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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.

1. 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 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.

2. Oxygen tension and embryonic development

Oxygen is a key environmental factor that controls developmental processes, tissue homeostasis, and cellular metabolism. The 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 of 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 tissue is heterogeneous, with 14.5% O_2 in alveoli, 12% O_2 in arterial blood, 5.3% O_2 in venous blood, and 1.1–9.5% 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 physiological normoxia for developing embryos. Before the establishment of utero–placental circulation in the second trimester, the oxygen

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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 development at 5% O_2, whereas high oxygen condition (20% O_2 or 40% O_2) resulted in abnormal morphogenesis of neural folds and failure of neural tube closure [12].

3. 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 [11,13–15]. 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) [16–19]. 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 [20]. 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 [21]. 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 [22–24].

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 [25,26].

4. HIF in embryonic development

The importance of the HIF pathway in development was demonstrated by the early embryonic lethality (E9.5-10.5) in both *Hif1α* and *Hif1β* deficient mice (see review [11,15]). In fact, abrogation of HIF activity impaired vascularization of the placental [27–29], cardiovascular morphogenesis and angiogenesis [30], heart development [31–33], and endochondrial bone formation during early embryogenesis [15,34,35]. In terms of nervous system development, HIFs are required for neural crest cell migration [36]. *Hif1α* and *Hif1β* knockout mice had abnormalities such as forebrain hypoplasia and neural fold closure defects [28,37]. Furthermore, neural-specific *Hif1a*-deficient mice exhibited hydrocephalus, reduction in neuronal cells, and impaired spatial memory [38]. Those studies demonstrated the indispensible role of HIF in embryonic brain development.

5. Methods to stabilize HIF

5.1. 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 min 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 the 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 h in 5% O_2. Lowering the oxygen to 2% resulted in a an even more robust HIF1α nuclear translocation [39].

5.2. Small molecules

Small molecules that activate or stabilize expression of 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.

5.2.1. Deferoxamine (DFO)

DFO stabilizes HIF through inhibition of PHD by chelating the cofactor Fe^{2+} [40,41]. 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 [42]. In human NPCs, HIF1α nuclear translocation can be seen after just 6 h of DFO treatment by immunostaining [39]. The effective dose of DFO ranges from 50 μM to 150 μM and the best result can be seen 24 h after treatment. However, extended DFO treatment inhibited cell proliferation in NPCs. After treatment with 50 μM, 100 μM or 200 μM of DFO for 72 h, the viability of NPCs were decreased by 40–70% in a dose-dependent manner [43]. 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 [44]. In another study, DFO administration after cerebral