Gel/

MountTM (Biomedica corp, Foster City, CA) and their fluorescent images were viewed with a microscope (Nikon E600 and Olympus DP70, Japan). For the quantitative analysis, we counted numbers of BrdU positive cells in a fixed area (700 x 1000 μ m) at the center of each well under the fluorescent-microscope. The number of BrdU positive cells was counted by an observer blind to the treatment condition and the counts were normalized to the total cell number (Hoechst 33258-stained nucleus).

TUNEL assay

The effect of oxygen concentrations on the cell death of NSCs was evaluated by TUNEL In Situ Cell Death Detection Kit, Fluorescein (Roche, Penzberg, Germany) (Studer et al. 2000). The single cell suspensions from primary neurospheres were prepared by centrifugation (1,200 rpm, 5 min) followed by mechanical dissociation. They were seeded at a cell density of 2.2×10^4 cells/350 μ l in N-2 medium containing EGF (20 ng/ml), which had been equilibrated to each oxygen concentration (0, 1, 2, 3, 4, 10 and 20 %), in Lab-Tek[®] Chamber SlideTM (Nunc, Inc., Naperville, IL, USA). The NSC cultures were placed in humidified portable isolation chambers (CO₂ multi-gas incubator, APMW-36, ASTEC, and AMP-30D, ASTEC, Japan). After 24 hr incubation at various concentrations of oxygen, cells were fixed with 4 % paraformaldehyde for 20 min at room temperature. Furthermore the cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, and incubated with 100 μ l TUNEL reaction mixture including 1.25 μ g/ml of Hoechst 33258 for 60 min at 37 °C. After sequential washing with PBS and water, the slides were mounted with Gel/ MountTM (Biomedica corp) and their fluorescent images were viewed

with a microscope (Nikon E600 and Olympus DP70). For the quantitative analysis, we counted the number of TUNEL positive cells in a fixed area (700 x 1000 μm) at the center of each well under the fluorescent-microscope. The number of TUNEL positive cells was counted by an observer blind to the treatment condition and the counts were normalized to the total cell number (Hoechst 33258-stained nucleus).

Immunocytochemistry

The effect of oxygen concentrations on the differentiation of NSCs into neurons and astrocytes was evaluated by immunocytochemical staining for Tuj-1 and GFAP, respectively after differentiation was induced by a low concentration (1 %) of fetal bovine serum (FBS) (Studer et al. 2000). Neuronal subtypes were also evaluated by immunocytochemical staining for GABA, glutamate, tyrosine hydroxylase (TH) and serotonin. After primary neurospheres were dissociated into the single cell suspensions in 1% FBS-containing N-2 medium lacking EGF in Lab-Tek[®] Chamber Slide[™], the NSCs were incubated for 2 days at various concentrations of oxygen (0, 1, 2, 3, 4, 10 and 20%) in humidified portable isolation chambers described above. The differentiated cells were fixed in PBS containing 4 % paraformaldehyde for 30 min at room temperature. Cells were incubated overnight with anti-Tuj-1 antibody (1:200; mouse monoclonal antibody, Sigma), anti-GFAP antibody (1:80; rabbit polyclonal antibody, Sigma), anti-GABA antibody (1:500; rabbit polyclonal antibody, Sigma), anti-Glutamate antibody (1:2000; rabbit polyclonal antibody, Sigma), anti-TH antibody (1:200; rabbit polyclonal antibody, Chemicon) and anti-Serotonin antibody (1:500; rabbit polyclonal antibody, Progen) in PBS containing 0.1 % Triton X-100 and 3 % normal goat serum at 4 °C. After removal of the primary antibody solution, cells were

washed with PBS three times and incubated with two secondary antibodies (Alexa Fluoro[®] 488 goat anti-mouse IgG, 1:200 and Alexa Fluoro[®] 568 goat anti-rabbit IgG, 1:200, Molecular Probes Inc., Eugene, OR) and 1.25 µg/ml of Hoechst 33258 for a nuclear counter staining for 4 hr at room temperature under light-shading conditions. After sequential washing with PBS and water, the slides were mounted with Gel/Mount[™] (Biomedica corp, Foster City, CA). For the quantitative analysis, we counted the number of Tuj-1-immunoreactive neurons, GFAP-immunoreactive astrocytes, GABA-immunoreactive neurons, glutamate-immunoreactive neurons, TH-immunoreactive neurons and serotonin-immunoreactive neurons in a fixed area (700 x 1000 µm) at the center of each well under the fluorescent-microscope (Nikon E600 and Olympus DP70). The number of immunoreactive cells was counted by an observer blind to the treatment condition and the counts were normalized to the total cell number (Hoechst 33258-stained nucleus).

Statistical analysis

Results are shown as the mean \pm S.E.M. Statistical difference was analyzed by One-way ANOVA followed by Fisher's PLSD test. Results were considered to be significant when the p value was less than 0.05.

Results

The effects of oxygen concentrations on the proliferation of the NSCs

When NSCs were treated with BrdU for 6 hr at 20% concentration of oxygen, the

proportion of BrdU-labeled cells was $10.3 \pm 0.8\%$ under our experimental conditions (Fig. 1). When compared to 20 % oxygen concentration, which is usually used in the traditional culture method, 10 %, 4 %, 3 %, 2 % and 1 % oxygen concentrations, but not 0% oxygen concentration, significantly increased the proportion of BrdU-labeled cells in the NSCs. The highest amount of proliferation was observed at 2% oxygen ($28.3 \pm 1.3\%$), which was significantly more than at 4% oxygen concentration ($24.3 \pm 0.7\%$) (Fig. 1).

The effects of oxygen concentrations on the cell death of the NSCs

With 20% oxygen concentration, the proportion of TUNEL-positive cells was $9.1 \pm 1.9\%$ under our experimental conditions (Fig. 2). The proportion of TUNEL-positive cells increased at lower oxygen concentration in a concentration-dependent manner (Fig. 2). When compared to 20 % oxygen concentration, 3 %, 2 %, 1 % and 0 % oxygen concentration significantly increased the cell death of the NSCs (Fig. 2).

The effects of oxygen concentrations on the differentiation of the NSCs

In the next series of experiments, we examined the effect of oxygen concentrations on the differentiation of NSCs. Two days after EGF withdrawal from N-2 medium and the concurrent addition of 1% FBS at 20% oxygen concentration, NSCs yielded abundant GFAP-immunoreactive astrocytes ($29.6 \pm 3.1\%$) and a small number of Tuj-1-immunoreactive neurons ($4.8 \pm 0.5\%$) (Fig. 3). However, when NSCs were differentiated at 3 %, 2 % and 1 % of oxygen concentrations, they produced significantly more Tuj-1-immunoreactive neurons when compared with culturing at

20% oxygen concentration (Fig. 3). The highest proportion of Tuj1-positive cells was observed at 2% oxygen. More interestingly, the tendency for NSCs to differentiate into Tuj-1-immunoreactive neurons was significantly higher in 2% oxygen concentration than in 4% oxygen concentration (2 % oxygen: 15.1 ± 1.0 %, 4 % oxygen: 7.9 ± 0.4 %; $p=0.04$). Moreover, when compared to 20 % oxygen, much lower oxygen concentration (0% and 1%) did not affect the proportion of Tuj-1-immunoreactive neurons (3.8 ± 0.7 % and 10.9 ± 2.3 % respectively). On the other hand, there was no difference in GFAP-positive cells between 20 % oxygen and any oxygen concentration examined (Fig. 3). The proportion of GFAP-positive cells significantly decreased in 0 % oxygen when compared to 3 % and 2 % oxygen concentrations (Fig. 3).

We next examined the effects of oxygen concentrations on neuronal subtype differentiation of the NSCs. As shown in Fig. 4 (A) and 4 (B), the proportion of GABA-positive neurons was significantly decreased in lower oxygen concentrations (10 %, 4 %, 3 %, 2 %, 1 % and 0 %) as compared to the 20 % oxygen concentration. On the other hand, the proportion of glutamate- positive neurons was significantly increased in lower oxygen concentrations (3 %, 2 %, 1 % and 0 %) as compared to the 20% oxygen concentration (Fig. 4 (C, D)). We could not find any TH-positive neurons or serotonin-positive neurons in our experimental condition (data not shown).

Discussion

In the present study, we demonstrated that oxygen concentration is critical for the proliferation and neural differentiation, as well as, cell death of the NSCs. The proliferation and neuronal differentiation of NSCs was enhanced under lowered oxygen

conditions, especially at a concentration of 2 %, which yielded significantly higher proportions of both BrdU-labeled cells and Tuj1-positive cells when compared with 20 % and 4 % oxygen. Also, the switching of the neuronal subtype differentiation from GABA-positive to glutamate-positive neurons was observed in lowered concentration of oxygen. Furthermore, these hypoxia-induced changes in the NSC functions were observed in the range of physiological and pathophysiological concentrations of oxygen (4 - 0 %). These findings raise the possibility that hypoxia might mediate the enhancement of proliferation and neural differentiation of the NSCs triggered by cerebral ischemia.

The lowered oxygen-induced enhancement of the NSC activity observed in our present study was consistent with previous reports demonstrating that the growth of neural crest stem cells, neuronal progenitor cells or mesencephalic precursor cells is enhanced in lowered oxygen (3-5%) when compared with high oxygen concentration (20%) (Morrison et al. 2000; Shingo et al. 2001; Storch et al. 2001; Studer et al. 2000). Thus, it seem to be common phenomenon that proliferation and neural differentiation of the NSCs or neuronal progenitor cells is higher under physiological oxygen than under non-physiologically high oxygen. However, these previous studies compared the effects of non-physiologically high and physiologically normal oxygen concentrations on NSC activities. In this study, we further examined the effects of much lower concentrations of oxygen (0, 1, 2, 3 and 4%), which occur physiologically and pathophysiologically in the brains *in vivo* (Harris et al. 1987; Liu et al. 2004; Studer et al. 2000). As previously described, we demonstrated that the proliferation and neural differentiation of NSCs was highest in the 2 % oxygen condition, which occurs in the penumbra, when compared to the other oxygen concentrations examined in this study including 4 %

oxygen, which is thought to occur in the normal brain. To our knowledge, this is the first report demonstrating that NSC proliferation and differentiation is enhanced under pathophysiological oxygen conditions occurring in cerebral ischemia. Since an interstitial oxygen concentration is reported to fall into 1 - 2 % in the ischemic penumbra (Harris et al. 1987; Liu et al. 2004; Studer et al. 2000), ischemia-induced facilitation of neurogenesis in the penumbra may be attributed to the direct actions of lowered oxygen concentration on the activities of the NSC.

In marked contrast to 2 % oxygen, 0 % oxygen failed to affect the proliferation and the neural differentiation of the NSCs, and rather increased the cell death approximately 4 fold when compared with 20 % oxygen concentration. This is different from the previous report suggesting that hypoxia reduced apoptosis of the NSCs derived from mesencephalon (Studer et al. 2000). This difference may be attributed to the regional brain differences in vulnerability against hypoxia/ischemia (Pulsinelli et al. 1982; Smith et al. 1984). Thus, the activation of ganglion-derived NSC induced by lowered oxygen is determined by the balance between proliferation followed by neural differentiation, and cell death. Since an interstitial oxygen concentration is reported to fall to around 0 % in the core region (Harris et al. 1987; Liu et al. 2004; Studer et al. 2000), ischemia-induced decrease in neurogenesis in the core region may be attributed to the direct actions of anoxia on the NSC activities. Ohta and colleagues showed that the number of oligodendrocyte progenitor cells increased in the ischemic penumbra after the ischemic insult, while decreasing in the ischemic core (Ohta et al. 2003). These phenomena observed *in vivo* supports our hypothesis that the ischemic penumbra environment may enhance NSC proliferation and neuronal differentiation.

It should be noted that the neuronal subtype differentiated from the NSCs switched from GABA-positive to glutamate-positive neurons under lowered oxygen concentrations. Similar to our findings, Studer and colleagues also demonstrated that neuronal precursors from E12 rat mesencephalon, which had been expanded in lowered oxygen (3%), differentiated into more dopaminergic and serotonergic neurons, while the differentiation to GABAergic and glutamatergic neuron was reduced under lowered oxygen conditions (Studer et al. 2000). Morrison and colleagues also reported lowered oxygen concentrations under 3-5% promotes dopaminergic differentiation from neural crest stem cells. Moreover, these differentiated neurons in low oxygen conditions could also synthesize and release dopamine and norepinephrine (Morrison et al. 2000). Storch and colleagues also reported the dopaminergic differentiation from human mesencephalic neural precursor cells in low oxygen conditions (Storch et al. 2001). These reports suggest that the oxygen reduction from non-physiological high condition to physiologically normal condition drastically enhances neuronal, especially projective neuronal, differentiation such as dopaminergic differentiation of CNS precursors *in vitro*. In the present study, the NSCs derived from the E15.5 ganglionic eminence failed to differentiate into dopaminergic or serotonergic neurons (Studer et al. 2000). Further study is required to elucidate the mechanisms underlying the hypoxia-induced switching of the neural subtype differentiation.

As described previously, hypoglycemia is another main factor mediating ischemic actions and both hypoxia and hypoglycemia additionally or synergistically contribute to the acute and delayed neural death (Siesjo 1992). Recently, we have shown that lowered glucose concentration suppresses the proliferation and increases the neuronal and astroglial differentiation of cultured NSCs (Horie et al. 2004). Thus, there are

some differences and some similarities in the effects on the NSC activities between hypoxia and hypoglycemia (Horie et al. 2004). The apparent difference is the effect on the proliferation; an increase following lowered oxygen and contrariwise a decrease following lowered glucose utilizing the same cells (derived from E15.5 ganglionic eminence) and culture conditions held constant. Another difference is that lowered glucose facilitated the differentiation into astrocytes, while lowered oxygen had little effect on it. A similar effect occurring with both lowered oxygen and lowered glucose concentration is the enhancement of the neuronal differentiation from NSCs. Thus, these two energy failures under ischemia have different effects on NSC proliferation and differentiation. The ischemia-induced activation of the endogenous NSCs is caused by the additional and synergic actions of the two main incidents; hypoxia and hypoglycemia. Because it is reported that hypoxia and hypoglycemia lead to distinctly different patterns of the intracellular pH (Knopfel et al. 1998) and gene expression (Brusselmans et al. 2001), these differences may explain the different actions on the NSC functions.

In conclusion, our study provides evidence that lowered oxygen concentration, known to occur under cerebral ischemia, facilitates NSC proliferation and differentiation, and that pathophysiological oxygen concentration (2%) in penumbra is found to be optimal for NSC to proliferate and neural differentiate into neurons.

Acknowledgements

The authors thank Anne-Lise Maag and William Slikker (Department of Neurosurgery, Stanford University) for editing the manuscript.

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Figure legends

Fig.1. The effects of oxygen concentrations on the proliferation of cultured NSCs. NSCs were treated with BrdU (10 μ M) for 6 hr under various oxygen concentrations and were processed for BrdU immunocytochemistry. The representative immunofluorescence images (A) and the proportions of BrdU-labeled cells (B) are shown. Arrows in (A) indicated the BrdU-labeled cells. When compared to 20 % oxygen, 10 %, 4 %, 3 %, 2 % and 1 %, but not 0% oxygen, significantly increased EGF-induced proliferation. The highest proliferation was observed at 2% oxygen. The asterisks indicate significant difference compared to 20 % ($p < 0.05$; One-way ANOVA followed by Fisher's PLSD test). $n = 8$

Fig.2. The effects of oxygen concentrations on cell death of the cultured NSCs. After incubation under various oxygen concentrations, NSCs were processed with the TUNEL assay and the representative immunofluorescence images (A) and the proportions of dead cells (B) are shown. Arrows in (A) indicated the TUNEL-positive cells. The low oxygen culture significantly increased the proportion of dead cells. The asterisks indicate significant difference compared to 20 % ($p < 0.05$; One-way ANOVA followed by Fisher's PLSD test). $n = 8$

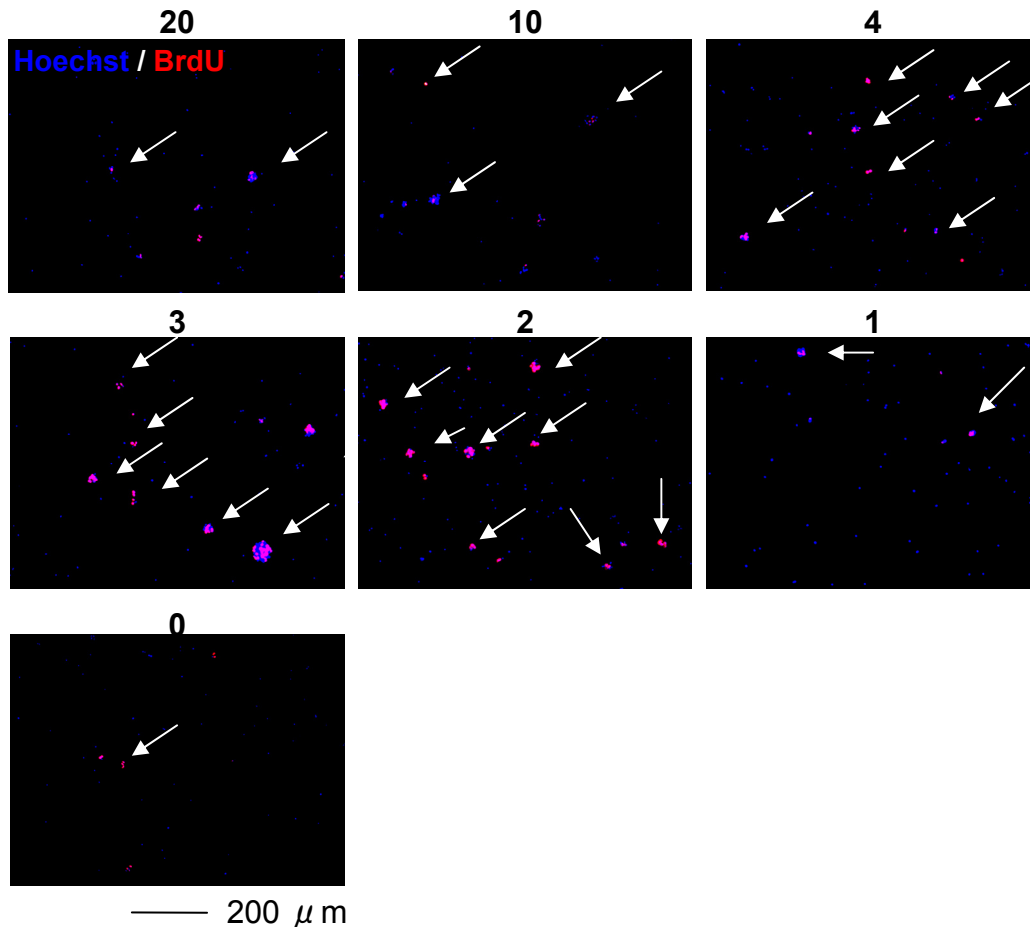
Fig.3. The effects of oxygen concentrations on the differentiation of cultured NSCs. After the NSCs were differentiated under various oxygen concentrations, they were processed for immunocytochemistry against a neuronal marker, Tuj1 and an astrocyte marker, GFAP, and the representative immunofluorescence images (A) and the

proportion of Tuj1- (B) and GFAP- (C) positive cells are shown. When compared to 20 % oxygen, 3 %, 2 % and 1 % significantly increased the proportion of Tuj1-positive cells. The highest proportion of Tuj1-positive cells was observed at 2% oxygen. In contrast, the proportion of GFAP-positive cells significantly decreased in 0 % oxygen when compared to 3 % and 2 % oxygen concentrations. The asterisks indicate significant difference compared to 20 % ($p < 0.05$; One-way ANOVA followed by Fisher's PLSD test). $n = 6$

Fig.4. The effects of oxygen concentrations on neuronal subtype differentiation of the cultured NSCs. After the NSCs were differentiated under various oxygen concentrations, they were processed for immunocytochemistry against a neuronal marker, Tuj1 and GABA (A, B) or glutamate (C, D). The representative immunofluorescence images of Tuj1 and GABA (A) and of Tuj1 and glutamate (C) are shown. The proportion of GABA-positive cells (B) and glutamate positive cells (D) against the number of Tuj1-positive cells are also shown. Arrows in (A) and (C) indicated the double-positive cells. The proportion of GABA-positive neurons was significantly decreased in lower oxygen concentrations, whereas the proportion of glutamate-positive neurons was significantly increased in lower oxygen concentrations. The asterisks indicate significant difference compared to 20 % ($p < 0.05$; One-way ANOVA followed by Fisher's PLSD test). $n = 8$

(A)

Oxygen concentration (%)



(B)

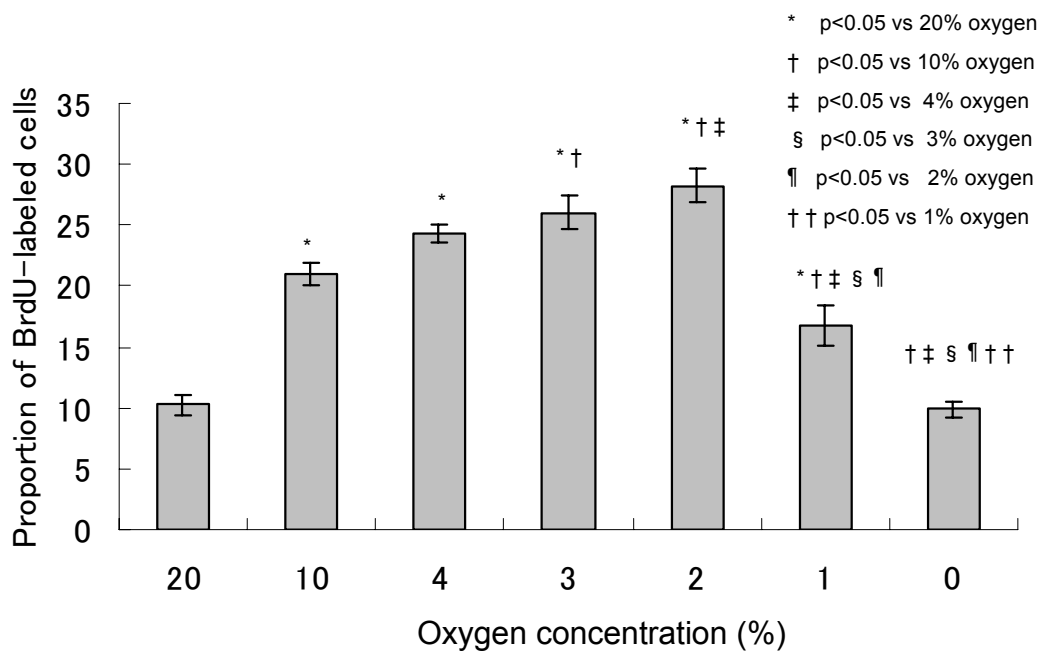


Fig. 1

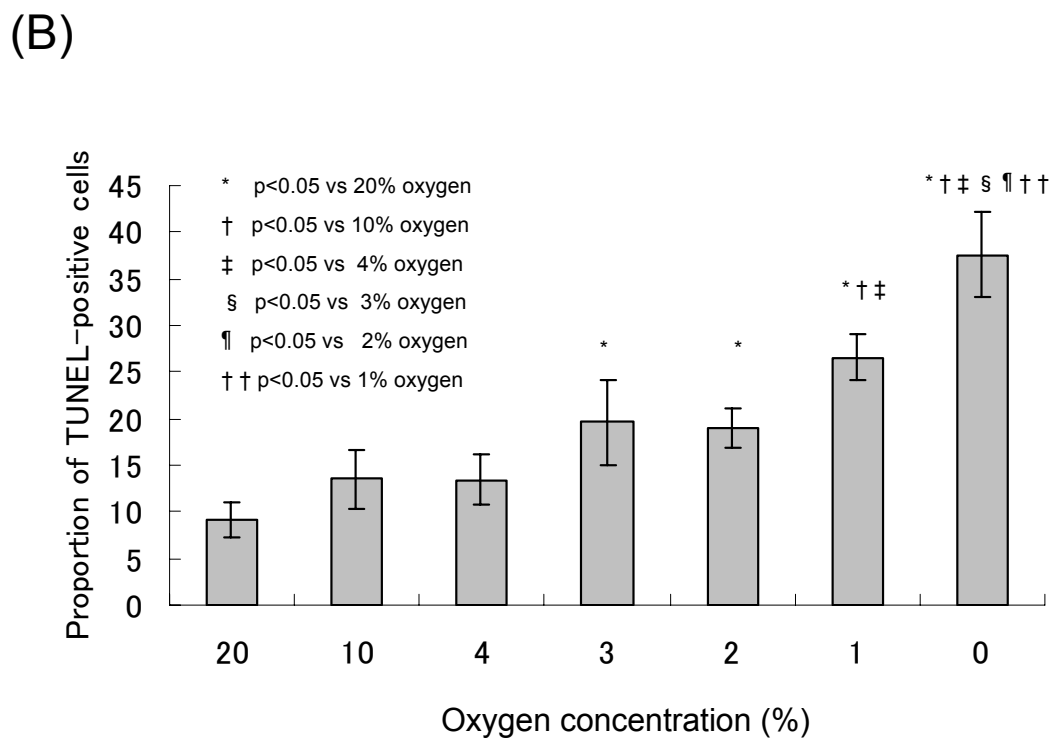
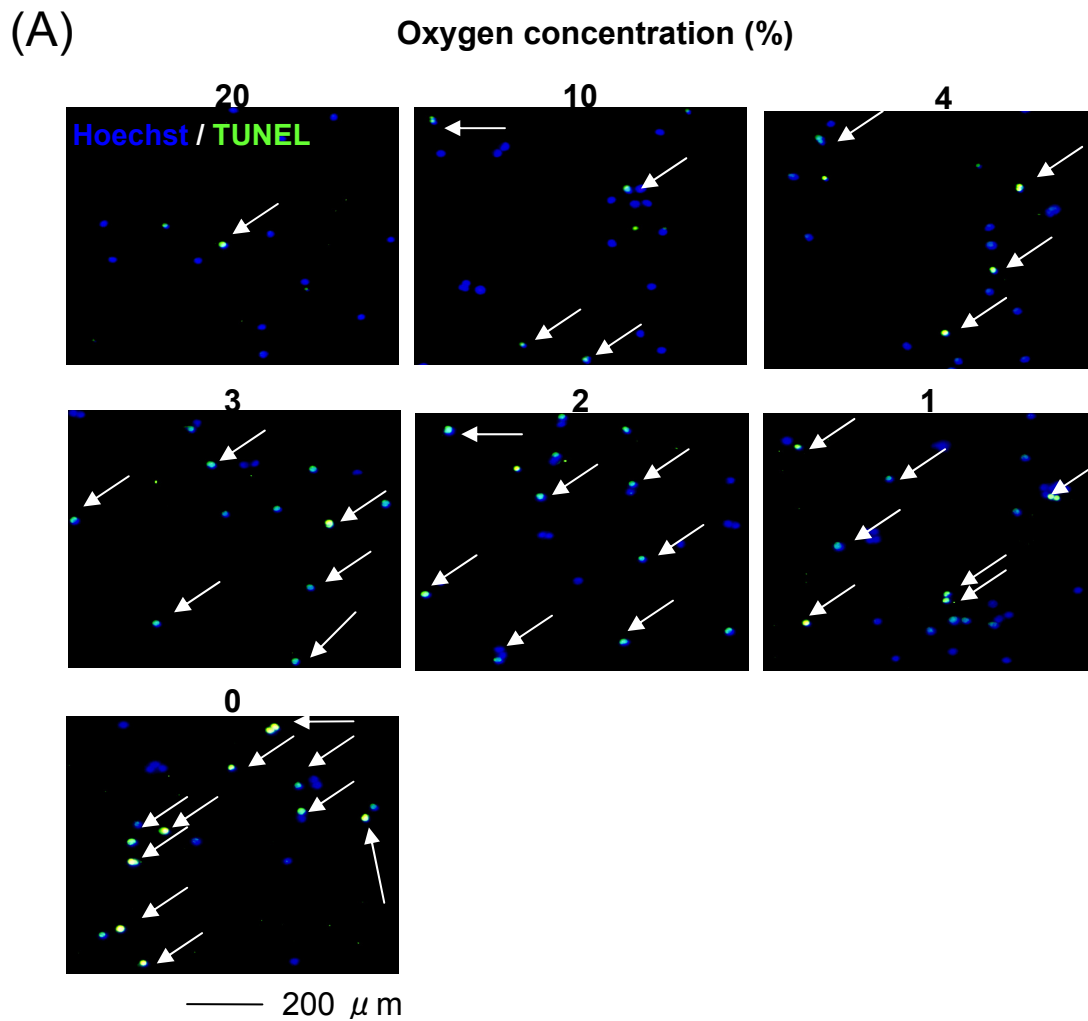
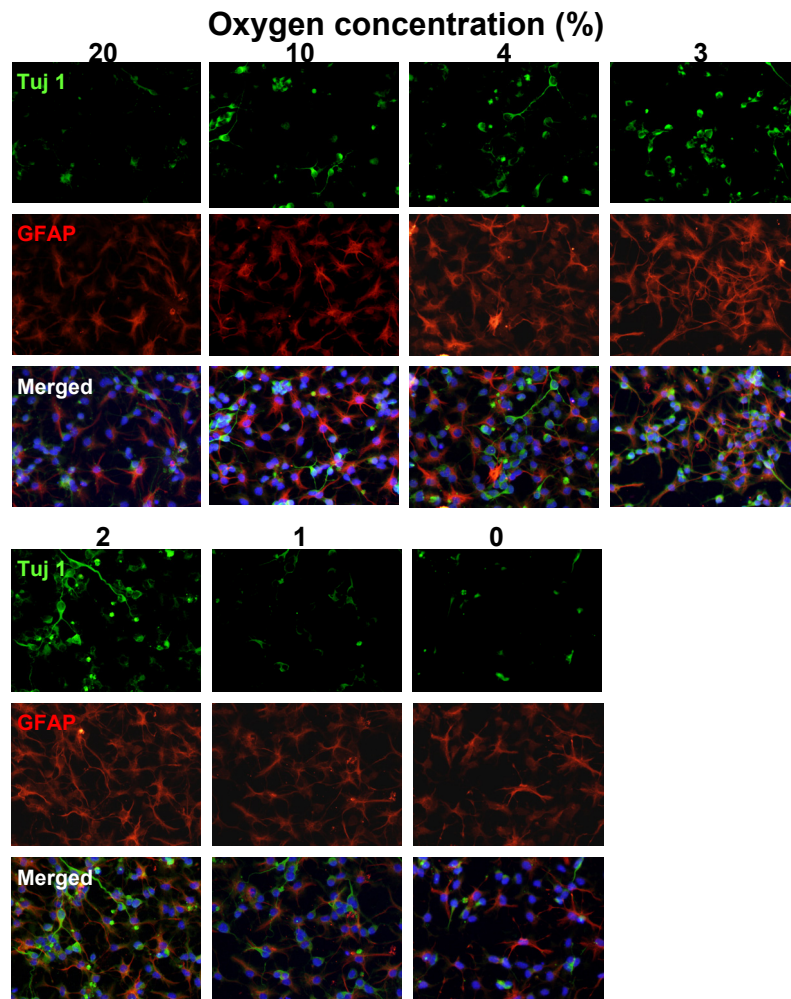
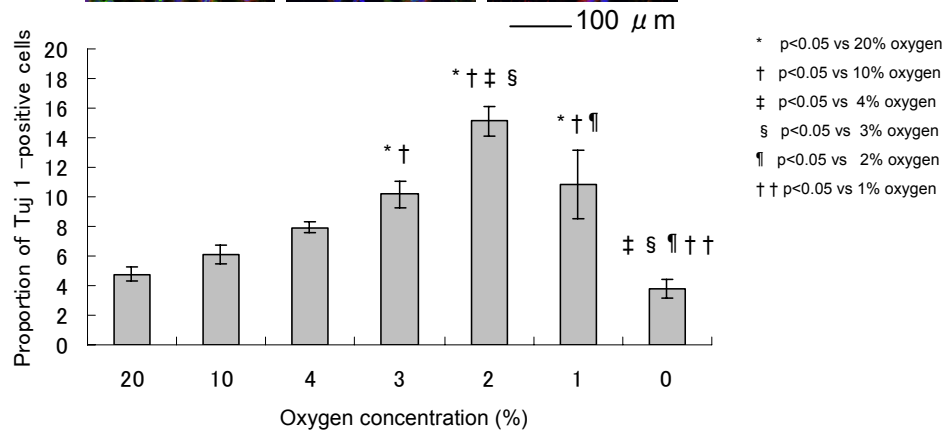


Fig. 2

(A)



(B)



(C)

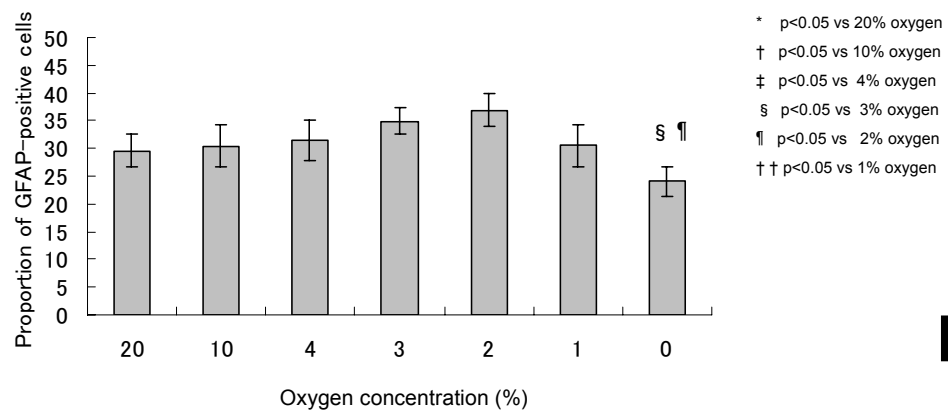


Fig. 3

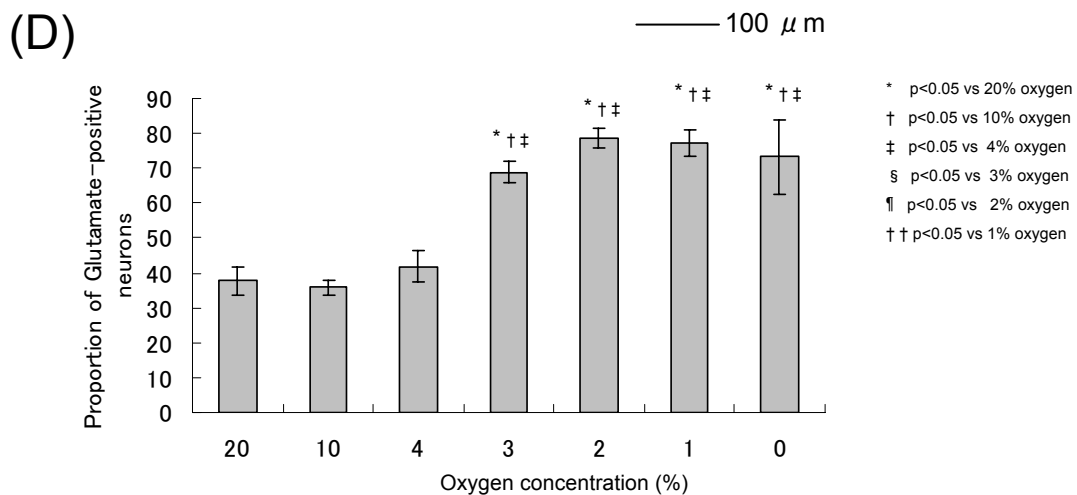
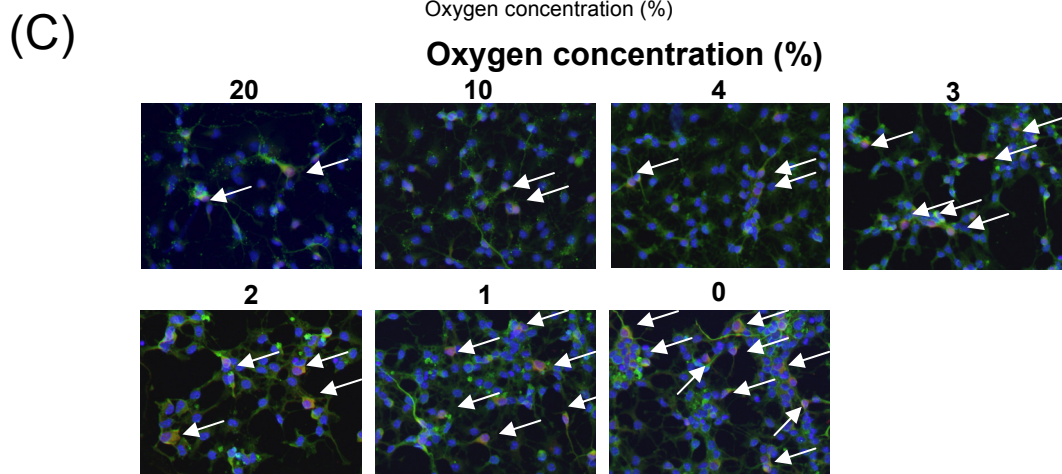
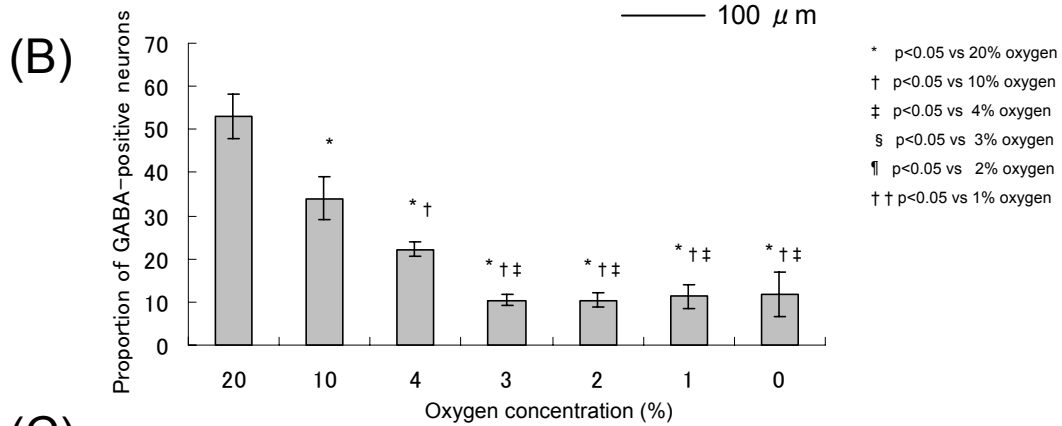
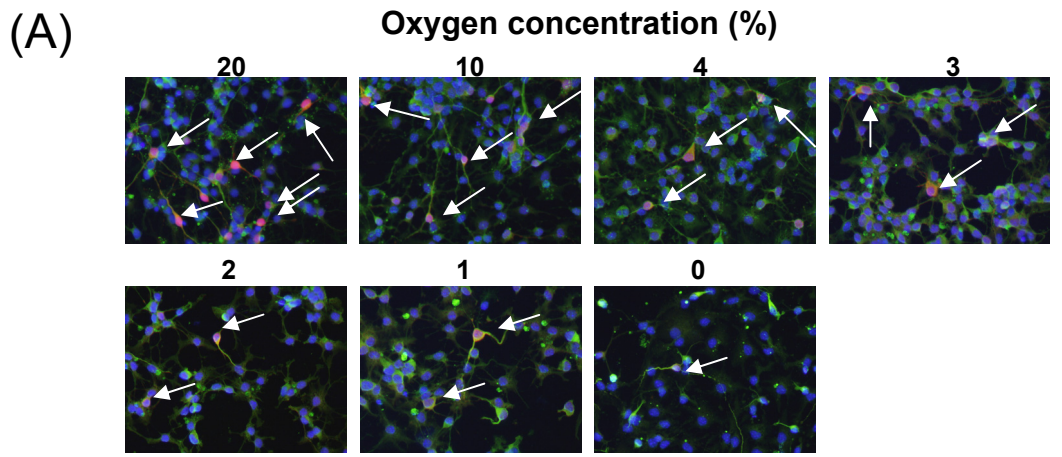


Fig. 4