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Manipulation of neural progenitor fate through the oxygen sensing pathway

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Abstract

Neural progenitor cells hold significant promise in a variety of clinical settings. While both the brain and spinal cord harbor endogenous neural progenitor or stem cells, they typically are not capable of repopulating neural populations in case of injury or degenerative disease. *In vitro* systems for the culture of neural progenitors has come a long ways due to advances in the method development. Recently, many groups have shown that manipulation of the oxygen-sensing pathway leading to activation of hypoxia inducible factors (HIFs) that can influence the proliferation, differentiation or maturation of neural progenitors. Moreover, different oxygen concentrations appear to affect lineage specification of neural progenitors upon their differentiation *in vitro*. Here we summarize some of these studies in an attempt to direct effort towards implementation of best methods to advance the use of neural progenitors from basic development towards clinical application.

Neural Progenitor Cells

Neural progenitor cells (NPCs) can be derived from a variety of sources. Neural stem cells (NSCs) reside in the subventricular and subgranular zones of mice [1–3], and can be isolated and expanded in well-established procedures that take advantage of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) to promote their proliferation. Cultured neural stem cells retain tri-lineage potential to differentiate into neurons, astrocytes, and oligodendrocytes and can be maintained for many generations in either neurosphere or adherent culture conditions [4, 5]. To isolate human neural progenitors, it is possible to

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derive them from human fetal brain or spinal cord tissue, or specify them from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) [6, 7]. Human neural progenitors are also thought to have tri-lineage potential and are also maintained in by addition of EGF and bFGF. Both murine and human neural progenitors share transcriptional profiles as well as epigenetic and metabolic features, despite being isolated from different contexts. This is not to say that they are identical, there are important differences outlined elsewhere. Here, we will describe how manipulation of HIF signaling affects the proliferation, specification or differentiation of neural progenitors, which we suspect will prove to be useful for both regulating the basic development of these cells as well as someday their clinical application.

Oxygen tension and embryonic development

Oxygen is a key environmental factor that controls developmental process, tissue homeostasis, and cellular metabolism. Majority of *in vitro* cell based studies are performed under atmospheric oxygen, with 95% air (contains 78% nitrogen and 21% oxygen) supplemented with 5% of carbon dioxide, resulting a final oxygen concentration to be 20%. However, the oxygen concentration in human tissues and organs is thought to be much lower than the atmospheric oxygen level. In fact, the oxygen level within adult human is heterogeneous, with 14.5% O_2 in alveoli, 12% O_2 in arterial blood, 5.3% O_2 in venous blood, and 1.1% to 9.5% O_2 in various other tissues [8]. Multiple studies suggested that the oxygen level in the human brain ranges from 2% to 4.4%, depending on the brain region and sample depth [8, 9].

In fact “Hypoxia” (less than 5% oxygen) is physiologically normoxia for the developing embryos. Before the establishment of utero–placental circulation in the second trimester, the oxygen level in uterine surface is around 2.36% O_2 [10]. As a result, embryogenesis before 10 weeks of gestation occurs under low oxygen [11]. The importance of low oxygen in mammalian nervous system development was first demonstrated by Morriss and New using *ex utero* rat embryos. Cultured E9.5 rat embryos underwent normal cranial neurulation at 5% O_2whereas high oxygen condition (20% O_2 or 40% O_2) resulted in abnormal morphogenesis of neural folds and failure of neural tube closure [12].

The oxygen-sensing pathway

Hypoxia-inducible factors (HIFs) belong to bHLH-PAS (basic Helix-Loop-Helix-per-Arnt-Sim) family of transcription factors that regulate cellular response to low oxygen. Besides oxygen sensing, they play a crucial role in regulating oxygen consumption, glucose uptake, metabolism, and development [13–16]. HIFs are heterodimeric transcription factors that are composed of an alpha-subunit (HIF1α, HIF2α, and HIF3α), and a constitutively expressed beta-subunit (HIF1β, also called aryl hydrocarbon receptor nuclear translocator, ARNT) [17–20]. The protein stability of alpha-subunit is regulated by prolyl hydroxylase domain proteins (PHD1–3, also known as EGLN1–3) in an oxygen-dependent manner. As a result, HIF-α subunits direct oxygen sensing in a linear range from 0.1 to 21% O_2 [21]. Specifically, when oxygen is abundant, HIF1α and HIF2α are hydroxylated by PhDs in the presence of Fe^{2+}. Hydroxylated HIF-α subunit is recognized by von Hippel-Lindau (VHL)

tumor suppressor protein, a recognition component of E3 ubiquitin ligase complex. Upon VHL binding, the HIF-α subunit is targeted for ubiquitination and rapid proteasomal degradation [22]. When O_2 concentration is less than 5%, decreased O_2 diminishes the enzymatic activity of PHDs. As a result, HIF1α and HIF2α proteins are stabilized, translocate into nucleus and dimerize with HIF1β subunit. The heterodimer then recruits the coactivators of P300/CBP, and bind to hypoxia response element (HRE) to transactivate specific target genes [23–25].

Besides activation by hypoxia, the PHD-HIF signaling pathway can be regulated by other micro-environmental factors, such as iron and TCA cycle intermediates. The hydroxylation of HIF-α subunit by PHD requires iron and ascorbate as cofactors. Thus, PHD enzymatic activity is affected by iron availability. Because oxygen and α-ketoglutarate (α-KG, also known as 2-oxoglutarate, or 2OG) are substrates, and succinate is the product of the hydroxylation reaction, the PHD activity can be inhibited by intracellular accumulation of succinate, or TCA cycle intermediates such as fumarate and malate, which serve as competitive inhibitors of α-KG [26, 27].

HIF in embryonic development

The importance of HIF pathway in development was demonstrated by the early embryonic lethality (E9.5–10.5) in both *Hif1α* and *Hif1β* deficient mice (see review [11, 16]). In fact, abrogation of HIF activity impaired vascularization of the placental [28–30], cardiovascular morphogenesis and angiogenesis [31], heart development [32–34], and endochondrial bone formation during early embryogenesis [16, 35, 36]. In terms of nervous system development, HIFs are required for neural crest cell migration [37]. *Hif1α* and *Hif1β* knockout mice had abnormalities such as forebrain hypoplasia and neural fold closure defects [29, 38]. Furthermore, neural-specific *Hif1a*-deficient mice exhibited hydrocephalus, reduction in neuronal cells, and impaired spatial memory [39]. Those studies demonstrated the indispensible role of HIF in embryonic brain development.

Methods to stabilize HIF

Manipulating Oxygen concentration

Manipulating oxygen concentration is the most straightforward and physiologically relevant way to stabilize HIF in neural stem cells. One way to establish a hypoxic cell culture environment is to use a multigas incubator with an oxygen sensor. Because atmospheric oxygen concentration is 21%, nitrogen is needed to decrease the oxygen level. One limitation of this method is that once the incubator door is opened, air containing high-level of oxygen flows in. In that case, the hypoxic environment is temporarily disturbed and has to be re-established. The fluctuation of oxygen concentration during cell culture and sample collection could cause inconsistency in experiments, and even false negative results. Moreover, HIF1α protein can be degraded within 5 minutes at atmospheric oxygen due to its inherent instability. Thus, hypoxic sample collection needs to be performed promptly. Another practical limitation is that the oxygen level can be set at just one concentration for an entire incubator. Thus, it requires multiple incubators to test multiple oxygen concentrations simultaneously.

Commercially available “sub-chamber” systems and “hermetical hypoxic workstations” can solve the problem of oxygen fluctuation. The “sub-chamber” includes a sealed secondary container that can transfer the cell culture plate, and an oxygen controlled hood that allow sample collection and cell feeding under low oxygen environment. An updated version is hermetical hypoxic workstation, which is a fully sealed, oxygen-controlled environment that contains an incubator for cell culture, and a glove box for experimental procedures. Thus, this system allows for complete manipulation of cells in hypoxic environment. Nuclear translocation of HIF1α by immunostaining can be used as an indicator for successful establishment of hypoxia. In our experience, HIF1α nuclear translocation can be seen in NPCs after 6 hours in 5% O_2. Lowering the oxygen to 2% resulted in a an even more robust HIF1α nuclear translocation [40].

Small molecules

Small molecules that activate HIF provide a cost-effective way to mimic hypoxia without having to physically establish a low oxygen environment. Small molecules can stabilize HIF by targeting the PHD-mediated HIF degradation pathway.

Deferoxamine (DFO)—DFO stabilizes HIF through inhibition of PHD by chelating the cofactor Fe^{2+} [41, 42]. DFO was the first FDA approved iron-chelating compound for the treatment of iron overload. Thus, the long-term outcome and side effects for DFO are well-characterized in patients. Significant side effects include local skin irritation, ophthalmological and auditory problems, and neurological symptoms at high doses [43]. In human NPCs, HIF1α nuclear translocation can be seen after just 6 hours of DFO treatment by immunostaining [40]. The effective dose of DFO ranges from 50 uM to 150 uM and the best result can be seen 24 hours after treatment. However, extended DFO treatment inhibited cell proliferation in NPCs. After treatment with 50 uM, 100 uM or 200 uM of DFO for 72 hours, the viability of NPCs were decreased by 40% to 70% in a dose-dependent manner [44]. Interestingly, DFO has been shown to have neuroprotective properties in several disease models. In an ischemia study using mouse model, pretreatment of DFO protected hippocampal neurons from cell death by activating HIF-1α signaling [45]. In another study, DFO administration after cerebral ischemia decreased brain damage and promoted functional recovery in rats [46]. Moreover, DFO was shown to ameliorate pathological symptoms and improve behavioral outcomes in mice with Parkinson’s disease (PD). Accumulation of iron in the Substantia Nigra (SN) is one of the defining characteristics of PD. Intranasal DFO treatment decreased iron-positive cells in the SN, prevented the degeneration of dopaminergic neurons, and efficiently alleviated behavioral deficits in the PD mouse [47].

Dimethyloxalylglycine (DMOG)—DMOG is an analogue of α-KG, the substrate of prolyl-4-hydroxylase. Thus, DMOG stabilizes HIF by inhibition of PHD by competition with its substrate [48, 49]. In our NPC cultures, 200 uM to 250 uM of DMOG was sufficient to induce nuclear translocation of HIF1α. In addition, the growth inhibition of DMOG at 250 uM is less than that of 100 uM DFO in human ESC or iPSC-derived NPCs. Milosevic et al reported that the viability of midbrain-derived human NPCs was decreased by 20% in 250 uM DMOG compared with untreated cells [44]. Similar to DFO, DMOG has shown

neuroprotective function after traumatic brain injury through HIF activation and subsequent angiogenesis [50].

Cobalt (II) Chloride ($CoCl_2$)—$CoCl_2$ inhibits PHD enzymes by replacement of Fe^{2+} from the catalytic core [42, 51]. In NPCs, 50 uM of $CoCl_2$ is sufficient to stabilize HIF1α protein under atmospheric oxygen and viability is not affected by up to 100 uM $CoCl_2$ [44]. Interestingly, $CoCl_2$ treated NPCs led to Increased numbers of TH^+ dopaminergic neurons upon differentiation for 1 week [44].

Other PHD inhibitors—FG-4497 and Ciclopiroxolamine (CPX, or FG-2229) stabilize HIF1α by blocking the active site of PHDs as well as chelating Fe^{2+} [52]. 10 uM of CPX can stabilize HIF1α protein but CPX is toxic to NPCs even at 1 uM, resulting in a 70% reduction of cell number in 72 hours [44]. On the other hand, 5 uM to 10 uM of FG-4497 is sufficient to stabilize HIF1a in 10–14 week fetal-derived mesencephalic NPCs [44]. FG-4497 is not cytotoxic and was shown to increase the number of NPCs by 40% after 72 hours. This treatment also increased dopaminergic differentiation of NPCs and protected dopaminergic neurons from MPP+ induced degradation [44].

Genetic activation of HIF

The recognition of HIF by pVHL depends on hydroxylation of several conserved proline residues in the HIF1α and HIF2α protein [53–55]. Mutating those residues to alanine, such as P564A [54], P577A/P402A [56], P577A/P402A/N813A [57], P402A/P564A in HIF1α, and P530A/P405A [56], P405A/P531A [58] in HIF2α, disables the VHL targeted degradation of HIF protein, and thus stabilizes HIF protein under atmospheric oxygen. Specifically, cells can be transfected (or infected) with the plasmids that express non-degradable HIF protein under control of a tetracycline-regulated promoter. Upon doxycycline induction, a rapid expression of HIF mRNA can be detected 4 hours after induction. Maximal mRNA levels can be reached at 20 hours post doxycycline-induction and the high levels of HIF mRNA can be sustained for at least 48 hours [56]. One limitation of this method is the low transfection/infection efficiency in human NPCs, making it difficult to get homogeneous HIF-overexpression. Moreover, one should keep in mind that the endogenous HIF1α mRNA expression level is not affected by doxycycline induction.

Whole animal hypoxia

While *in vitro* tissue culture provides the flexibility for manipulation, whole animal hypoxia model could be a more physiologically relevant method to study development and diseases. For example, chronic hypoxemia caused by chronic obstructive pulmonary disease (COPD) or chronic altitude sickness can lead to serious neurological complications. Wistar rats exposed to 10% O_2 for 10 days develop a syndrome similar to the blood oxygen tension in COPD patients, and thus can be used as an animal model to study COPD caused neurological disorders [59]. A hermetically sealed chamber with O_2 control can be used to establish the hypoxic environment for the whole animal hypoxia study. Most animals cannot survive over 15 minutes in hypoxic environment (5% O_2 or less). However, a recent study by Park et al reported that the African naked mole-rat can tolerate 5% O_2 for 5 hours. It even survived 18 minutes of anoxia (0% O_2) without apparent injury [60]. The authors discovered

that those naked mole-rat switched to anaerobic metabolism that used fructose to fuel vital organs like brain and heart during anaerobic periods [60]. Therefore, the naked mole-rat is an important model to study the whole animal's response to extreme low oxygen going forward.

Effects of oxygen tension on NSC proliferation and differentiation

Oxygen tension in control of NSC proliferation

The influence of low oxygen on proliferation and self-renewal of neural stem cells has been studied by various groups over the past decade. Different oxygen levels have diverse effects on NPC proliferation (Table 1 and 2). Specially, mild hypoxia (3%-6% O_2) increased the proliferation of both rodent NSCs [61–64] and human NSCs [65–68] compared with the cells cultured in atmospheric (20%) oxygen. Whereas severe hypoxia (1–2% O_2) and anoxia (<1% O_2) decreased the proliferation of primary fetal tissue derived cortical progenitors [66], MYC-immortalized fetal NPCs [68], and human ESC or iPSC-derived NPCs [40]. Interestingly, the NPCs originated from different brain regions responded differently to different oxygen conditions. Storch et al reported that fetal mesencephalic precursors grew well under 2% O_2but failed to proliferate at 20% O_2. The forebrain precursors derived from the same samples, however, expanded equally well under 2% and 20% O_2 [65] (Figure 1).

Oxygen tension in control of NSC differentiation

Mitogens, such as EGF and bFGF, are key factors to maintain viability and pluripotency of NSCs. In the absence of the mitogens, *in vitro*-cultured neural progenitors spontaneously differentiate into neurons, astrocytes and oligodendrocytes. The phenomenon that neurogenesis proceeds gliogenesis *in vitro* is consistent with the established temporal NPC specification in mammalian neocortical development [69, 70]. Mitogen withdrawal initiates NSC differentiation. Oxygen plays an important role in this process to direct NPC specification towards specific lineages as discussed below (Figure 1).

Mild hypoxia to promote neurogenesis and oligodendrogenesis

Upon NPCs differentiation, mild hypoxia (2.5–6% O_2) induces the number of neuronal and oligodendrocyte lineage compared with atmospheric oxygen (Tables 1 and 2). The role of mild hypoxia in promoting neurogenesis was first identified in rat neural progenitors. Studer et al demonstrated that 3% O_2 increased the number of tyrosine-hydroxylase positive (TH+) dopaminergic neurons in *in vitro* cultured rat mesencephalic precursors upon differentiation [61]. In a separate study, Morrison et al demonstrated that mild hypoxia promoted the generation and survival of TH+ and sympatoadrendal-1 (SA-1) positive neurons derived from rat neural crest stem cells in peripheral nervous system [62]. Later studies further demonstrated the pro-neurogenesis effect of mild hypoxia in progenitors derived from embryonic brain tissues of different developmental stages in human [66, 68, 71, 72] and mouse [64, 73].

Mild hypoxia also promotes oligodendrogenesis. When human post-natal subventricular zone neural precursors were induced to differentiation, precursors expanded in 5% O_2 generated 17-fold more galactocerebroside-C positive (GalC+) oligodendrocytes than those

in 20% O_2. When precursors were expanded at 5% oxygen and then differentiated at 20% oxygen, oligodendrocyte maturation was further enhanced 2.5-fold [67]. A similar pro-oligodendrogenesis effect was demonstrated in 2.5% and 5% oxygen using immortalized human fetal cortical precursors [68]. Moreover, transplantation of hypoxia-cultured rat NSCs into the adult rat hippocampus showed a survival advantage and increased specification towards the oligodendrocyte lineage compared with those cultured in 20% O_2 [63]. Stacpoole et al further demonstrated that 3% O_2 is a suitable culture condition for oligodendrocyte differentiation from human ESCs, and they identified the pathways involved in forebrain oligodendrocyte specification [74].

Severe hypoxia promotes astrogliogenesis

While mild hypoxia enhanced NSC proliferation and promoted the generation of neurons and oligodendrocytes, severe hypoxia (1–2% O_2) induced the differentiation of human NSCs towards astrocytes lineage upon growth factor withdrawal [40, 66, 68]. We reported that the ratio of astrocytes to neurons can be strongly induced by 2% O_2 compared with 20% O_2 in human-ESC or human-iPSC derived NPCs [40]. In a another study, 1% or less oxygen promoted astrogliogenesis in human cortical progenitors at the expense of GABAergic neurons and O4+ oligodendrocytes [66], whereas 3% O_2 promoted neurogenesis and oligodendrocyte in those cortical NSCs [66, 68]. The observation that lowered oxygen promotes astrogliogenesis also holds true in mouse neural progenitors. Two separate studies using mouse E11.5 and E13.5 NPCs demonstrated more GFAP+ astrocytes were generated under 2% O_2 compared with 20% O_2 [73, 75]. Furthermore, the astrogliogenesis effect was more significant under 2% oxygen than 5% oxygen [73], supporting the pro-astrogliogenic effect in lower oxygen. It is important to note that the time required to differentiate into astrocytes *in vitro* is typically much longer than that of neurons. Depending on the maturity of NPCs, it typically takes 1 week to generate astrocytes in mouse [73, 75], versus 2–4 weeks to get astrocytes with human NPCs [40, 66, 67]. Thus, in studies with short term of differentiation, GFAP+ astrocytes are not detected [61] (Tables 1 and 2).

It is worth noting that that neural progenitors behave similarly both *in vitro* and *in vivo*. Using whole animal hypoxia model, XA de Tassigny et al demonstrated that prolonged exposure to severe hypoxia (1% O_2) inhibited the survival of newly generated Tuj1+ neurons and O4+ oligodendrocytes in rat subventricular zone, whereas the number of GFAP+ astrocytes was not affected *in vivo* [59]. This finding is consistent with the *in vitro* studies discussed above that severe hypoxia inhibits neurogenesis and oligodendrogenesis, but promotes astrogliogenesis.

The mechanism of HIF signaling in NSC under hypoxia

How does HIF activation lead to NSC proliferation and self-renewal?

In pluripotent stem cells, under mild hypoxia, HIF signaling can induce expression of OCT4 and Wnt/beta-catenin signaling, which are positive regulators of stem cell self-renewal and proliferation. Binding directly to OCT4 promoter, HIF2α can transactivate OCT4 and regulates mouse ESC proliferation and maintenance [76]. Silencing of HIF2α resulted in a significant decrease in hESC proliferation and down-regulation of pluripotency genes, such

as OCT4, SOX2 and NANOG [77]. In neural stem cells, the hypoxia-mediated proliferation is mediated by nuclear orphan receptor TLX. Under low oxygen tension, TLX is recruited to OCT4 promoter to augment its transcription, and thus promotes the proliferation of adult hippocampal neural progenitors [78].

Multiple studies suggested that mild hypoxia enhanced the proliferation and neurogenesis of NSCs through Wnt/β-catenin signaling [66, 79–81]. Activation of Wnt/β-catenin signaling in NSCs promote neurogenesis, whereas inhibition of Wnt signaling by Dkk1 promotes astrogliogenesis [82]. Under low oxygen, HIF-1α enhanced the activation of Wnt/β-catenin pathway and downstream effectors LEF-1 and TCF-1, which led to increased proliferation in NSCs [80]. Ortega et al further demonstrated that mild hypoxia (3% O_2) induced WNT7A expression in radial glia cells (RGCs), resulting in increased proliferation and neurogenesis, whereas severe hypoxia (1% or less oxygen) reduced WNT7A level and diminished RGCs proliferation and neuronal differentiation [66].

HIF signaling in NSC differentiation

Hypoxia mediated neural progenitor survival and neuronal differentiation can also be attributed to the HIF induced soluble factors, such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF). EPO increased dopaminergic neuron differentiation and survival in hypoxic conditions. Addition of EPO in 20% O_2 partially recapitulated the pro-neurogenesis effect under mild hypoxia [61]. VEGF acts as a ‘neurogenic factor’ that significantly augmented the proliferation and neuronal specification of NPCs during embryonic development and adult neurogenesis (see review [83]). Moreover, VEGF and EPO are beneficial to the neuronal survival, migration, and functional recovery in pathological conditions [84, 85]. EPO increases neurogenesis and oligodendrogenesis of subventricular zone precursor cells after neonatal stroke [85]. A separate study showed that transplantation of NPCs into ischemic mouse brain attenuated apoptosis of surrounding neurons by VEGF-mediated neuronal protection [86].

HIF promotes the astrocyte lineage differentiation through diverse mechanisms. Low oxygen induced demethylation of GFAP promoter in a HIF-dependent manner, presumably as part of a program to facilitate the differentiation into astrocytes in both human [87] and mouse NPCs [75]. Notch activation was required for the hypoxia-mediated acquisition of astrogliogenic potential by NPCs [75]. Xie et al demonstrated that 2% O_2 promoted astrogliogenesis through HIF mediated regulating of the evolutionarily conserved LIN28/let-7 axis. Specifically, Lin28 blocks the maturation of *let-7* miRNAs, and let-7 suppresses Lin28 translation [88]. When NPCs mature, the level of LIN28 drops leading to an increase of let-7 miRNAs. Increased *let-7* miRNAs promoted neural progenitors maturation as measured by increased gliogenic potential [90, 91]. Under low oxygen, HIF inhibits *LIN28* transcription through the competitive inhibition of MYC in LIN28 promoter [40]. In previous work, it was also shown that decreased LIN28 enables the generation of let-7 microRNAs, and results in maturation of NPC with increased astrocytes upon differentiation [91]. Furthermore, inhibition of *let-7* blocked the low oxygen mediated astrogliogenesis in NPC stage, further confirming the relationship of HIF and *let-7* in promoting NSC specification towards the astroglial lineage under severe hypoxia [40] [91]. Others have also

shown that *let-7* is known to promote NPC differentiation through the TLX/microRNA-9 circuit [89].

Conclusions

Clearly, oxygen tension and HIF activity can play important diverse roles in the biology of neural stem and progenitor cells. These roles depend strongly on the context in which HIF activity is modulated and the degree of manipulation. In addition, because some of these manipulations are compatible with clinical-grade production of cells, the use of DFO, DMOG, or oxygen regulation will allow for Good Manufacturing Process (GMP)-compatible promotion of proliferation, specification or differentiation as outlined here.

Glossary

DA	Dopamine
DAT	Dopamine transporter
DBH	Dopamine beta-hydroxylase
DCX	Doublecortin
GABA	gamma-Aminobutyric acid
Glu	Glutamate
MAP2	Microtubule-associated protein 2
SA1	Sympathoadrenal lineage-specific marker
TH	Tyrosine hydroxylase
Tuj1	Neuron-specific Class III β-tubulin
GFAP	Glial fibrillary acidic protein
S100β	S100 calcium-binding protein B
Vim	Vimentin
GalC	Galactocerebroside
MBP	Myelin basic protein
NG2	Neuron-glial antigen 2
O4	Oligodendrocyte marker O4
Olig2	Oligodendrocyte transcription factor 2
PDGFRa	Platelet-derived growth factor receptor alpha
bFGF	Basic fibroblast growth factor
BMP2	Bone morphogenetic protein 2

EGF	Epidermal growth factor
FBS	Fetal bovine serum
GDNF	Glial cell line-derived neurotrophic factor
IL1-b	Interleukin 1 beta
IL11	Interleukin 11
LIF	Leukemia inhibitory factor
NGF	Nerve growth factor
NT-3	Neurotrophin-3
PDGF	Platelet-derived growth factor

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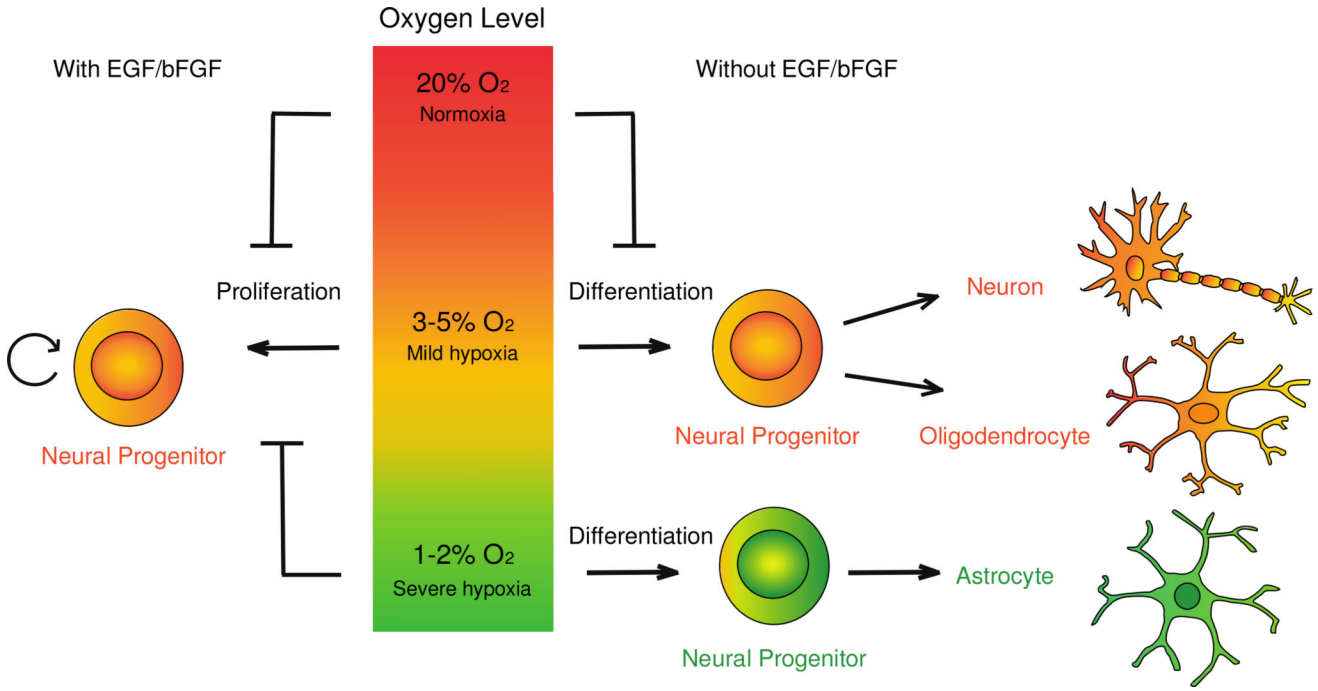

Figure 1. The model describing the influence of oxygen level on the proliferation and lineage specification of NPCs

In the presence of growth factors (EGF and bFGF), the proliferation capacity of NPCs is higher in mild hypoxia (3–5% O_2) than in 20% O_2. Severe hypoxia (1–2% O_2), however, slows down the proliferation of NPCs. In the absence of growth factors, the differentiation potential of NPCs is affected by different level of oxygen. Mild hypoxia promotes neurogenesis and oligodendrogenesis, whereas severe hypoxia favors the generation of astroglial lineage.

Table 1

Effects of lowered oxygen on proliferation, survival, and differentiation of human neural progenitor cells compared to atmospheric oxygen

References	Human cells	Oxygen (%)	Method of expansion	Days of expansion	Effect on proliferation	Effect on apoptosis	Method of differentiation	Days of differentiation	Effect on lineage specification		
									Neurons	Astrocytes	Oligodendrocytes
Storch et al. [65]	Fetal 6–9 week mesencephalic precursors	3	Neurosphere cultured in DMEM/F12 media with bFGF (20 ng/ml) EGF (20 ng/ml). Passaged every 18–27 day.	90–240	Significant Increase	Not tested	EGF/bFGF withdrawal with IL1-b, IL11, LIF, and GDNF	10	Converted to dopaminergic neurons (TH+, DAT+)	Detected (GFAP+)	Not tested
	Fetal 6–9 week forebrain precursors				Modest increase	Not tested			Not inducible to dopaminergic neurons	Not tested	Not tested
Liu et al. [71]	Fetal 11–15 week mesencephalic progenitors	3	Neurospheres cultured in DMEM/F12 media with bFGF (20 ng/ml), EGF (10 ng/ml), and LIF (10ng/ml). Passaged weekly.	35–42	Not tested	Not tested	EGF/bFGF withdrawal	28	Increased (Tuj1+, TH+, DA+)	Not tested	Not tested
Ortega et al. [66]	Fetal 14–19 week cortical progenitor cells	<1	Fetal cortical cells were dissected and cultured in DMEM/F12, B27 supplement, bFGF (10 ng/ml), EGF (10 ng/ml).	7	Decrease	No differences	EGF/bFGF withdrawal, with 1day in <1% oxygen and 6 days in 20% oxygen	7	Decreased (Tuj1+, DCX+, GABA+)	Increased (S100β, VIM+)	Decreased (O4+, Olig2+)
		1			Decrease	Increase	EGF/bFGF withdrawal	7	No differences (DCX+, GABA+)	Increased (S100β, VIM+)	No differences (Olig2+)
		3			Increase	Decrease	EGF/bFGF withdrawal	21	Increased (Tuj1+, GABA+)	No differences (VIM, S100β, GFAP+)	No differences (Olig2+)
Pistollato et al. [67]	Postnatal brain subventricular zone CD133+ Nestin+ precursors	5	Cells cultured in DMEM/F12 media with bFGF (20 ng/ml), EGF (10ng/ml), BMP2(10ng/ml), 1% BSA, 10 μg/ml rh insulin, 200 μg/ml transferrin. Passaged weekly.	7	Increase	No differences	EGF/bFGF withdrawal	21	No differences (Tuj1+)	No differences (GFAP+)	Significantly increased(GalC+)
Giese et al. [72]	MYC-immortalized fetal ventral mesencephalic progenitor cell line (ReNcell VM, Millipore Inc)	3	Cells cultured in DMEM/F12 media with bFGF (10ng/ml), EGF(20ng/ml), Glutamax, B27 supplement, heparin sodium salt.	3	No differences	Decrease	EGF/bFGF withdrawal	4	Increase (Tuj1+)	Not tested	Not tested
Santilli et al. [68]	v-MYC immortalized 10.5-week fetal	1	Neurospheres cultured in growth media with bFGF	7 to 10	Decrease	Increase	EGF/bFGF withdrawal	10	No differences (Tuj1+, MAP2+, GABA+)	Increased (GFAP+)	No differences (GalC+)

References	Human cells	Oxygen (%)	Method of expansion	Days of expansion	Effect on proliferation	Effect on apoptosis	Method of differentiation	Days of differentiation	Effect on lineage specification		
									Neurons	Astrocytes	Oligodendrocytes
	diencephalic and telencephalic precursors	2.5	(20ng/ml) and EGF. Passaged every 7–10 days.		Increase	No differences	EGF/bFGF withdrawal	10 or 17	Increased (Tuj1+, MAP2, GABA+)	No differences (GFAP+)	Increased (GalC+)
		5			Increase	No differences	EGF/bFGF withdrawal	10 or 17	Increased (Tuj1+, GABA+)	No differences (GFAP+)	Increased (GalC+)
Xie et al. [40]	Human ESC or iPSC derived neural progenitors	2	Cells cultured in DMEM/F12, N2 and B27 supplement, FGFb (20ng/ml) and EGF (50ng/ml).	7	Decrease	No differences	EGF/bFGF withdrawal	21, 28, 35 or 42	No differences (Tuj1+, MAP2+)	Increased (GFAP+)	Not tested

Table 2

Effects of lowered oxygen on proliferation, survival, and differentiation of murine neural progenitor cells compared to atmospheric oxygen

References	Rodent cells	Oxygen (%)	Method of expansion	Days of expansion	Effect on proliferation	Effect on apoptosis	Method of differentiation	Days of differentiation	Effect on lineage specification		
									Neurons	Astrocytes	Oligodendrocytes
Studer et al. [61]	Rat E12 mesencepha lic precursors	3	Cells dissected from rat embryonic day 12 (E12) mesencephalon were mechanically dissociated, plated on DMEM/F12 medium with N2 supplement and bFGF (10 ng/ml).	4 to 6	Increase	Decrease	bFGF withdrawal	5	Increased (TH+); Decreased (GABA+, Glu+)	Not detected (GFAP+)	Not tested
Morrison et al. [62]	Rat E14.5 neural crest stem cell (in peripheral nervous system)	3 to 6	DMEM-low glucose medium with 15% chick embryo extract, bFGF (20 ng/ml), N2 and B27 supplement, 2-mercaptoethanol (50 M), retinoic acid (35 ng/ml).	6	Increase	No differences	Reduced bFGF (10ng/ml) and chick embryo extract (1%), with or without BMP2	5 to 6	Increased neurons	Increased (GFAP+)	Not applicable
							Reduced bFGF (10ng/ml) and chick embryo extract (1%), with BMP2, forskolin, NGF, NT-3	12	Increased (TH+, DBH+, SA1+); increased dopamine and norepinephrine release	Not tested	Not applicable
Stacpoole et al. [74]	Rat postnatal P0-P2 cortical NPCs	3	Neurospheres cultured in DMEM/F12, N2 and B27 supplement, FGFb (20 ng/ml), EGF (20 ng/ml), and Heparin (5μg/ml).	7	Increase	Not tested	Not applicable	0	No differences (Tuj1+)	No differences (GFAP+)	No differences (O4+)
		3		7			Reduced bFGF (10ng/ml) with PDGF	5	No differences(Tuj 1+)	Slight decrease (GFAP+)	increased (O4+)
		3		28			Reduced bFGF (10ng/ml) and PDGF	2	No differences(Tuj 1+)	Decreased (GFAP+)	increased (O4+, MBP+, Olig2+)
		3		28			Transplantation into rat brain	2	Not tested	Increased (GFAP+)	Increased oligodendrocyte progenitor (NG2+)
Mutoh et al. [75]	Mouse E11.5 telencephalic NPCs	2	Cells cultured in DMEM/F12, N2 supplement, FGFb (10 ng/ml).	4	Not tested	Not tested	4-day bFGF withdrawal plus 4-day with LIF	8	Decreased (Map2+)	Increased (GFAP+)	Not tested
Chen et al. [73]	Mouse E13.5 cortical NPCs	5	Cells cultured in N2 medium with FGFb (20 ng/ml).	5	Increase	Decrease	bFGF withdrawal with NT-3	6	Slightly decreased (Tuj1+, MAP2+)	Increased (GFAP+)	Increased (O4+, PDGFRa+)
		2			Increase	Not tested	bFGF withdrawal with NT-3	6	Decreased (Tuj1+, MAP2+)	Significant increased (GFAP+)	Significant increased (O4+, PDGFa+)
Horie et al. [64]	Mouse E15.5 ganglionic eminence-derived NPCs	0	Neurospheres cultured in N-2 medium (DMEM/F12 with	5	No differences	Increase (highest)	EGF withdrawal with 1% FBS	2	Decreased (GABA+)	Decreased (GFAP+)	Not tested

References	Rodent cells	Oxygen (%)	Method of expansion	Days of expansion	Effect on proliferation	Effect on apoptosis	Method of differentiation	Days of differentiation	Effect on lineage specification		
									Neurons	Astrocytes	Oligodendrocytes
		1	36.4mM glucose, 23ug/ml insulin, 92ug/ml transferrin, 55u< putrescine, 27.5nM sodium selenite, 20nM progesterone, and 50U/ml penicillin-streptomycin) with EGF (20ng/ml).		Increase	Increase			Increased (Tuj1+, Glu+); Decreased (GABA+)	No differences (GFAP+)	Not tested
		2			Increase (highest)	Increase			Increased (Tuj1+, Glu+); Decreased (GABA+)	No differences (GFAP+)	Not tested
		3			Increase	Increase			Increased (Tuj1+, Glu+); Decreased (GABA+)	No differences (GFAP+)	Not tested
		4			Increase	No differences			Decreased (GABA+)	No differences (GFAP+)	Not tested
		10			Increase	No differences			Decreased (GABA+)	No differences (GFAP+)	Not tested
Kim et al. [92]	Mouse ESC-derived NPCs	3.5	Cells cultured in DMEM/F12 with N2 supplement, FGFb (20 ng/ml).	6	Not tested	Not tested	bFGF withdrawal with ascorbic acid (200 μM), IL1-b, IL11, LIF, and GDNF	6	Increased (TH+, Tuj1+)	No differences (S100β+)	No differences (Olig2+)