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Oxygen tension controls the expansion of human CNS precursors and the generation of astrocytes and oligodendrocytes

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Human neural precursor proliferation and potency is limited by senescence and loss of oligodendrocyte potential. We found that in vitro expansion of human postnatal brain CD133$^+$ nestin$^+$ precursors is enhanced at 5% oxygen, while raising oxygen tension to 20% depletes precursors and promotes astrocyte differentiation even in the presence of mitogens. Higher cell densities yielded more astrocytes regardless of oxygen tension. This was reversed by noggin at 5%, but not 20%, oxygen due to a novel repressive effect of low oxygen on bone morphogenetic protein (BMP) signaling. When induced to differentiate by mitogen withdrawal, 5% oxygen-expanded precursors generated 17-fold more oligodendrocytes than cells expanded in 20% oxygen. When precursors were expanded at 5% oxygen and then differentiated at 20% oxygen, oligodendrocyte maturation was further enhanced 2.5-fold. These results indicate that dynamic control of oxygen tension regulates different steps in fate and maturation and may be crucial for treating neurodegenerative diseases.
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Introduction

Oxygen is the central energy source in oxidative cell metabolism (Bruick, 2003) and is tightly regulated in the metabolically active brain (Hoge and Pike, 2001). Central nervous system (CNS) tissue PO_2 values are conserved among mammalian species and range from as low as 0.55% (4.1 mm Hg) in the midbrain to 8.0% (60 mm Hg) in the pia. The PO_2 of cortical grey matter in rodents is 2.53–5.33% (19–40 mm Hg) (Erecinska and Silver, 2001). Measurements in human brain show a mean PO_2 varying from 3.2% (23.8±8.1 mm Hg) at 22–27 mm below the dura to 4.4% (33.3±13.3 mm Hg) at 7–12 mm below the dura (Dings et al., 1998). Since normal alveolar oxygen tension is 14% (Guyton and Hall, 2006), these measurements suggest that a steadily decreasing oxygen gradient is formed as blood reaches the brain tissues. Tissue oxygen perfusion is often disrupted in pathological states, such as ischemia–reperfusion (Saito et al., 2005) and head injury (Menon et al., 2004), and may be altered in hyperbaric therapies (Carson et al., 2005) (Fig. 1A).

In addition to its well-described actions in cell metabolism, oxygen also regulates cell fate. Lowered oxygen in the 2–5% range promotes the generation of specific neural lineages in rat. Culture of rat ventral midbrain precursors at lowered oxygen promotes the generation of tyrosine hydroxylase-positive (TH$^+$) dopaminergic neurons, a cell type that is lost in Parkinson's disease (Studer et al., 2000). Culture of rat neural crest stem cells in lowered oxygen promotes both survival and the differentiation of TH$^+$ sympathoadrenal cells (Morrison et al., 2000). Additionally, culture of human and mouse ventral midbrain precursors at low oxygen lowers cell death and senescence and also yields greater proportions of TH$^+$ dopamine neurons (Milosevic et al., 2005; Storch et al., 2001), suggesting that these mechanisms may be common to many species. While these previous studies showed that low oxygen increases the percentage of specific neuronal types, low oxygen also represses neuronal differentiation (Gustafsson et al., 2005). This suggests that oxygen may control the competence of precursors to generate these fates in addition to controlling their differentiation per se.

A related parameter, intracellular reduction–oxidation (RedOx) balance, has been implicated in controlling oligodendrocyte progenitor proliferation and survival. As measured by a RedOx-sensitive dye, O2A progenitors with a more reduced state have a greater likelihood of self-renewal and a larger clone size compared to cells with a more oxidized state. Pharmacologically raising or lowering the RedOx state blocks the actions of mitogens or differentiating factors, respectively (Smith et al., 2000). This suggests that RedOx state is a complex integrator of many inputs, perhaps including O_2 tension, which regulate cell fate during

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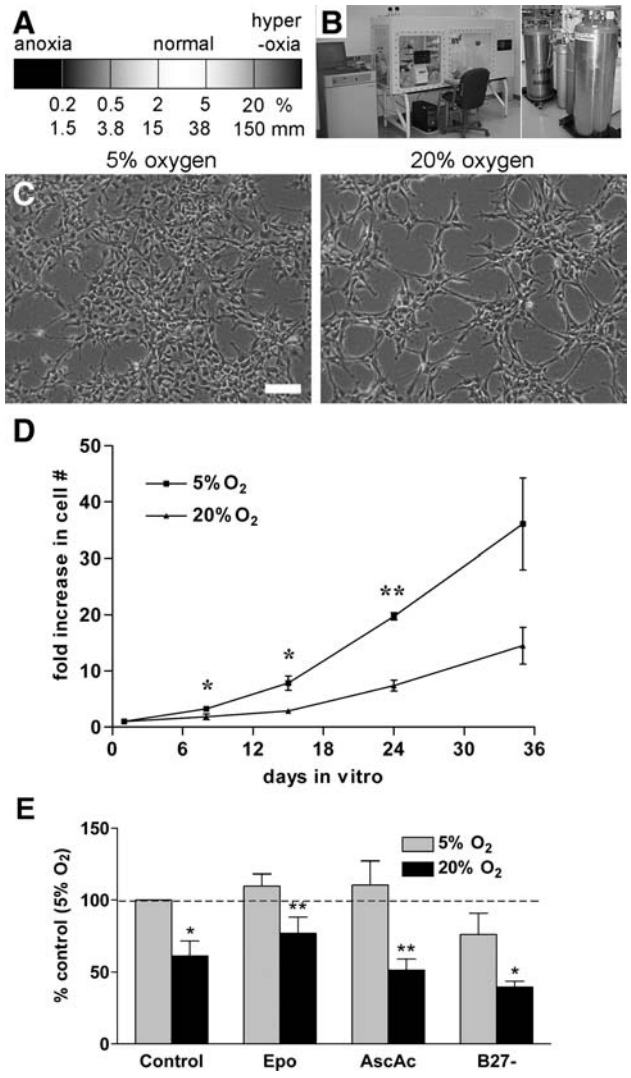

Fig. 1. Lowered oxygen promotes expansion of human CNS subventricular precursors over extended times. (A) Physiological oxygenation (normoxia, light shade) of different adult mammalian brain regions ranges from 0.5% to 8% oxygen (3.8 to 61 mm Hg), while 21% room oxygen tension is hyperoxic (from Erecinska and Silver, 2001). (B) Environmental control system used for these studies. (C) Expansion of human SVZ cells, plated at equivalent densities and expanded 7 days at either 5% or 20% oxygen; bar = 100 μm. (D) Quantitation after successive passaging, mean ± S.E.M., $n = 3$. (E) Human precursor cells were expanded at 5% or 20% oxygen alone or with erythropoietin (EPO), ascorbic acid (AscAc) or B27 without (–) retinoic acid. None of these extracellular factors duplicates the enhancement of expansion by lowered oxygen; mean ± S.E.M., $n = 4$, $^{*}p < 0.05$; $^{**}p < 0.01$, paired t-test for all graphs.

development. However, the regulation of oligodendrocyte fate is difficult to assay for human CNS precursors, since their ability to generate oligodendrocytes rapidly diminishes during extended in vitro expansion (Chandran et al., 2004; Kim et al., 2006; Wright et al., 2006). Furthermore, while human neural precursors can sometimes expand for extended periods of time (Walton et al., 2006), cellular senescence is often a limiting factor (Wright et al., 2006).

To address the role of oxygen at defined steps in human precursor expansion and differentiation, we utilized a novel system to control gas composition during incubation, microscopy-aided recording and experimental manipulation (Fig. 1B). We found that 5% oxygen, which is within the physiologically measured range, specifically promotes the proliferation of nestin^{+} human postnatal CNS precursors. Expansion in 5% oxygen increases the proportion of these cells with a stem cell phenotype, as measured by CD133/CD24 expression and the ability of the precursors to generate all three CNS lineages, including oligodendrocytes. In contrast, 20% oxygen causes precursors to differentiate to astrocytes. Subsequently increasing oxygen tension during cell differentiation promotes the maturation of oligodendrocytes. These novel findings show that oxygen tension exerts multiple effects on human CNS cell fate at different stages during proliferation and differentiation. In addition to defining the physiological actions of oxygen on human precursors, this suggests a systematic method to enrich for human CNS stem cells and oligodendrocytes for clinical purposes.

Results

Human CNS precursor expansion increases at lowered oxygen tension

Fetal and postnatal human CNS precursors proliferate extensively in the presence of the mitogens bFGF and EGF (Caldwell et al., 2001; Carpenter et al., 1999; Ostenfeld and Svendsen, 2004; Walton et al., 2006). We observed a significant increase in human CNS precursor numbers when cultured at 5% compared with 20% oxygen (Fig. 1C). These differences were seen upon the first passage and became greater in subsequent passages (Fig. 1D). The enhancement of precursor expansion in lowered oxygen may be due to minimized oxidative stress or to the secretion of trophic factors. We tested this hypothesis by culturing precursor cells at 5% or 20% with standard medium alone or with [1] erythropoietin (Epo), which is induced under low oxygen conditions (Studer et al., 2000) and is required for neural precursor survival and expansion in vivo (Yu et al., 2002); [2] the anti-oxidant ascorbic acid; or [3] B27 supplements, which contain a number of anti-oxidants and survival factors; we used a variant without retinol, which is known to promote neuronal differentiation. None of these additives promoted expansion as did 5% oxygen (Fig. 1E). Epo had no effect on SVZ precursors at 10 ng/ml (Fig. 1E) or 30 ng/ml (not shown), unlike its effect on ventral midbrain cells (Studer et al., 2000), nor did ascorbic acid. Another antioxidant, *N*-acetylcysteine, also failed to promote expansion in two replicate experiments (not shown). While this was not an exhaustive test of all candidate factors, the results suggest that addition of anti-oxidants or growth factors is suboptimal in duplicating the growth-promotion effects of 5% oxygen.

Interaction of oxygen tension and density in the expansion of nestin^{+} precursors

We observed an enhancement of human CNS precursor expansion at 5% compared with 20% oxygen at most plating densities tested (Fig. 2A), but particularly at lower densities. Cells still survived at low density in 20% oxygen, but these had a more flattened appearance than cells in 5% oxygen. One characteristic of extended culture at 5% oxygen was an increase in the number of small bipolar or tripolar cells relative to more flattened cells, which we suspected were neural precursors and glial cells, respectively.

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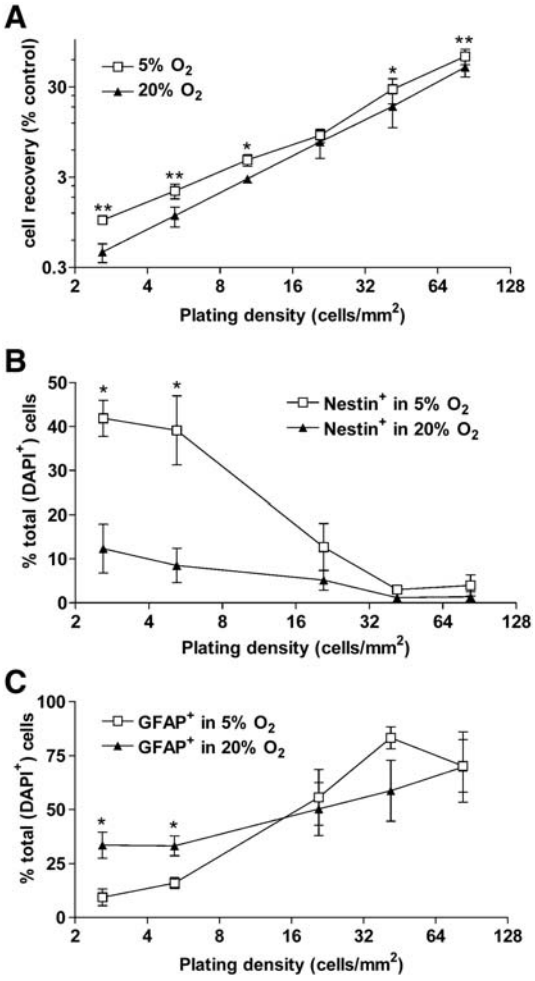

Fig. 2. Higher oxygen and high densities increase GFAP$^+$ cells at the expense of nestin$^+$ cells. (A) Human SVZ cells were plated at varying plating densities and expanded 7 days at either 5% or 20% oxygen. Expansion of precursors is higher at 5% versus 20% oxygen at nearly all densities tested (normalized to recovery at lowest density in 5% oxygen). (B) Percentage nestin$^+$ precursors is higher at 5% oxygen than 20% oxygen, but increasing cell densities leads to decreasing nestin$^+$ cells for both oxygen tensions. (C) At the lowest densities, percentage GFAP$^+$ precursors are higher at 20% oxygen than 5% oxygen, but there is no significant difference at higher densities. Thus, 20% oxygen promotes GFAP$^+$ cells numbers at the expense of nestin$^+$ precursors; higher densities also promote glial fates and mask the enhancement of nestin$^+$ precursor numbers by 5% oxygen. Mean±S.E.M., n=4–5 (total cells), n=3–4 (nestin$^+$ and GFAP$^+$), *p<0.05; **p<0.01, paired t-test of 5% against corresponding 20% at each density.

We stained these cultures for nestin, a general marker of neural precursor cells (Tohyama et al., 1992), and glial fibrillary acidic protein (GFAP), a marker of astrocytes and radial glia (Casper and McCarthy, 2006; deAzevedo et al., 2003). Nestin predominantly stained smaller cells while GFAP predominantly stained the flattened cells. We found that these cultures were heterogeneous under all conditions, but that both density and oxygen tension affected the ratio of nestin$^+$ and GFAP$^+$ cells. In 5% oxygen, low density cultures contained 46% nestin$^+$ cells (Fig. 2B) with less than 8% GFAP$^+$ cells (Fig. 2C). The majority of the remaining cells were nestin$^+$ βIII-tubulin$^+$ with some nestin$^+$ GFAP$^+$ (not shown). With increasing plating density, cells reached confluence and the percentage of nestin$^+$ cells dropped to near-zero, while GFAP$^+$ cells eventually made up 80% of the population. This is consistent with our previous data showing that high densities promote GFAP$^+$ glial differentiation by a contact-dependent mechanism (Rajan et al., 2003; Tsai and McKay, 2000).

In contrast, 20% oxygen reduced both the recovery of cells and the percentage of these cells that were nestin$^+$ (Fig. 2B). This was most pronounced at the lowest densities that in 5% oxygen produced high percentages of nestin$^+$ cells. GFAP$^+$ cells were four times higher at the lowest densities and increased further at higher plating densities (Fig. 2C). Thus, GFAP$^+$ cells are enhanced by both higher oxygen tensions and increasing density, while nestin$^+$ precursors are enhanced at lower oxygen tensions. Higher densities promote a glial fate and mask the enhancement of nestin$^+$ precursor numbers by 5% oxygen.

Distinct sensitivity of precursors to BMP activity at 5% versus 20% oxygen

The interaction between oxygen tension and density in promoting GFAP$^+$ differentiation led us to explore what signals could be involved. Bone morphogenetic proteins (BMPs) are strong inducers of astroglial fates (Chen and Panchision, 2007); we previously showed that the combination of high density and signaling by BMPs promote glial differentiation by an mTOR-dependent pathway (Rajan et al., 2003; Sailer et al., 2005). In contrast, the endogenously secreted BMP-antagonist, noggin (Zimmerman et al., 1996), limits glial differentiation and directs postnatal stem cells to generate neurons (Lim et al., 2000). We tested whether similar signals operate in human cells and whether oxygen interacts with this pathway. We plated human SVZ cells at 49 cells/mm^2 (medium–high density) and expanded 6 days at either 5% or 20% oxygen, with or without BMP2 or noggin. At 20% oxygen, there was surprisingly little effect of noggin or BMPs on total cell numbers (Fig. 3A). At 5% oxygen, however, noggin strongly promoted precursor expansion. We then analyzed cell phenotype in these cultures (Fig. 3B). Whereas 5% oxygen permitted the inhibition of BMP signaling by Noggin (measured as an increased percentage of Nestin$^+$ precursors and decreased GFAP$^+$ glia), it blocked the gliogenic effect of BMP2 on precursor cells. In contrast, 20% oxygen limited the effect of Noggin, but strongly enhanced the effect of BMPs in promoting a glial fate. Caspase3-mediated apoptosis was not a prominent response to BMP treatment (not shown), unlike that reported in previous studies in rodents (Chen and Panchision, 2007; Mabie et al., 1999; Panchision et al., 2001). We hypothesized that oxygen could regulate BMP signaling at multiple steps, including SMAD activation and transcriptional induction of target genes like *Id1* (Lopez-Rovira et al., 2002), which is involved in gliogenesis (Nakashima et al., 2001; Vinals et al., 2004; Yanagisawa et al., 2001). To control for the possibility that differing BMP responses were due to long-term effects of oxygen on cell fate, we started with cells that had been cultured extensively in 5% oxygen and only switched to 20% oxygen 4 h prior to beginning BMP treatment. By measuring serine phosphorylation of Smads1/5/8, a key step in BMP signal transduction (Shi and Massague, 2003), we found that activation occurs more rapidly, in more cells and for a longer duration in 20% oxygen than in 5% oxygen (Fig. 3C). Furthermore, expression of Id1 protein by immunocytochemistry was increased within 24 h of BMP2 treatment in 20% oxygen but not 5% oxygen (Fig. 3D). These results indicate that lowered oxygen tension represses BMP signaling and subsequent glial differentiation

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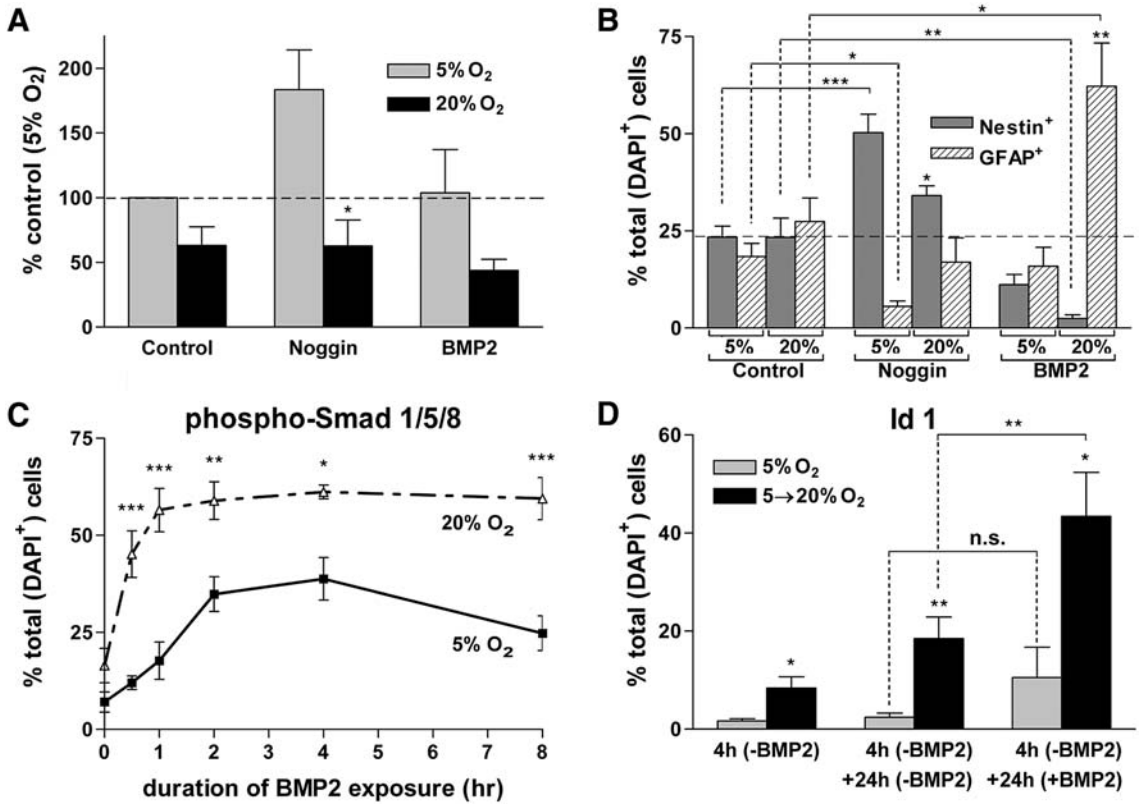

Fig. 3. Lowered oxygen represses the gliogenic effect of BMP signaling. (A) Human SVZ cells were plated at 49 cells/mm^2 and expanded 6 days at either 5% or 20% oxygen. At 20% oxygen, neither BMP stimulation, nor BMP inhibition by noggin, has an effect on total cell numbers. At 5% oxygen, however, noggin exposure increases cell numbers. (B) At 5% oxygen, noggin increases nestin$^+$ precursors while decreasing GFAP$^+$ glia as a percentage of total cells; BMP2 has no significant effect on either cell type. In contrast, at 20% oxygen, noggin has no significant effect while BMP2 decreases nestin$^+$ cells and increases GFAP$^+$ cell numbers. Mean±S.E.M., $n=4$. (C) Time course of SMAD activation after 10 ng/ml BMP2 treatment in 5% versus 20% oxygen. (D) *Id1* induction after 10 ng/ml BMP2 treatment in 5% oxygen versus 20% oxygen. For both C and D, cells were extensively cultured in 5% oxygen; after passaging, half of replicate wells were transferred to 20% for 4 h prior to BMP2 treatment. Mean±S.E.M., $n=3$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s., not significant, paired *t*-test for C, one-way ANOVA with Tukey's post-hoc test for others.

of CNS precursor cells, while a higher oxygen tension promotes BMP signaling.

20% oxygen promotes differentiation of human CNS precursor cells

The effect of oxygen on precursor cell numbers could reflect an alteration of either proliferation status or cell death. To address this, we analyzed these cells by: [1] Ki67 expression, marking actively cycling cells; [2] nestin expression, identifying all neural precursor cells; [3] activated, proteolytically cleaved caspase3 to identify apoptosis; and [4] pyknotic nuclei, which identify dead cells and can be easily distinguished by their round, intensely bright DAPI$^+$ appearance. We also analyzed the expression of the neuronal marker PSA-NCAM and the astrocytic marker GFAP, which identify progenitor as well as differentiated cells of these lineages (Bernier et al., 2000; Casper and McCarthy, 2006; deAzevedo et al., 2003). We observed higher percentages of Ki67$^+$ and nestin$^+$ cells at both 5% compared with 20% oxygen (Fig. 4A). Activated caspase3 was not significantly increased at 20% oxygen and pyknotic cell numbers were not changed. In contrast, the neuronal lineage was slightly but significantly promoted, as measured by PSA-NCAM$^+$ cells, and GFAP$^+$ glial cells were more strongly increased at 20% oxygen. These results indicate that the principal response to 20% oxygen exposure is terminal differentiation of precursors to an astrocytic fate.

To determine how quickly precursors responded to increased oxygen tension, we initially cultured precursors at 5% oxygen for 3 days, then acutely exposed cells to 20% oxygen and fixed cells 24 h or 48 h later. We saw an increase in the percentage of cells expressing total p53 (not shown) and phosphorylated forms of p53 at the Ser37 and Ser15 residues within 24 h of exposure to 20% oxygen (Figs. 4B, D). There was no change in the cell number or localization of p53 phosphorylation at the Ser6, Ser9, Ser20 and Ser392 residues. This is notable since high oxygen tension also increases p53 activity via Ser15 and Ser37 phosphorylation in lung adenocarcinoma cells (Das and Dashnamoorthy, 2004). Since p53 activity can activate either apoptosis or terminal differentiation, we looked at the expression of p21^{cip1}, a cyclin-dependent kinase inhibitor that is induced by p53 and is a direct effector of mitotic arrest. We found that p21^{cip1+} cell numbers were not changed at 24 h but doubled at 48 h after exposure to 20% oxygen (Figs. 4C, E). Thus, exposure to 20% oxygen induces a significant activation of p53 phospho-Ser15/Ser37 within 24 h and p21^{cip1}-mediated mitotic arrest by 48 h in human SVZ precursors. The principal result of this mitotic arrest appears to be astrocytic differentiation.

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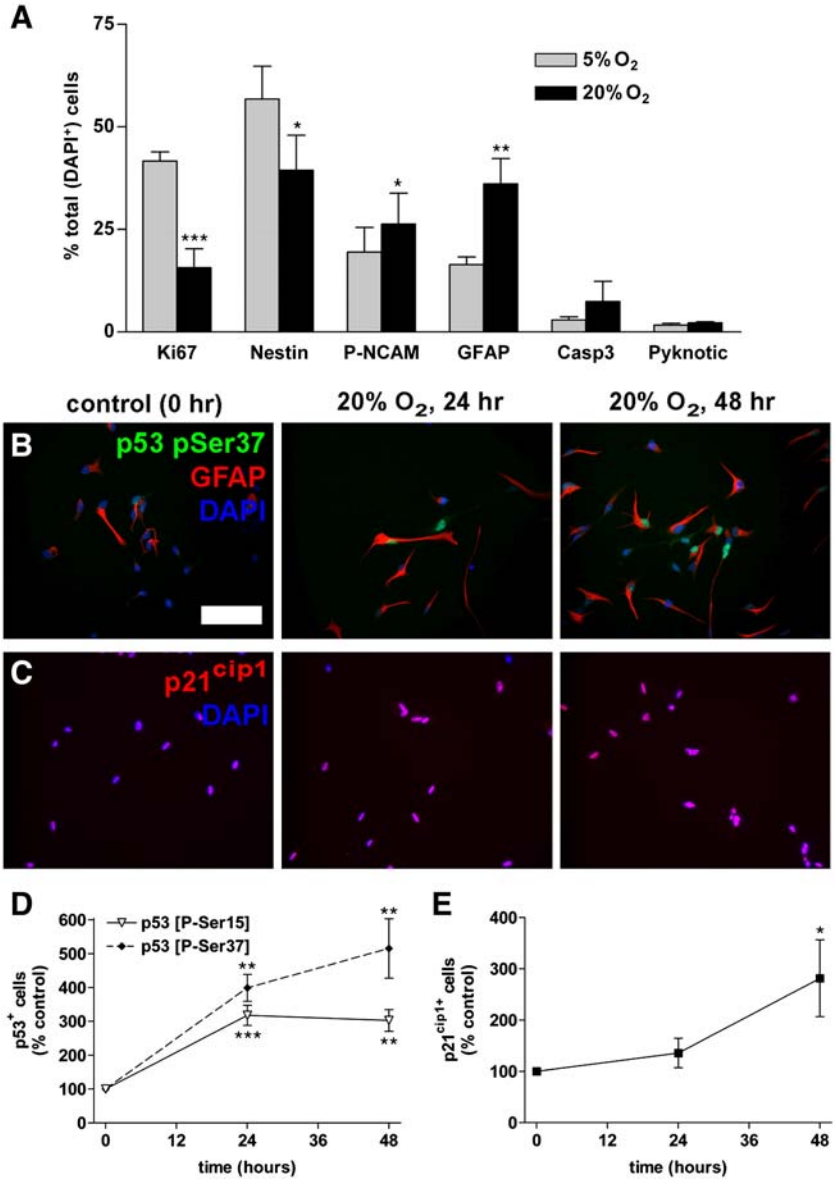

Fig. 4. 20% oxygen promotes differentiation of human precursor cells. (A) Expansion of human SVZ precursor cells (8.6 cells/mm^2 expanded for 7–10 days) is enhanced at 5% oxygen as measured by Ki67 and nestin expression. In contrast, 20% oxygen decreases these markers and increases GFAP$^+$ cells, suggesting glial differentiation. The neuronal phenotype increases modestly as measured by PSA-NCAM (P-NCAM) staining. Cell death is not significantly affected, as measured by activated caspase3 (Casp3) staining and pyknotic cell number. Mean±S.E.M., $n=4$–8. (B–C) After initial expansion at 5% oxygen, acute exposure to 20% oxygen increases serine phosphorylation of p53 (B) and expression of p21^{cip1} (C), an effector of cell cycle arrest. p53 is known to promote either differentiation, via p21^{cip1} induction, or apoptosis. (D, E) Quantitation of p53 phosphorylation at Ser15 and Ser37 (D) and p21^{cip1} induction (E) after acute exposure to 20% oxygen for 0, 24 or 48 h; mean±S.E.M, $n=4$; *$p<0.05$, **$p<0.01$, ***$p<0.001$, paired t-test for all experiments.

Lowered oxygen promotes the generation of multiple lineages

While 20% oxygen promotes mitotic arrest and astrocyte differentiation, we wanted to see if 5% oxygen simply prevented mitotic arrest or if it affected the competence of precursors to generate neurons, astrocytes and oligodendrocytes. We expanded precursors with mitogens for 7–10 days and then differentiated cells by mitogen withdrawal for 21 days (Figs. 5A, B). As expected, total cell number was higher at 5% oxygen compared with 20% oxygen, but the percentage of cells expressing the neuronal marker βIII-tubulin or the astrocyte marker GFAP was not significantly altered. This may reflect the substantial numbers of precursor cells that already express these markers (Fig. 4A) and which may be committed progenitors. However, the number of galactocerebroside-C$^+$ (GalC$^+$) oligodendrocytes generated was 17-fold higher at 5% oxygen than at 20% oxygen (Figs. 5A, B, E).

To account for these results, we proposed two alternative mechanisms: (1) 20% oxygen tension inhibits the expansion of all precursors equally, but also selectively inhibits the maturation of oligodendrocytes, or (2) 20% oxygen is detrimental to the expansion of multipotent precursors, but not neuronal- or astrocyte-restricted precursors.

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Higher oxygen promotes oligodendrocyte differentiation

We tested these hypotheses by including replicate wells in which we switched oxygen exposure 2 days after mitogen withdrawal (Figs. 5C–E). Surprisingly, switching from 5% to 20% (5→20%) oxygen caused a further increase in the number of GalC$^+$ oligodendrocytes (Figs. 5C, E). This number was over 2-fold higher than in cultures differentiated at 5% oxygen and over 40-fold higher than cultures maintained at 20% oxygen during both expansion and differentiation. However, switching from 20% to 5% (20→5%) oxygen during differentiation did not rescue the generation GalC$^+$ cells (Figs. 5D, E). These results indicate that the failure to generate oligodendrocytes at 20% oxygen is primarily due to negative precursor selection rather than delayed maturation of oligodendrocyte progenitors. This negative effect may be on stem cells and/or oligodendrocyte-committed progenitors. However, once oligodendrocytes are generated, late stages of differentiation are enhanced by higher oxygen tensions. This may be partly due to accelerated mitotic arrest as seen by greater proportions of Ki67$^-$ cells (not shown).

To determine whether oligodendrocyte progenitors were present in cultures at 5% oxygen, we stained for platelet derived growth factor receptor alpha (PDGFRα), a marker for immature oligodendrocyte progenitors (Blakemore et al., 2002; Zhang et al., 2000), and O4, a marker for late oligodendrocyte progenitors and early post-mitotic oligodendrocytes (Zhang et al., 2000). We found that PDGFRα$^+$ cells were present in these precursor cultures (Fig. 5F); however, even in the presence of continuous bFGF/EGF, exposure to 20% oxygen decreased the proportions of PDGFRα$^+$ cells in these cultures within 4 days. Simultaneously, the total expression of the later marker O4 was unchanged; however, the proportion of these cells co-expressing p21^{cip1} increased, indicating that these oligodendrocyte progenitors had undergone mitotic arrest. We could not detect O4$^+$ cells co-expressing activated caspase3, suggesting that apoptosis was not a significant contributor to the changes in the oligodendrocyte lineage. Thus, while 5% oxygen permits the generation and maintenance of oligodendrocyte progenitors, 20% oxygen actually drives mitotic arrest and promotes the differentiation of oligodendrocytes even in the presence of mitogens, thereby depleting the supply of oligodendrocyte progenitors.

Enhanced precursor expansion at 5% oxygen involves greater clonogenicity and higher CD133$^+$ cell numbers

To test the second hypothesis, we measured clone formation by plating cells at 0.2 cell/well in 96-well dishes, which ensures high confidence that no well will have more than one cell. 3-week expansion in 5% oxygen yielded a 14-fold higher frequency of clones than in 20% oxygen (Fig. 6A). Analysis of some of these clones after differentiation indicated that they contained cells expressing markers for neurons, astrocytes and oligodendrocytes (not shown). This is consistent with a positive effect of 5% oxygen on self-renewal and multipotency, two defining properties of stem cells. We also analyzed precursor cells for markers that predict stem cell properties of human CNS cells. This included the cell

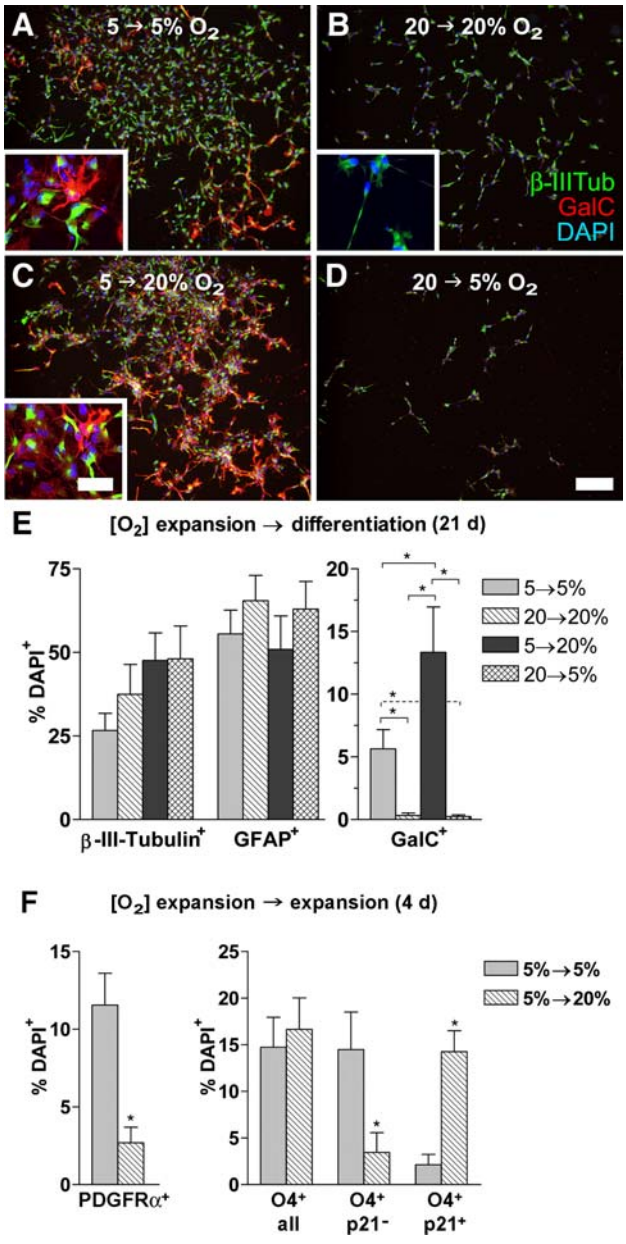

Fig. 5. Lowered oxygen promotes multi-lineage competence, while increasing oxygen after mitogen withdrawal promotes oligodendrocyte maturation. Human SVZ precursors were cultured for 7 days expansion and 21 days differentiation, entirely at 5% oxygen (5→5%, A), entirely at 20% oxygen (20→20%, B), at 5% oxygen during expansion and 20% oxygen during differentiation (5→20%, C), or at 20% oxygen during expansion and 5% oxygen during differentiation (20→5%, D). Colonies containing neurons (βIII-tubulin), astrocytes (GFAP) and oligodendrocytes (GalC) were generated at 5% oxygen (A). In contrast, almost no oligodendrocytes were generated at 20% oxygen (B). Switching from 5% to 20% (5→20%) oxygen after mitogen withdrawal caused a further increase in GalC$^+$ oligodendrocytes (C). Conversely, switching from 20% to 5% (20→5%) after withdrawal still did not restore the generation of oligodendrocytes (D). Thus, 5% oxygen maintains precursor capability to generate oligodendrocytes, while subsequent exposure to 20% oxygen enhances oligodendrocyte differentiation. (E) Quantitation, mean±S.E.M., $n=4$ for βIII-tubulin and GFAP, $n=3$ for GalC. (F) Early oligodendrocyte progenitors are reduced by acute exposure to increased oxygen tension for 4 days, as measured by PDGFRα staining. O4$^+$ late progenitors are not decreased but rather show increased expression of p21^{cip1}, indicating mitotic arrest; mean±S.E.M., $n=3$. *$p<0.05$, **$p<0.01$, ***$p<0.001$, one-way ANOVA with Tukey's post-hoc test. Bar in main images=200 μm; bar in insets=50 μm.

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surface antigens CD133 and CD24; adult human CNS subventricular zone cells that express high levels of CD133 and low CD24 have high clonogenic capacity and multipotency, while cells expressing high levels of CD24 have limited clonogenic capacity (Uchida et al., 2000) and may be a transit-amplifying precursor population (Doetsch et al., 2002). Cells grown in mass culture were analyzed for co-expression and intensity of these markers by flow cytometry. We saw that in 5% oxygen, the majority of cells expressed high levels of CD24, but that a distinct population of $CD133^{+}CD24^{-}$ cells was present (Fig. 6B). This latter expression pattern is consistent with extensively self-renewing, multipotent stem cells (Uchida et al., 2000). In cells expanded in 20% oxygen,

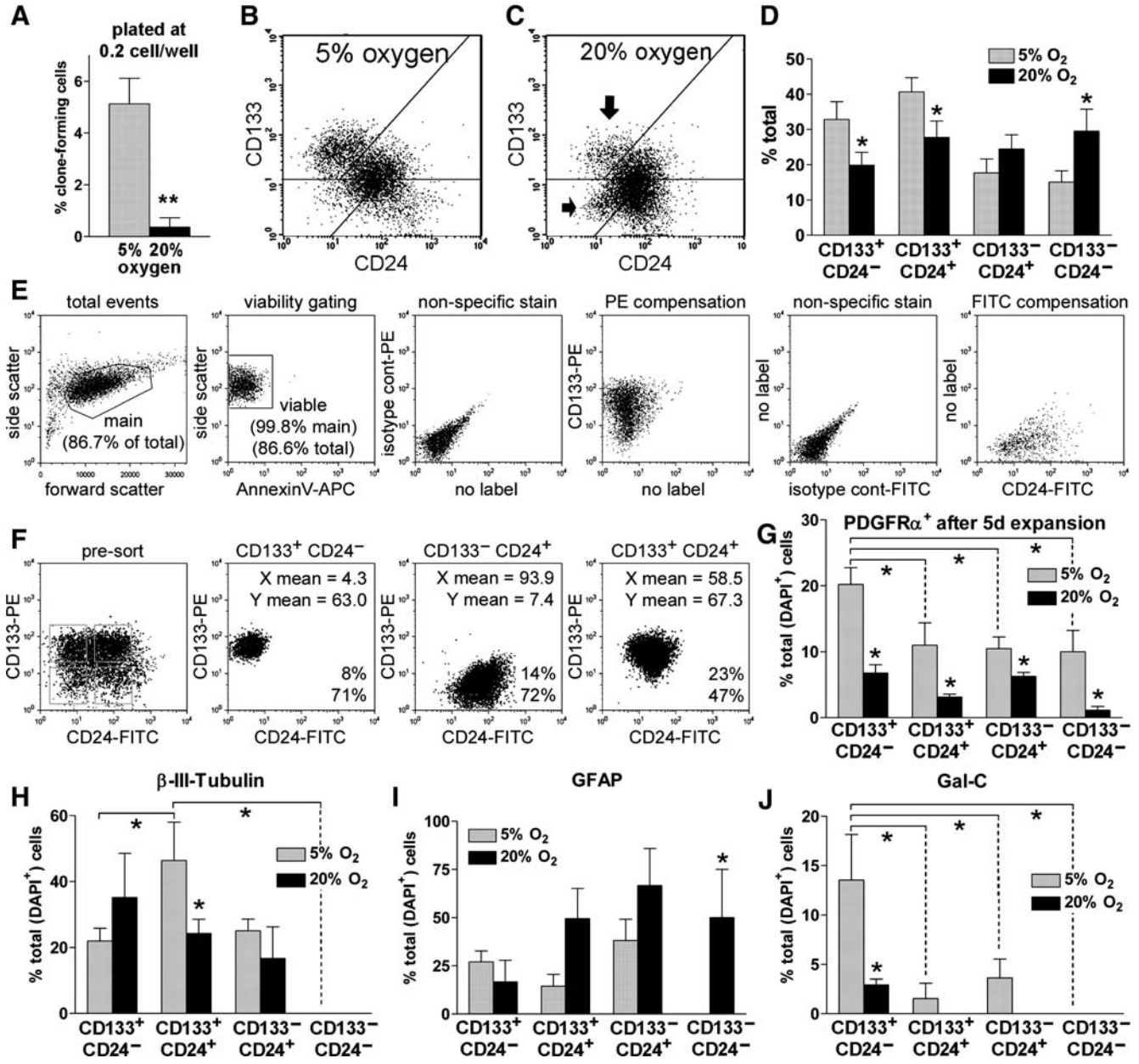

Fig. 6. 5% oxygen promotes clonogenicity and increased numbers of $CD133^{+}$ multipotent cells. (A) Clone formation of precursors plated at 0.2 cells/well and expanded 3 weeks in either 5% or 20% oxygen, mean ± S.E.M., $n=3$. (B–C) Human SVZ precursors were expanded for 7 days at 5% (B) or 20% (C) oxygen, passaged and labeled with antibodies against CD133 and CD24, then analyzed by flow cytometry. The dot plots show that long-term exposure to 20% oxygen decreases the number of $CD133^{+}CD24^{+}$ cells (long arrow) and increases the number of $CD133^{-}/CD24^{-}$ cells (short arrow). (D) Quantitation, mean ± S.E.M., $n=6$. (E–J) Prospective isolation experiment of cells initially expanded at 5% oxygen; (E) gating for intact cells (main population, polygon) and Annexin-V exclusion (viable cells, rectangle), followed by isotype control antibody labeling to control for non-specific binding and individual CD133 and CD24 antibody binding to compensate overlapping signal. (F) Gray rectangles denote sort gates overlaid on CD133/CD24 expression profile of viable cells; example of purity analysis showing cell enrichment after sorting for $CD133^{+}CD24^{-}$, $CD133^{-}CD24^{+}$ and $CD133^{+}CD24^{+}$; each plot shows mean relative fluorescent intensity of CD24 (*X* mean) and CD133 (*Y* mean) for enriched population, along with percentage cells within gate area before (top) and after (bottom) sorting. (G) Proportion of $PDGFR\alpha^{+}$ cells identified after 5 days expansion of sorted cells. (H–J) sorted cells expanded for 5 days, differentiated for 20 days and stained for neurons (H, βIII-tubulin), astrocytes (I, GFAP) and oligodendrocytes (J, GalC); mean ± S.E.M., $n=3$ for F–I, $n=2$ for J; $^{*}p<0.05$, $^{**}p<0.01$, paired *t*-test for all experiments.

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there was a decrease in the percentage of both $CD133^+CD24^-$ and $CD133^+CD24^+$ cells (Figs. 6C, D), consistent with a progressive loss of stem cells and transit amplifying cells. In contrast, 20% oxygen caused an increase in the number of $CD133^-CD24^-$ cells.

To determine whether this change in cell composition reflected a change in differentiation potential, we passaged cells that had been extensively expanded in 5% oxygen, prospectively isolated them based on CD133 and CD24 expression (Figs. 6E, F) and cultured them at low density. Analysis of βIII-tubulin and GFAP, which also mark precursors of the human astrocyte and neuronal lineage, respectively, showed that after 5 days expansion the $CD133^+CD24^+$ and $CD133^-CD24^+$ populations contained the majority of $\beta III\text{-tubulin}^+$ precursors and the fewest $GFAP^+$ precursors (not shown). We did not see a significant difference in the proportions of these cells within the first 5 days at 5% versus 20% oxygen (not shown), although it is likely that some of these cells underwent mitotic arrest (Fig. 4). However, $CD133^+CD24^-$ cells expanded in 5% oxygen had the highest percentage of cells expressing PDGFRα. Exposure of precursors to 20% oxygen for 5 days of expansion was sufficient to eliminate the majority of $PDGFR\alpha^+$ cells (Fig. 6G). After differentiating by mitogen withdrawal for 3 weeks, cells were stained for markers of neurons (βIII-tubulin), astrocytes (GFAP) and oligodendrocytes (GalC). In 5% oxygen, the highest percentage of neurons were generated from $CD133^+CD24^+$ cells, the putative transit-amplifying population; these neurons were preferentially lost when the precursors were expanded and differentiated in 20% oxygen (Fig. 6H). $GFAP^+$ astrocytes were more frequently generated from $CD133^-$ cells, particularly in 20% oxygen (Fig. 6I). Enriched $CD133^-CD24^-$ cells did not expand, unlike other sorted cell groups, suggesting that they are unlikely to be precursors. Surprisingly, these isolated cells either died or lifted from the surface at 5% oxygen and generated morphologically flattened cells that only expressed GFAP when exposed to the differentiating effect of 20% oxygen. These results are consistent with glial differentiation seen in unsorted cultures at 20% oxygen (Fig. 4). $GalC^+$ oligodendrocytes (Fig. 6J), like their $PDGFR\alpha^+$ progenitors (Fig. 6G), were generated most frequently from $CD133^+CD24^-$ precursors (a 2.5-fold GalC enrichment compared with unsorted cells in Fig. 5B), although they were also generated in smaller numbers from $CD24^+$ cells. Exposure of precursors to 20% oxygen for 5 days of expansion and 3 weeks of differentiation was sufficient to eliminate the majority of $GalC^+$ oligodendrocytes. These combined results indicate that the majority of tri-lineage differentiation occurs from $CD133^+CD24^-$ precursors, consistent with previous reports that these are self-renewing, multipotent stem cells (Uchida et al., 2000). Exposure to 20% oxygen reduces the expansion of these cells and the proportion of $PDGFR\alpha^+$ precursors included in or generated by this cell population.

Discussion

The clinical use of neural stem cell to replace those lost through trauma or disease requires systematic methods to enrich for the cell of interest. Recent studies implicate oxygen tension and RedOx state as regulators of neural precursor metabolism, survival and fate. Lowered oxygen in the range of 2–5% promotes the generation of dopaminergic neuronal fates from rat and human precursors (Morrison et al., 2000; Storch et al., 2001; Studer et al., 2000), increases the expansion of neural crest stem cells and promotes adrenergic differentiation (Morrison et al., 2000). Lowered oxygen or a reduced RedOx state also prevents neuronal differentiation of rat precursors (Gustafsson et al., 2005) and O2A oligodendrocyte progenitors (Smith et al., 2000), suggesting that oxygen has differing effects depending on precursor type. Here we show for the first time that multi-lineage competence of human CNS precursors and the selective differentiation of oligodendrocytes are regulated by dynamic changes in oxygen tension. Lower oxygen tensions maintain and expand $nestin^+$ precursors that exhibit enhanced stem cell properties, while higher oxygen tensions promote p53 phosphorylation, $p21^{cip1}$ induction and astrocytic differentiation. Furthermore, a novel interaction between oxygen tension and BMP signaling is involved in the control of neural precursor differentiation.

A critical process in development is the interaction of proliferating embryonic tissues with oxygen and nutrients supplied by growing vasculature, first maternally by the placenta and continuing with the developing vascular plexus of the embryo. There is evidence that dynamic regulation of oxygen tension contributes to the normal process of self-renewal and fate choice during the development of non-CNS tissues. Analysis of HIF1α, ARNT and VEGF-deficient mice indicated that the generation of hemangioblasts from embryonic stem (ES) cells is promoted by lowered oxygen and that oxygen sensing is required for proper hematopoietic development (Adelman et al., 2000; Ramirez-Bergeron et al., 2004). Recent analysis of the HIF2α-over-expressing and null mouse indicates that this oxygen-sensitive signaling molecule induces Oct4, a positive regulator of pluripotency, and is required to maintain germ cell numbers (Covello et al., 2006). An intriguing possibility is that similar mechanisms may work in the development of the CNS. In support of this, lowered oxygen represses neuronal differentiation of rat precursors by the combined activity of HIF1α and Notch, while 20% oxygen down-regulates these signals and can lead to neuronal differentiation (Gustafsson et al., 2005). Likewise, we find that differentiation is the principal response of human SVZ precursors to 20% oxygen. It is also possible that senescence occurs after 20% oxygen exposure, as has been shown in long-term cultures of human fetal cortical precursors (Wright et al., 2006). Unlike that study, our cultures do not growth-arrest after extended culture in 20% oxygen, but the proportion of $GFAP^+$ glia increases. Thus, we cannot rule out that senescence is occurring selectively in the $nestin^+$ $GFAP^-$ precursor population, but our results clearly show that long-term expansion of $nestin^+$ cells in enhanced in lowered oxygen.

Our study is the first to show higher proportions of oligodendrocytes generated from human CNS precursor cultures grown in lowered oxygen and an enhanced differentiation of these cells after switching to 20% oxygen. Oligodendrocytes can be generated at 20% oxygen from freshly isolated human brain with short-term expansion (Windrem et al., 2004), or from human embryonic stem (ES) cells (Keirstead et al., 2005; Nistor et al., 2005). However, extensively expanded human cortical precursors generate few oligodendrocytes (Kim et al., 2006; Wright et al., 2006) in standard expansion conditions. The use of suspension culturing may have reduced oxygen tension in the dense core of the neurospheres (Tokuda et al., 2000) or exerted a protective anti-oxidant effect that limits apoptosis or senescence of these cells (Itahana et al., 2004; Limoli et al., 2004; Madhavan et al., 2006). However, any short-term benefit may be lost with repeated passaging and expansion. Addition of factors that promote the oligodendrocyte lineage, such as platelet-derived growth factor (PDGF) or tri-iodothyronine (T_3) (Keirstead et al., 2005; Nistor et al., 2005), may also provide pro-survival effects and even induce hypoxia-response proteins like HIF1α (Schultz et al., 2006). This is in contrast to our monolayer cultures, in which

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each cell had more equivalent exposure to ambient oxygen tensions. This enhanced oxygen sensitivity of the oligodendrocyte lineage is consistent with previous reports showing that anoxia (Back et al., 2002), oxidative stress (Back et al., 1998; Fern and Moller, 2000) and hyper-oxia (Gerstner et al., 2006) preferentially damage immature oligodendrocytes but not their more differentiated derivatives. We find that the number of $O4^+$ cells is not decreased by acute exposure to 20% oxygen but that $p21^{cip1}$ is induced in these cells, indicating that increasing oxygen tension depletes oligodendrocyte progenitors primarily through terminal differentiation.

Surprisingly, we find that oxygen also modulates BMP signaling. Noggin leads to even greater expansion of $nestin^+$ precursors at 5% oxygen but not 20% oxygen, while BMP2 promotes *Id1* expression and gliogenesis at 20% but not 5% oxygen. Our results are notable since BMPs are strong gliogenic factors (Chen and Panchision, 2007; Gross et al., 1996; Rajan et al., 2003; Sailer et al., 2005). Human precursor cultures are often highly enriched in $nestin^+$ $GFAP^+$ cells (Walton et al., 2006), which may be multipotent radial glia (Zecevic, 2004) or glial-committed progenitors. Our results indicate that the synergistic actions of low oxygen and noggin reduce BMP signaling below the gliogenic threshold, suggesting that endogenous BMP activity may be generating both $nestin^+$ $GFAP^+$ and $GFAP^+$ cells while inhibiting the generation of oligodendrocytes (Mekki-Dauriac et al., 2002; Samanta and Kessler, 2004). Although an interaction between oxygen and BMPs has not previously been reported in CNS precursors, hypoxia acts through a C-terminal-binding protein (Ctbp1)-dependent mechanisms to repress BMP-responsive genes in pulmonary cells (Wu et al., 2006). Unlike that study, we find that in CNS precursors oxygen acts in a novel manner to directly repress SMAD activation (Fig. 3C). Our future studies will determine if oxygen tension uses a HIF-dependent or novel mechanism to regulate BMP-mediated fate choice in human CNS precursors.

Oxygen may be an important regulator of cell function during development, but there is currently little data on how tissue oxygenation changes during gestation and postnatally. While microdialysis catheters have been used for more than a decade to determine local concentrations of neurotransmitters, growth factors and metabolites in a variety of disease states (Goodman et al., 1996; Hlatky et al., 2004; Ungerstedt, 1997; Vespa et al., 2003), brain tissue oxygen monitors have been developed only recently to accurately assess the brain oxygen content in either damaged or at-risk tissue. At present, the desired and optimal concentration of oxygen within both damaged and non-damaged tissue is widely debated (Menon et al., 2004; Stiefel et al., 2005), but PO_2 measurements in humans are consistent with the 0.55% (4.1 mm Hg) to 8.0% (60 mm Hg) ranges measured in other mammals (Erecinska and Silver, 2001).

In contrast, preparation and analyses of isolated precursor cells are typically performed in a near-room atmosphere of 20% oxygen balanced with 5% carbon dioxide, conditions which far exceed oxygen tensions measured in vivo (Erecinska and Silver, 2001). The principal exception has been in vitro fertilization studies. Experiments on both human and mouse pre-implantation embryos indicate that survival and proliferation is maximized in lowered oxygen (Adam et al., 2004; Catt and Henman, 2000; Kilani et al., 2003). Moreover, in vitro analysis of $CD34^+$ cells from human cord blood revealed that culture in 3% oxygen has a minimal effect on the expansion of colony-forming cells (a transit-amplifying population), but promotes the expansion and maintenance of the long-term reconstituting stem cell population as compared to 20% oxygen culture (Ivanovic et al., 2004). It was noted in this study that 3% oxygen is similar to the oxygen tensions measured in bone marrow.

Recent progress in stem cell biology has improved the possibility of treating patients by transplanting new cells that can replace those lost through trauma or disease. We show that oxygen tension regulates human CNS precursors and the oligodendrocyte fate at multiple stages, suggesting that regulating oxygen may be critical to cell replacement for demyelinating disorders. Additionally, recent studies support an endogenous oxygen-sensitive regenerative capacity in the CNS (Arvidsson et al., 2002; Nakatomi et al., 2002), suggesting that clinical modulation of oxygenation in stroke patients may affect stem cell recruitment, neurogenesis and gliogenesis (Androutsellis-Theotokis et al., 2006). Finally, tumor hypoxia is positively correlated with poor clinical prognosis (Postovit et al., 2002). Since some cancers are initiated by cancer stem cells (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Singh et al., 2004), lowered oxygen may actually promote tumorigenesis (Jogi et al., 2002) and thus be an important variable to control for cancer therapies.

Experimental methods

Atmosphere-controlled incubation

For culture in 20% oxygen, cells were incubated in a Forma Series II CO_2 incubator; CO_2 was added to maintain a 20% O_2, 5% CO_2 75% N_2 balance. For culture in 5% oxygen, cells were incubated in a customized, computer-controlled system (Biospherix, Ltd., Syracuse, NY). This system controls temperature and gas levels during all phases of culturing and experimentation (5% O_2, 5% CO_2, 90% N_2 balance in this study) and eliminates artifacts introduced by periodic high oxygen exposure, such as might occur during examination of cells under the typical laboratory microscope. The system consists of (1) a modular remote chamber for maintaining cultures; (2) a glove box for cell manipulation, and (3) an attached chamber containing a microscope (Axiovert 10, Zeiss), with a mounted digital camera (QColor3, Olympus), for cell visualization and recording (Fig. 1B).

Expansion and differentiation of human CNS SVZ precursors

Institutional Review Board approval was obtained at both Children's National Medical Center and Children's Hospital of Orange County for the acquisition of human brain tissue. Normal neural precursor cells were derived from brain subventricular zone (SVZ) tissue of a premature neonate that died shortly after birth from pulmonary failure; the continuous culture from this tissue is denoted SC30 (Schwartz et al., 2003). For the present experiments, cells were cultured on fibronectin-coated dishes in DMEM/F12 (Irvine Scientific, Irvine, CA) supplemented with BIT9500 (1% bovine serum albumin, 10 μg/ml rh insulin, 200 μg/ml human transferrin; Stem Cell Technologies, CA), 20 ng/ml basic fibroblast growth factor, (bFGF) and 10 ng/ml epidermal growth factor (EGF, both from R&D Systems, Minneapolis, MN). For continuous expansion, one-half of this medium was replaced every day and cultures were passaged every 7th day using Cell Dissociation Buffer (Invitrogen, Carlsbad, CA). In some experiments, cultures were supplemented with erythropoietin (10 ng/ml; R&D Systems), ascorbic acid (200 μM; Sigma, St. Louis, MO), B27 without retinoic acid (2% v/v; Invitrogen), bone morphogenetic protein 2 (BMP2, 10 ng/ml; R&D Systems) or noggin (200 ng/ml; R&D Systems).

For terminal fate and oxygen switching experiments, cells were expanded for 7 days and then precursor cell differentiation was induced by culturing cells in DMEM/F12/BIT9500 in the absence of mitogens. After 2 days post-mitogen withdrawal, cells were supplemented with 2% B27 (Invitrogen), 10 ng/ml neurotrophin-3 and 10 ng/ml ciliary neurotrophic factor (both from R&D Systems) to promote maturation of post-mitotic cells. After an additional 19 days (21 days differentiation total), cells were fixed in cold 4% paraformaldehyde for 15 min, rinsed and stored prior to analysis.

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Immunocytochemistry

Immunofluorescence was performed using primary antibodies against Ki67 (mouse, 1:100; Dako), nestin (rabbit, 1:100; McKay lab), activated caspase3 (CM-1, rabbit, 1:4000; Idun), glial fibrillary acidic protein (GFAP, mouse, 1:1000; Sigma), polysialated neural cell adhesion molecule (PSA-NCAM, mouse, 1:400; Chemicon), βIII-tubulin (rabbit, 1:2000; Covance), galactocerebroside-C (GalC, mouse, 1:300; Chemicon), PDGFRα (rabbit, 1:100; Santa Cruz), O4 (mouse, 1:100; Sigma), $p21^{cip1}$ (mouse, 1:800; LabVision), phospho-p53 Sampler Kit (as directed; Cell Signaling), phospho-Smad1/5/8 (rabbit, 1:100; Cell Signaling) or Id1 (rabbit, 1:50; Santa Cruz). After incubation, cells were washed and incubated with species-specific fluorescent secondary antibodies (Alexa dyes; Invitrogen). Cells were nuclear-counterstained with 4′,6-diamidino-2-phyenylindole (DAPI) to measure total cell number. Staining was visualized by epifluorescence (BX60 upright microscope, Olympus) and images compiled for figures using Photoshop 7.0 (Adobe). Data was quantified as total cell number or marker-expressing cells as a percentage of total (nuclear DAPI-stained) cells.

Flow cytometry and fluorescence-activated cell sorting (FACS)

After culture at 5% or 20% oxygen, cells were passaged and resuspended in flow cytometry buffer, consisting of 1× HBSS, pH 7.2, containing 1.55 g/l glucose and 0.1% fraction V of bovine serum albumin (BSA; Sigma). Cells were counted and diluted to a density of 4×10^6 cells/ml buffer; analysis was performed with 25 μl aliquots containing 1×10^5 cells. For surface marker analysis, we used antibodies against human CD133 (clone AC133/2-PE, as directed; Miltenyi) and human CD24 (FITC-conjugated mouse IgG_{2a}; BD Biosciences). Cells were incubated at 4 °C for 30 min, washed in buffer and resuspended in 400 μl buffer. For viability analysis, we added 7-amino-actinomycin-D (7-AAD, 50 ng/ml final; BD Biosciences) or Annexin-V-APC (1:200, as directed; BD Biosciences) prior to analysis. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) as previously described (Panchision et al., in press). Fluorescent intensities for cells in the population were point-plotted on 2-axis graphs or histogram using CellQuest software (BD Biosciences).

For FACS, cells were labeled as described above, then run on fluorescence-activated cell-sorter (FACS), either a FACSAria (BD Biosciences) or Influx (Cytopeia) sorter as previously described (Panchision et al., in press). Single-viable cells were gated based on Annexin-V exclusion and pulse-width, then physically sorted into 96-well plates or into collection tubes for plating. Post-sort purity analysis was performed on aliquots from each sort group. FACSDiva or FlowJo software was used for analysis.

Statistical analysis

Graphs and statistical analyses were prepared using Prism 3.03 (Graph Pad). All values presented as mean±standard error of the mean (S.E.M.). Statistical significance was measured by simple paired *t*-tests or one-way ANOVA with post-hoc Tukey's test, $*p<0.05$, $**p<0.01$, $***p<0.001$. For all graphs, an asterisk directly above a bar indicates a significant difference with its 5% oxygen counterpart; an asterisk over a bracket indicates a significant difference with another variable as indicated.

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