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Oxygen Tension Controls the Expansion and Differentiation of Normal and Tumor-derived Human Neural Stem Cells.

Role of oxygen in BMP responsiveness

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APPENDIX

PAGE

SUMMARY

During neural development the generation of diverse cell types involves the response of precursor cells to a wide variety of environmental cues, like soluble factors, the extracellular matrix and oxygen tension. Among these, oxygen tension and oxidation state in particular are important biophysical parameters that control neural precursor proliferation, survival and fate determination, so the dynamic control of oxygen availability regulates self renewal and the generation of cell diversity during development and throughout the life of the organism. However, the mechanisms by which oxygen acts in this manner are poorly understood.

Current evidence suggests that oxygen levels in both developing and mature brain are much lower than the 20% oxygen used in standard mammalian cell culture (Erecinska M. et al. 2001).

Previously it has been found that the sensitivity to oxygen tension is greater in mouse than in rat cells. Precursor survival and expansion is greatly improved at an oxygen tension (2-5%) closer to that measured in vivo and far lower than conditions typically used (20%) in culture studies. In the last decade much research has focused on the detrimental effect of anoxia during ischemic episodes and these results indicate that atmospheric oxygenation is incompatible with long-term precursors cell survival. Furthermore, one of the main effort is directed to maximize the culture efficiency of neural precursors for replacement therapies and also in vitro fertilization research has focused on oxygenation in order to optimize viability of the early post-fertilization embryo. It will be very important to understand the signals that control survival, proliferation and fate choice of precursor cells and it will also be necessary to investigate what subtypes of precursors are preferentially selected under normal in vitro condition. Indeed, the addition of antioxidants and other free radical scavengers is likely to be no more than a sub-optimal surrogate for culturing in lowered oxygen.

Moreover, it has been recently shown that hypoxia is a crucial component of the brain tumor niche as it positively correlates with tumor aggressiveness and over-activity of Hypoxia Inducible Factor-1α (HIF-1α) reinforces tumor progression.

Thus, the main aim of the project is to understand the role of oxygen tension in proliferation and lineage determination of Human CNS (Central Nervous System) and brain tumor derived precursor cells. We sought to understand if a lower oxygen tension (2-5%), compared to environmental 20% oxygen, promotes the expansion of a more premature and actively proliferating subtype of precursor cells, affecting cell multipotency, and which could be the molecular pathways modulated by oxygen tension.

Our results indicate that dynamic control of oxygen tension regulates different steps in fate and maturation and may be crucial for treating neurodegenerative diseases (i.e. demyelinating diseases). They also suggest that the maintenance of brain tumors stem-ness, particularly in high Grade Glioma (HGG) tumors, is correlated to a hypoxic microenvironment in which BMP signaling pathway and the pro-differentiating effects mediated by BMP are down-regulated.

SOMMARIO

Durante lo sviluppo cerebrale le cellule progenitrici dei diversi sottotipi cellulari, rispondono ad un'ampia varietà di stimoli ambientali, come fattori solubili, la matrice extracellulare e la tensione di ossigeno. Fra questi, la tensione di ossigeno e lo stato ossidativo in particolare sono importanti parametri biofisici per il controllo della proliferazione dei precursori cerebrali, della loro sopravvivenza e della determinazione del destino cellulare, quindi il controllo dinamico della disponibilità di ossigeno regola l'autorigenerazione cellulare e il differenziamento sia durante lo sviluppo embrionale che durante l'intera vita dell'organismo. Ad ogni modo, i meccanismi con i quali la tensione di ossigeno modula queste risposte non state ancora del tutto compresi.

Recenti scoperte, hanno evidenziato che i livelli di ossigeno nel cervello in fase di sviluppo e in quello adulto sono inferiori rispetto ai livelli ambientali comunemente usati anche nella coltura in vitro di cellule di mammifero. Precedentemente è stato visto che cellule cerebrali di topo sono molto più sensibili di quelle di ratto alla tensione di ossigeno. La sopravvivenza e la proliferazione delle cellule progenitrici è ampiamente incrementata da una tensione di ossigeno più simile a quella misurata in vivo (2-5%) e decisamente più bassa di quella comunemente usata per colture cellulari (20%). Nell'ultimo decennio molti studi si sono focalizzati sugli effetti deleteri dell'anossia durante episodi di tipo ischemico e i risultati ottenuti indicano che l'ossigeno atmosferico è incompatibile con la sopravvivenza a lungo termine dei precursori cellulari. Inoltre molti tentativi di miglioramento della efficienza di coltura delle cellule staminali utili per terapie di trapianto e anche la ricerca sulla fertilizzazione in vitro si sono concentrate sulla modulazione dell'ossigenazione, anche per ottimizzare la vitalità dell'embrione ottenuto in seguito alla fertilizzazione. Attualmente è di grande importanza capire quali segnali controllano la sopravvivenza, la proliferazione e il differenziamento dei precursori cellulari e sarà anche necessario comprendere quali siano sottotipi di cellule progenitrici che più ampiamente vengono selezionati nelle normali condizioni di coltura in vitro. Inoltre l'aggiunta di antoossidanti si è rivelata essere una condizione subottimale rispetto alla coltura in vitro con un inferiore livello di ossigeno (5%).

Inoltre è stato scoperto che l'ipossia (0.2%-2%) è un componente cruciale del microambinete dei tumori cerebrali in quanto correla positivamente con l'aggressività del tumore, e l'iperattivazione del Hypoxia Inducible Factor-1α (HIF-1α) rinforza la progressione tumorale.

Quindi, lo scopo principale del nostro progetto è stato quello di capire quali effetti la tensione di ossigeno esercitasse sulla proliferazione e sulla determinazione del lineage delle cellule progenitrici del sistema nervoso centrale e di cellule ottenute da biopsie di tumori cerebrali. Si è cercato di comprendere se una più bassa tensione di ossigeno (2-5%), rispetto a quella ambientale (20%) promuovesse l'espansione di una sottopopolazione più immatura e attivamente proliferante di precursori cerebrali, se influisse sulla multipotenzialità e sulla capacità di differenziamento cellulare se vi fossero particolari pathway molecolari modulati dall'ossigeno. I risultati ottenuti indicano che il controllo dinamico della tensione di ossigeno risulta essere cruciale nella regolazione della crescita e del differenziamento delle cellule progenitrici neurali, soprattutto al fine di un miglioramento delle terapie di trapianto per malattie neurodegenerative (es. malattie demielinizzanti). Inoltre suggeriscono che il mantenimento della staminalità dei tumori cerebrali, in particolare dei glomi di alto grado (High Grade Gliomas, HGGs), è correlata alla presenza di un microambiente ipossico, all'interno del quale i segnali prodifferenziativi mediati dalle Bone Morhogenetic Proteins (BMPs) vengono downregolati.

INTRODUCTION

I PART: oxygen tension modulation of human postnatal subventricular zone (SVZ) derived precursors cells proliferation and differentiation.

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).

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 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).

In this study 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.

II PART: oxygen tension modulation of brain tumor derived cells proliferation.

Central Nervous System (CNS) tumors represent the 22% of all pediatric malignancies up to 14 years and 10% of tumors in 15-19 year-olds and they are one of the leading causes of cancer death in children. Grade III anaplastic astrocytoma and grade IV glioblastoma multiforme (GBM) make up the high-grade gliomas (HGG), which are commonly referred

to as malignant glioma to reflect their invasive nature and capacity to disseminate (Rood BR and MacDonald TJ, 2005). Nowadays, outcome for children and adults with primary HGG is still poor, thus understanding the intrinsic properties that characterize HGG tumor cells, particularly the more immature cells (also called cancer stem cells) and the identification of the microenvironmental signals that regulate tumor cells proliferation and survival, result to be critical for the development of successful and more selective chemotherapies.

High grade gliomas are highly vascularized, with the tendency to infiltrate and they are characterized by extensive areas of necrosis and hypoxia. Recent literature shows that hypoxia positively correlate with tumor aggressiveness (Azuma Y et al. 2003) (Helczynska K et al. 2003) (Jogi A et al 2002) and over-activity of Hypoxia Inducible Factor-1α (HIF-1α) (Smith K et al. 2005) is implicated in tumor progression. The correlation between hypoxia and tumor aggressiveness has been causally linked to increased genomic instability (Koshiji M et al. 2005), but it is also related to increased survival of proliferating cells by suppression of p53 and its associated cell growth control (Zhang L, Hill RP. 2004). Importantly, hypoxia has been shown to promote de-differentiation of neuroblastoma cells (Jogi A et al. 2002) (Jogi A et al. 2003) (Jogi A et al. 2004), suggesting that it may reinforce an environment for aggressive tumor growth. It may also prevent a pre-existing stem cell population from differentiating, which is important in light of increasing evidence that cancer is initiated by dysfunctional stem cells (Al-Hajj M et al. 2003) (Bonnet D et al. 1997) (Singh SK et al. 2004).

Additionally, it is known the involvement of signalling molecules, such as bone morphogenetic proteins (BMPs) as crucial mediators of stem cell self renewal and cell fate determination, which appear disrupted in brain cancers. Particularly, BMPs have been shown to be strong inducers of astroglial fates (Chen and Panchision, 2007) while 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) (Fig.1A).

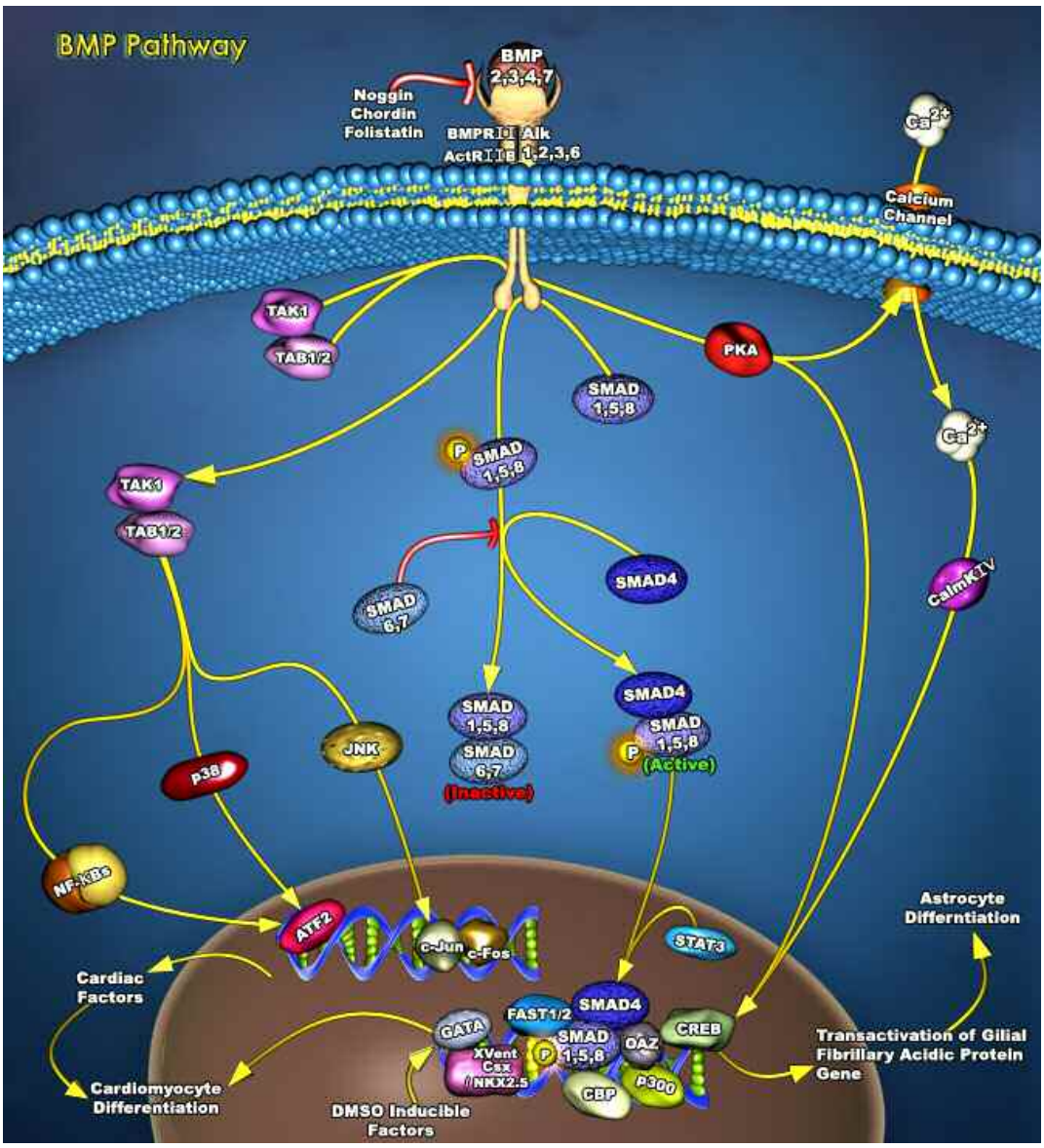


Figure 1A. BMP Signaling Pathway.

Recently, Piccirillo et al. showed that BMP4 (and analogously BMP2) can modulate glial differentiation through increase in GFAP expression in primary cultures of glioblastoma (GBM), in particular in the brain tumor inducing population, thus indicating BMPs as potentially useful candidate molecules to promote differentiation of the tumor-initiating cells.

In the results described in the 1st part of this study (part I) we have related BMP2 signaling activation in normal postnatal sub-ventricular zone (SVZ) derived cells, to oxygen tension; in particular, we found that 5% oxygen, which is in the physiological range, inhibits BMP2 signaling and consequentially activation of phospho-Smad 1/5/8 and Id1 expression (inducer of astroglial fate, downstream target of BMPs stimulation) are diminished. These results suggest that oxygen is an important biophysical parameter in the regulation of normal neural precursor proliferation and differentiation.

In light of these considerations, in the 2nd part of our research we investigated the role of oxygen tension, by comparing a lowered oxygen tension (5% and also 2% oxygen, to test a much stronger hypoxic condition) versus environmental 20%, in the modulation of High Grade Glioma (HGG) derived cells proliferation and their response to BMP in vitro stimulation. Our results indicate that HGG cells tend to expand in vitro in all the oxygen tensions tested, even though for a higher number of passages under lowered oxygen, but at 20% oxygen, expansion of more differentiated GFAP+ cells at the expense of nestin+ precursors increases and mitotic arrest is induced. Importantly, the astroglial commitment seen under 20% oxygen seems to be related to endogenous activation of the BMP signaling pathway, as shown after rapid 20% oxygen exposure.

Furthermore, treatment with BMP2 has a specific anti-proliferative and pro-differentiating action toward HGGs cells, effects that are diversely modulated by oxygen tension, while not promoting any significative effect in normal cells. Finally, Hypoxia Inducible Factor-α (Hif1-α), which appears to be highly expressed in HGG cells even regardless the presence of hypoxic condition, is down-regulated by BMP2 treatment in both the oxygen tensions tested.

Thus, based on these results, we suggest that a hypoxic microenvironment in the brain tumor niche maintains stem-ness also by down-regulating BMP signaling pathway and its pro-differentiating effects.

Besides studying HGG derived cells as described, we also investigated the role of oxygen tension in the modulation of medulloblastoma (MDB) cells proliferation and their response to BMP in vitro stimulation and Notch signaling activation and inhibition. It is known that hypoxia requires notch signalling (Fig. 1B) to maintain the undifferentiated cell state in a mouse myogenic cell line C2C12 and mouse embryonic teratocarcinoma cell line P19

(Maria V. Gustafsson et al. 2005) and notch pathway inhibition in DAOY cells, by pharmacologic inhibitors of γ-secretase, depletes stem-like cells and blocks engraftment (Fan X et al. 2006).

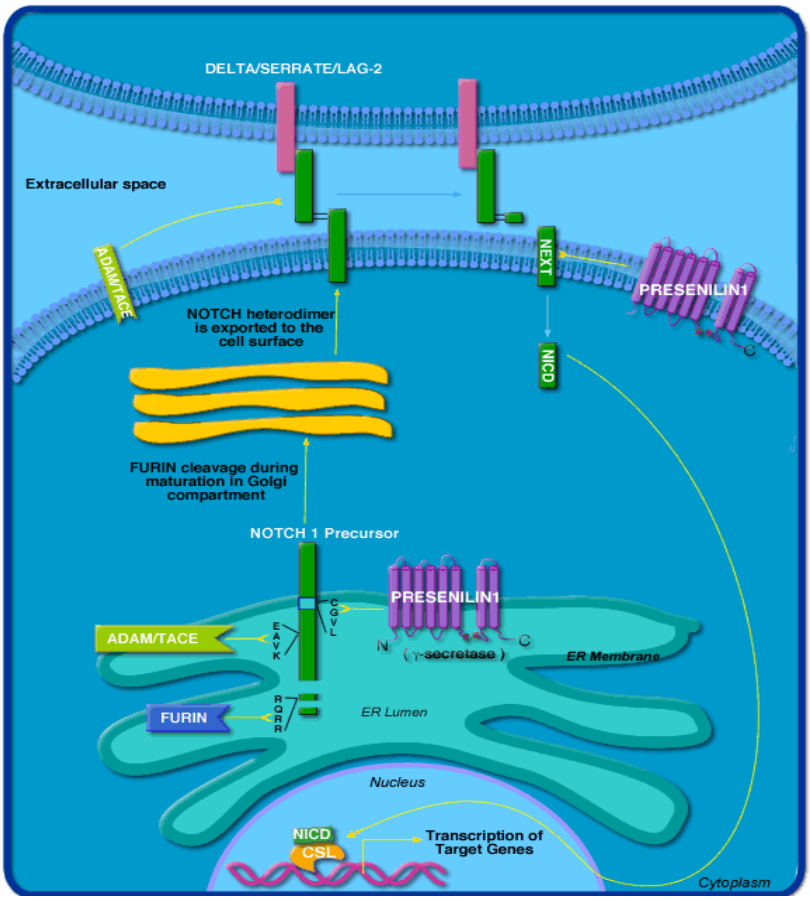


Figure1B. Notch Signaling Pathway.

Medulloblastoma is the most common cancerous brain tumor of childhood. It accounts for 20% to 25% of all childhood tumors, and they are malignant, invasive embryonal tumor of the cerebellum, predominantly displaying neuronal differentiation, and an inherent tendency to metastasize via cerebrospinal fluid pathways. Although it is thought that medulloblastomas originate from immature or embryonal cells at their earliest stage of development, the exact cell of origin, or "medulloblast" has yet to be identified. It is currently thought that medulloblastoma arises from cerebellar "stem cells" that have been prevented from dividing and differentiating into their normal cell types.

Additionally, it has been reported that in medulloblastoma cells there is a higher endogenous Notch activation (Raffel C et al. 1997) (Fan X et al 2004) (Hallahan AR et al 2004), (Fan X et al 2006). Notch is known to promote the survival and proliferation of nonneoplastic neural stem cells and to inhibit their differentiation (Reya T, et al 2001) (Solecki DJ, et al 2001). Signaling is initiated by ligand binding, followed by intramembranous proteolytic cleavage of the Notch receptor by the γ-secretase complex. Inhibitors of this complex slow the growth of Notch-dependent tumors such as medulloblastoma and T-cell leukemia (Fan X, et al. 2004) (Hallahan AR, et al. 2004). The results we obtained indicate that MDB derived cells, can be expanded successfully in vitro only when cultured at 2-5% oxygen, while at 20% oxygen cells differentiate and eventually die after undergoing mitotic arrest. In MDB derived cells BMP2 does not seem to induce any major effect but inhibition of BMP2 with noggin, while does not induce any notable effects under hypoxic conditions, seems to increase the already high vulnerability of these tumor cells to high oxygen tension; importantly we also found that inhibition of notch activation by a γ-secretase inhibitor induces differentiation in different ways comparing normal and MDB derived cells and DLL4-mediated notch activation is differentially modulated by oxygen tension.

We also performed grafting in the motor cortex of NOD SCID mice of the tumor derived cells tested as above, a routinely used in vivo model to test tumor expansion after cortical engraftment. This set of experiments has been important to validate tumor-initiating cell populations and assay interactions between tumor stem cells and a more physiologically similar microenvironment (i.e. murine brain cortex).

Indeed, recent evidence indicates that tumors consistently arise from a specific subset of cells defined as cancer stem cells. Two studies (Bonnet D et al. 1997) (Blair A et al. 1997) found that cells with the highest capacity to re-initiate new tumors exhibit properties nearly identical to those of stem cells. This has been shown for acute myeloid leukemia and for breast carcinoma tumors by using cell surface selectable markers. Dissociated CNS tumor cells express primitive markers (i.e. CD133, Sox2, Musashi1, Bmi1 and Nestin) and exhibit multipotency when placed in defined serum-free stem cell medium (Singh SK et al. 2003)

(Hemmati HD et al. 2003) (Ignatova TN et al. 2002) (Guzman ML, Jordan CT, 2004). Additionally, $CD133^{hi}$ cells show the highest frequency of initiating new glioblastoma and medulloblastoma tumors when grafted in NOD/SCID mice (Singh SK et al. 2004). Although many tumors contain cells that display stem cell like-features, the identity of the stem cell that acquires the initial genetic mutation leading to tumor formation has remained elusive. A normal stem cell is defined by its dual properties of self-renewal and multilineage differentiation potential, and serves to repopulate the mature cells pool of its host organ system. A cancer stem cell would function in a similar way to sustain the growth and spread of tumors, however, it would not be subjected to the same intrinsic and extrinsic controls as normal stem cells. By using magnetic cell sorting and flow cytometry analysis, based on expression of the cell surface antigen CD133, we isolated the cancer stem cell population reproducibly from primary cultures of both HGG and MDB. This population has been routinely grafted into NOD/SCID mice brain parenchyma and was able to reliably reconstitute the original tumor phenotype. Interestingly, we also found that MDB derived cells, which are normally poorly growing in vitro, were able to acquire a proliferative capacity considerably higher after in vivo transplantation, with a strong increase in nestin+ and Ki67+ cells when compared to their in vitro counterpart.

MAIN AIMS OF THE STUDY

To address the role of oxygen at defined steps in human neural precursors and human brain tumor derived cells expansion and differentiation, we utilized a novel system to control gas composition during incubation, microscopy-aided recording and experimental manipulation (Fig. 2B). Our main aim was understanding how oxygen tension regulates proliferation and fate choice of normal human postnatal subventricular zone (SVZ) derived precursor cells (Part I). In particular we sought to elucidate which were the molecular pathways involved in oxygen tension sensitivity and also which specific lineage steps were affected by oxygen.

Based on the results obtained in part I, in the second part of our study (Part II) we investigated the role of oxygen tension, by comparing an even lowered oxygen (2%) tension versus environmental 20%, in the modulation of High Grade Glioma (HGG) derived cells proliferation and their response to BMP in vitro stimulation and also of Medulloblastoma (MDB) derived cells, and their response to Notch signaling activation and inhibition.

We also performed in vivo grafting of brain tumor derived cells and CD133+ isolated brain tumor stem cells with the main goal to validate tumor-initiating cell populations and assay interactions between tumor stem cells and a more physiologically similar microenvironment.

RESULTS (PART I)

The results obtained for part I and partially for part II have been collected during the first 2 years of doctorate in the laboratories of the Center for Neuroscience Research, in Children's National Medical Center, Washington, DC, where I worked as a visiting PhD student with Dr. Panchision, PhD. Results in part I have been published (Pistollato, F., et al. Mol Cell Neurosci. 2007 Jul;35(3):424-435. Epub 2007 Apr 12).

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. These differences were seen upon the first passage and became greater in subsequent passages (Fig 2C). 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 antioxidants 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. 2E). Epo had no effect on SVZ precursors at 10 ng/ml (Fig. 2E) 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.

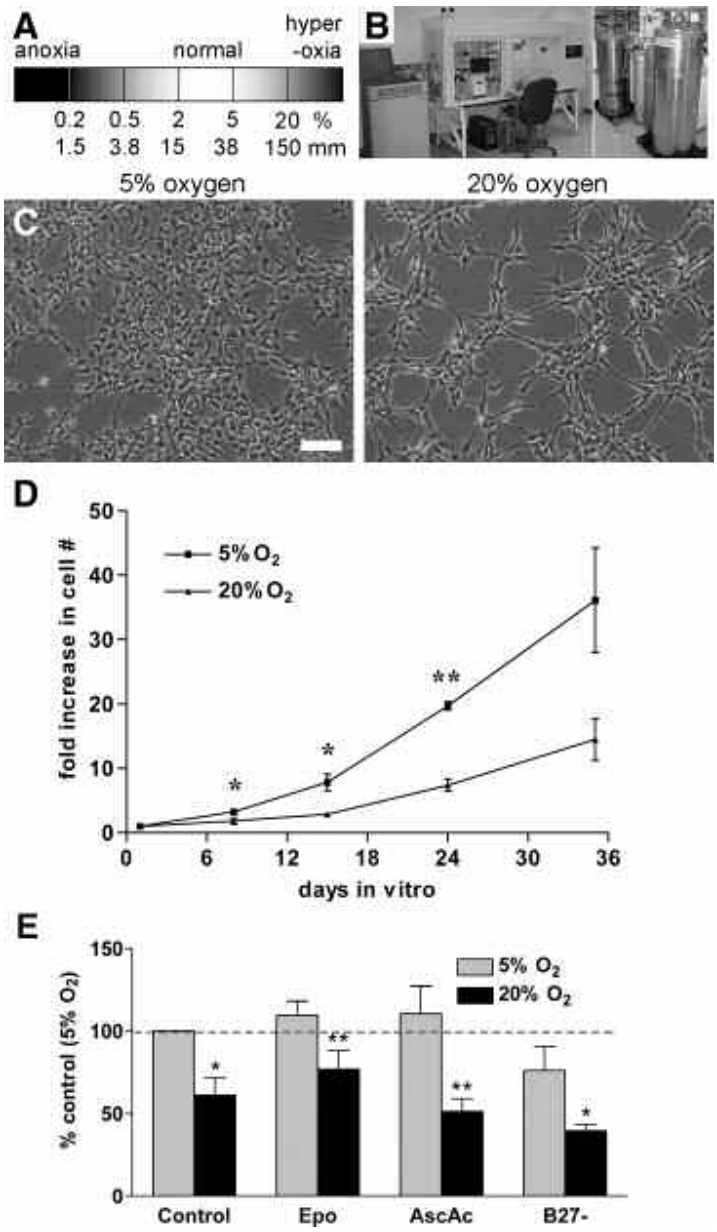


Figure 2. Lowered oxygen promotes expansion of human CNS subventricular precursors over extended times. (A) Physiological oxygenation (normoxia, light shade) of different adult mammalian brain regions ranges from 0.5% to 8% oxygen (3.8 to 61 mm Hg), while 21% room oxygen tension is hyperoxic (from Erecinska and Silver, 2001). (B) Environmental control system used for these studies. (C) Expansion of human SVZ cells, plated at equivalent densities and expanded 7-10 d 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.

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. 3A), 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. So 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. 3B) with less than 8% GFAP$^+$ cells (Fig. 3C). The majority of the remaining cells were nestin$^+$βIII-tubulin$^+$ with some nestin$^+$GFAP$^+$ (not shown). With increasing plating density, at almost 100% confluence, the percentage of nestin$^+$ cells dropped to near-zero, while GFAP$^+$ cells eventually made up 80% of the population. This is consistent with 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. 3B). This was most pronounced at the lowest densities that otherwise, 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 3C). Thus, GFAP$^+$ cells are enhanced by both higher oxygen tensions (even at low plating densities) and increasing density, while nestin$^+$ precursors are enhanced at lower oxygen tensions. In conclusion, higher densities promote a glial fate and mask the enhancement of nestin$^+$ precursor numbers by 5% oxygen.

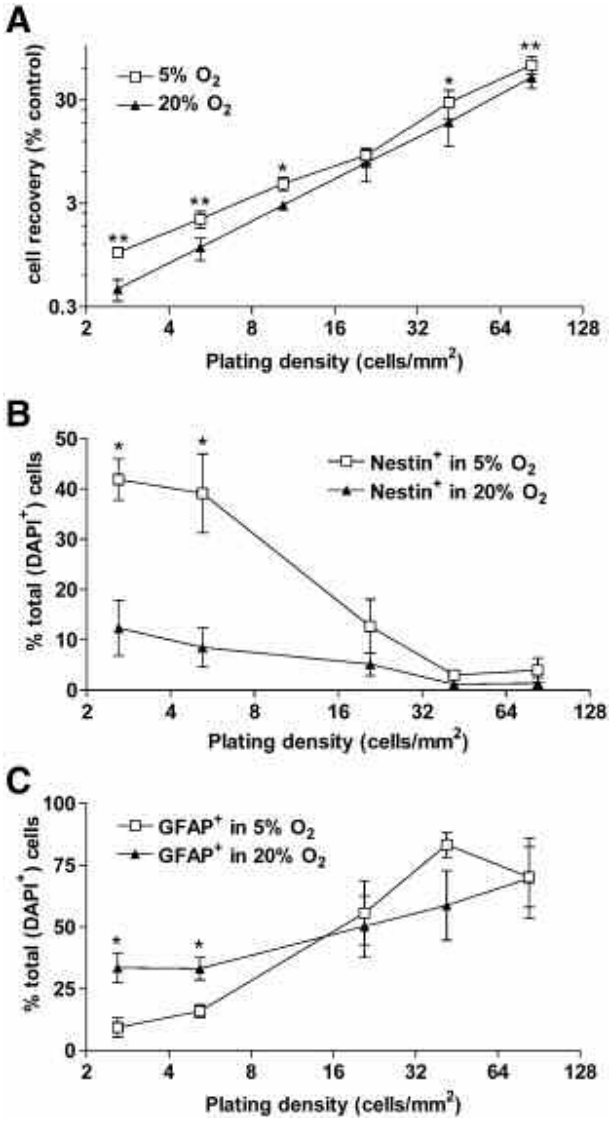


Figure 3. Higher oxygen and high densities increase GFAP$^+$ cells at the expense of nestin$^+$ cells. (A) Human SVZ cells were plated at varying plating densities and expanded 12d at either 5% or 20% oxygen. Expansion of precursors is higher at 5% versus 20% oxygen at nearly all densities tested (normalized to recovery at 5% oxygen, lowest density). (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 is 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.

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); it has been 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 medium-high density (49 cells/mm^2) and expanded 7 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. 4A). At 5% oxygen, however, noggin strongly promoted precursor expansion. We then analyzed cell phenotype in these cultures (Fig. 4B). Given the modestly high plating density there was no difference in the percentages of $Nestin^+$ and $GFAP^+$ cells comparing the control groups in the two oxygen tensions (as elucidated in fig 3B-C). 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 BMP2 in promoting a glial fate. Apoptosis, measured as cleaved-Caspase3 expression and pyknotic nuclei count, 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., 2001), 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 hours 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 4C). Furthermore, expression of Id1 protein by immunocytochemistry was increased within 24 hours of BMP2 treatment in 20% oxygen but not 5% oxygen (Fig 4D). These results indicate that lowered oxygen tension represses BMP signaling and subsequent glial differentiation of CNS precursor cells, while a higher oxygen tension promotes BMP signaling.

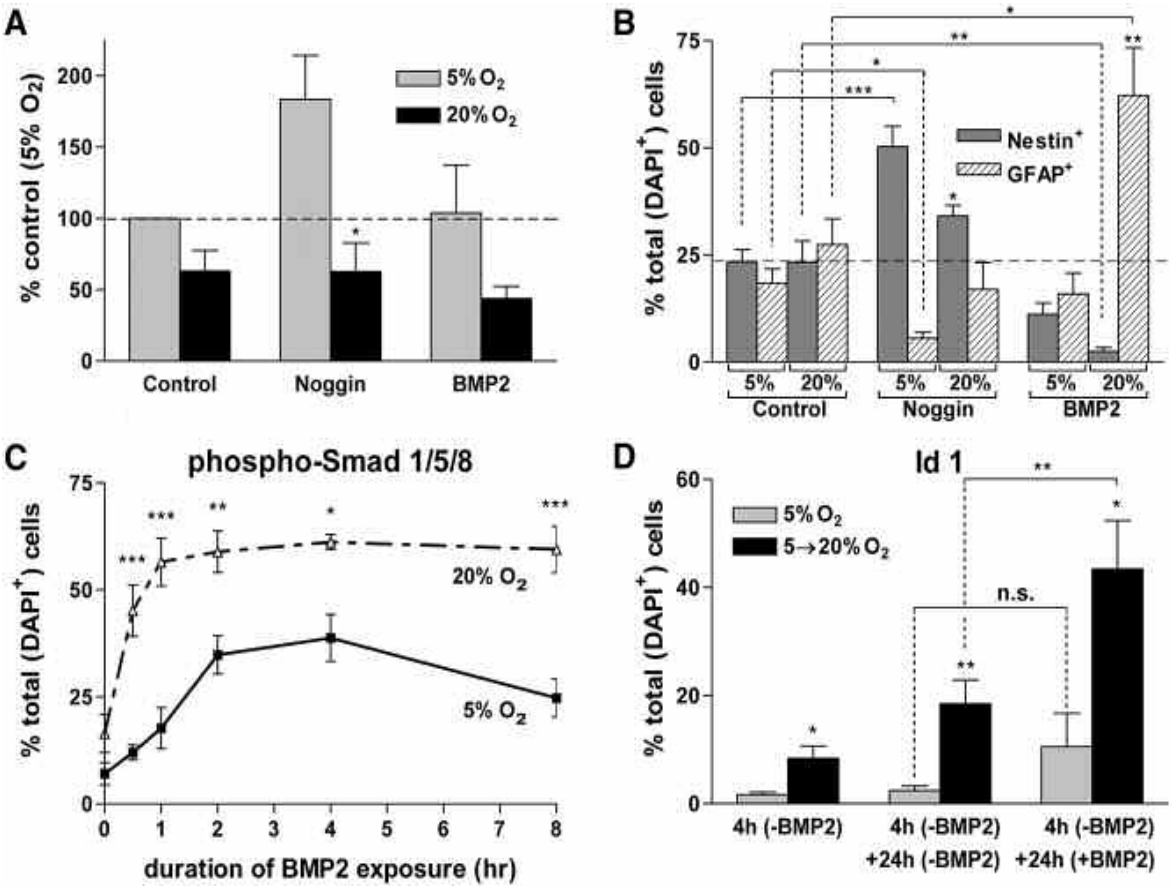


Figure 4. Lowered oxygen represses the gliogenic effect of BMP signaling. (A) Human SVZ cells were plated at medium density (49 cells/mm^2) and expanded 7 d at either 5% or 20% oxygen. At 20% oxygen, neither BMP stimulation, nor BMP inhibition by noggin, has an effect on total cell numbers. At 5% oxygen, however, noggin exposure increases cell numbers. (B) At 5% oxygen, noggin increases nestin$^+$ precursors while decreasing GFAP$^+$ glia as 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, cells are plated in replicate wells, half of which are transferred to 20% oxygen; 4 hr later cells are treated with BMP2 for the indicated time points. 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.

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 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. 5A), in cells plated at reasonable low density (8.6 cells/mm^2). Activated caspase3 was not significantly increased at 20% oxygen and pyknotic cell numbers were not changed. While the neuronal lineage was just slightly promoted as measured by $PSA\text{-}NCAM^+$ cells, $GFAP^+$ glial cells were significatively increased at 20% oxygen. These results indicate that the principal response to 20% oxygen exposure is terminal differentiation of precursors toward 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 hours or 48 hours 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 hours of exposure to 20% oxygen (Fig. 5B,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 hours but doubled at 48 hours after exposure to 20% oxygen (Fig 5C,E). Thus, exposure to 20% oxygen induces a significant activation of p53 phospho-Ser15/Ser37 within 24 hours

and p21^{cip1}-mediated mitotic arrest by 48 hours in human SVZ precursors. The principal result of this mitotic arrest appears to be astrocytic differentiation.

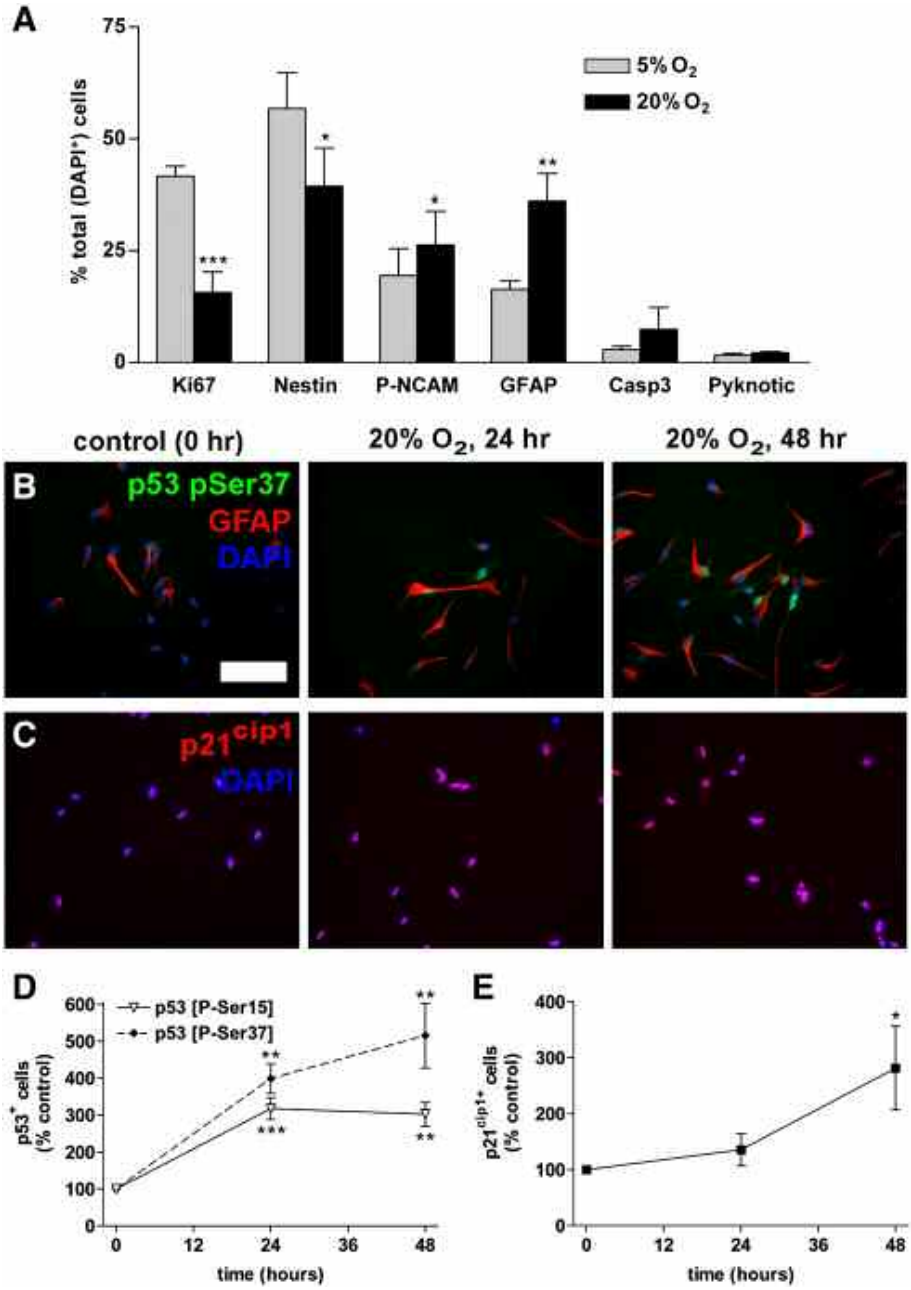


Figure 5. 20% oxygen promotes differentiation of human precursor cells. (A) Expansion of human SVZ precursor cells, plated at low density (8.6 cells/mm^2) and expanded for 10 days is enhanced at 5% oxygen as measured by Ki67 and nestin expression. In contrast, 20% oxygen decreases these markers and selectively increases GFAP$^+$ cells, suggesting glial differentiation. The neuronal phenotype changes 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 hr; 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 (Fig. 6A,B). As expected, total cell number was higher at 5% oxygen compared with 20% oxygen, but the percentage of cells expressing the neuronal marker β-III-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. 5A) 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 (Fig. 6A,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.

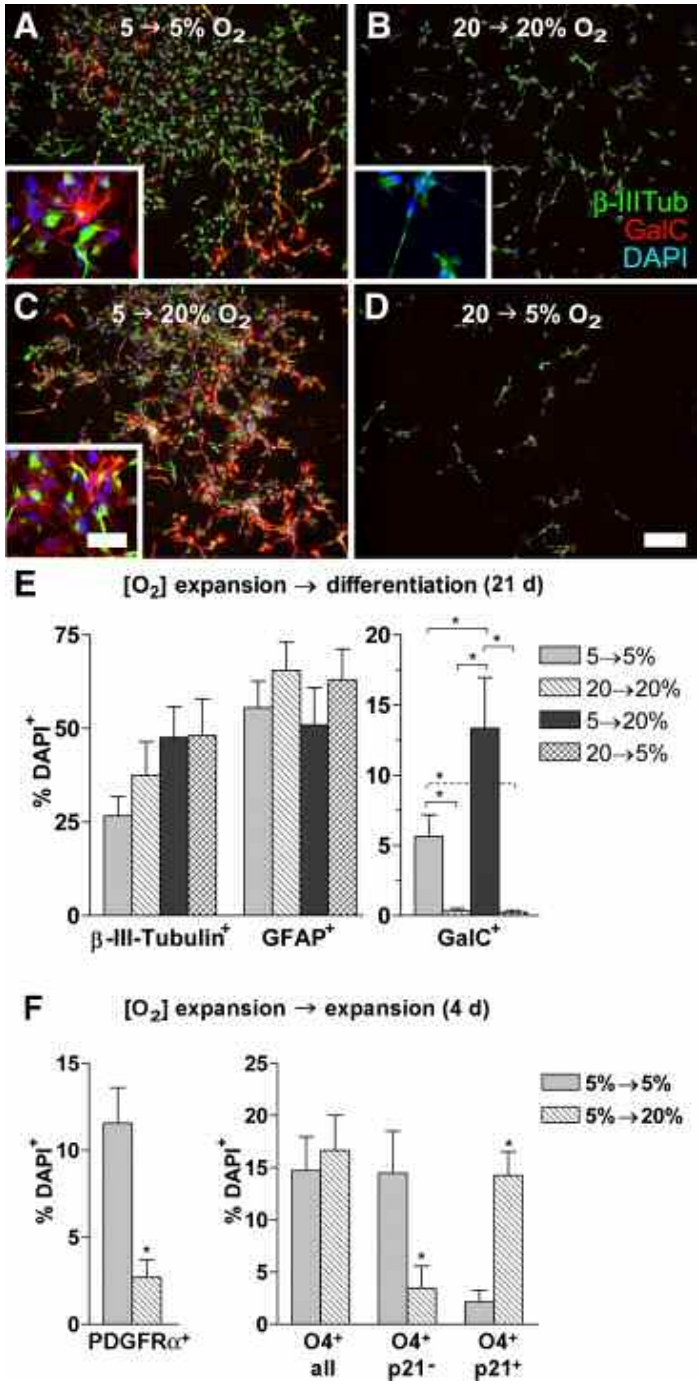


Figure 6. Lowered oxygen promotes multi-lineage competence and, after mitogen withdrawal, a subsequent increase in oxygen promotes oligodendrocyte maturation. Human SVZ precursors were cultured for 7 d expansion and 21 d differentiation, entirely at 5% oxygen (5 → 5%, A), entirely at 20% oxygen (20 → 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 of $GalC^+$ 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. *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. the comment for fig 5F is not written: (F) Oligodendrocyte progenitors are significatively reduced after 20% oxygen exposure for 4d in expansion (i.e. in presence of mitogens), as shown by PDGFRα staining. Furthermore, the total expression of the later marker O4 does not change, but the proportion of these cells co-expressing $p21^{cip1}$ increase, indicating that these oligodendrocyte progenitors had undergone mitotic arrest. Quantitation, mean ± S.E.M., n = 3

Higher oxygen promotes oligodendrocyte differentiation

We tested these hypotheses by including replicate wells in which we switched oxygen exposure 2 days after mitogen withdrawal (Fig. 6C,D,E). Surprisingly, switching from 5% to 20% (5→20%) oxygen caused a further increase in the number of $GalC^+$ oligodendrocytes (Fig. 6C,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 (Fig. 6D,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 $PDGFR\alpha^+$ cells were present in these precursor cultures (Fig 6F); 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.

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. Three weeks expansion in 5% oxygen yielded a 14-fold higher frequency of clones than in 20% oxygen (Fig 7A). 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 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 7B). This latter expression pattern is consistent with extensively self-renewing, multipotent stem cells (Uchida et al., 2000). In cells expanded in 20% oxygen, there was a modest decrease in the percentage of CD133^{+}CD24^{-} and larger decrease in CD133^{+}CD24^{+} cells (Fig. 7C, 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 (Fig 7E-F) and cultured them at low density. While the percentages of GFAP and β-III-tubulin did not significatively change after 5 days in expansion comparing the four sorted groups in the two oxygen tensions (data not shown), importantly we found that $CD133^{+}CD24^{-}$ 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 7G). After differentiating by mitogen withdrawal for 3 weeks, cells were stained for markers of neurons (β-III-tubulin), astrocytes (GFAP) and oligodendrocytes (Gal-C). 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 7H). $GFAP^{+}$ astrocytes were more frequently generated from $CD133^{-}$ cells, particularly in 20% oxygen (Fig 7I). Enriched $CD133^{-}CD24^{-}$ cells expanded poorly compared with other sorted cell groups, suggesting that they are unlikely to be precursors; these 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 5). The percentage of $Gal\text{-}C^{+}$ oligodendrocyte (Fig 7J), like their $PDGFR\alpha^{+}$ progenitors (Fig 7G), was higher in $CD133^{+}CD24^{-}$ precursors (a 2.5-fold Gal-C enrichment compared with unsorted cells in Fig 6E), 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 $Gal\text{-}C^{+}$ 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.

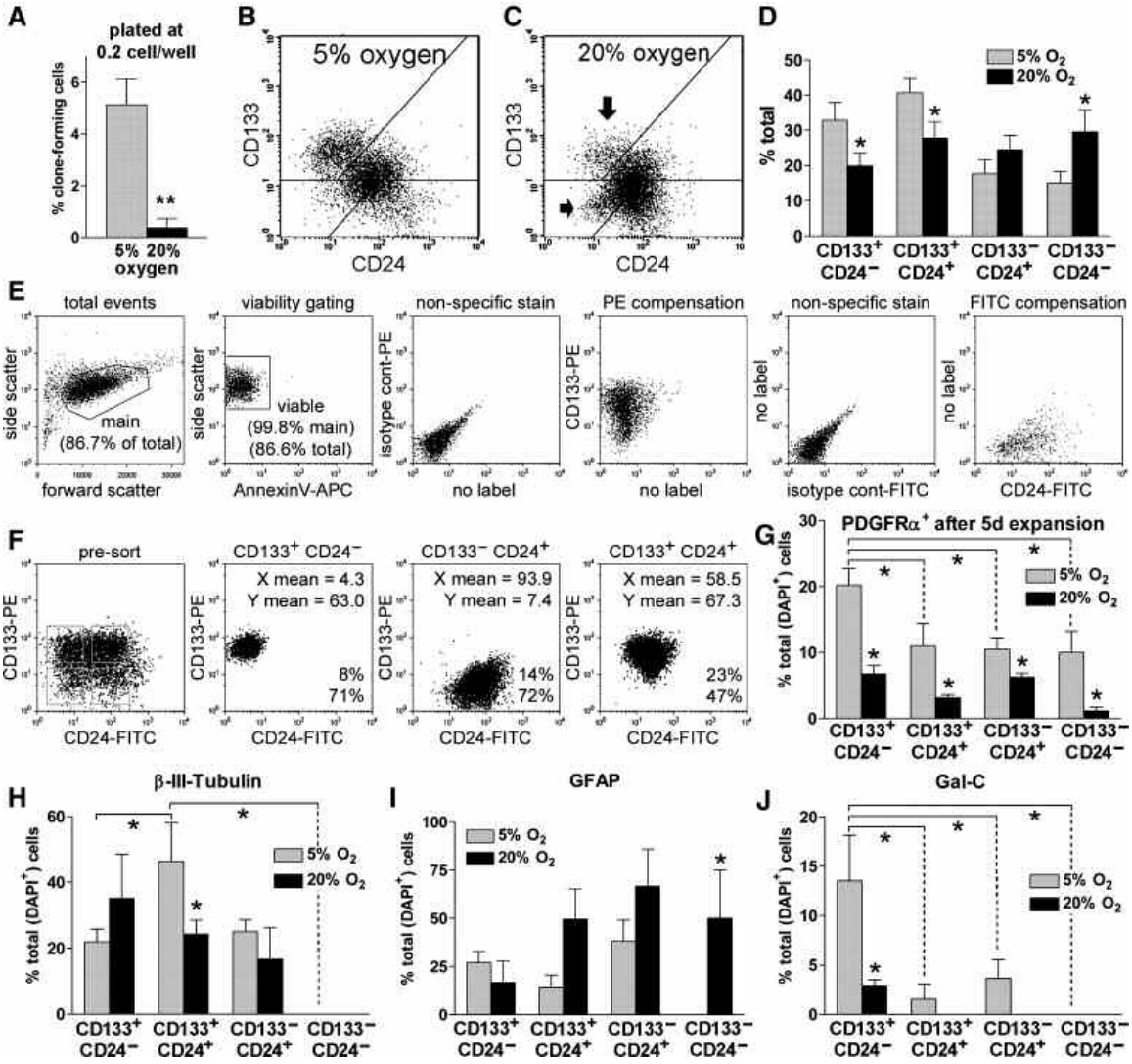


Figure 7. 5% oxygen promotes clonogenicity and increased numbers of CD133$^+$ multipotent cells. (A) Clone formation of precursors plated at 0.2 cell/well and expanded three weeks in either 5% or 20% oxygen, mean ± S.E.M., n = 3. (B-C) Human SVZ precursors were expanded for 7 d at 5% (B) or 20% (C) oxygen, 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$^+$ cells (long arrow) and strongly increases the number of CD133$^-$/CD24$^-$ cells (short arrow). (D) Quantitation, mean ± S.E.M., n = 5. (E-J) prospective isolation experiment of cells extensively expanded at 5% oxygen; (E) gating for intact cells (main population, polygon) and AnnexinV 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) 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 PDGFRα+ cells identified after 5 d expansion of sorted cells. (H-J) sorted cells expanded for 5 d, differentiated for 20 d and stained for neurons (H, β-III-Tubulin), astrocytes (I, GFAP) and oligodendrocytes (J, Gal-C); mean ± S.E.M., n = 2 for H, I, J, n=3 for G; *$p < 0.05$, **$p < 0.01$, paired t-test for all experiments.

RESULTS (PART II)

The results described in part II have been obtained during part of my experience in Children's National Medical Centre, Washington DC, and during the third year of doctorate in the laboratories of Oncohaematology, Paediatric Department of the University of Padova (Prof. Basso). The results here reported are going to be submitted for publication.

Brain tumor-derived precursors require a lower oxygen tension for optimal expansion than normal precursors

Based on the previous results described in part I, (Pistollato et al. 2007), it has been demonstrated that human postnatal SVZ derived precursor cells proliferate better under lowered physiological oxygen tensions (i.e. 5% oxygen), tension that is in the physiological range. Looking at the in vitro effects mediated by a lowered PO_2 in human central nervous system (CNS) tumor derived precursor cells, in particular 7 paediatric and 2 adult High Grade Gliomas (especially glioblastomas, 5 out of 9) we found that the expansion of tumor-derived precursors increased as a function of decreasing oxygen tension, but only at 2% oxygen it was significantly higher than at 20% oxygen. By comparison, normal SVZ-derived precursors showed a significantly greater expansion at 5% oxygen but did not increase further at 2% oxygen (Fig 1). This suggested that tumor precursors may require lower oxygen tensions than normal precursors for maximal expansion.

From Washington, DC	Studied by Immuoncitochemistry		
	TUMOR TYPE	**Age**	**Sex**
HuTu10	Ependymoma	15	Female
HuTu14	Glioblastoma	3 months	Female
HuTu21	Glioblastoma	12	Male
HuTu24	Ependymoma	4	Female
HuTu29	Astrocytoma	13	Male
HuTu30	Ependymoma	5	Female
From Padova	Studied by Immunocitochemistry, Flow cytometry, Western blotting, RQ-PCR		
HuTuP01	Glioblastoma	64	Male
HuTuP02	Glioblastoma	42	Male
HuTuP05	Glioblastoma	9	Male

Table1. High Grade Gliomas Samples

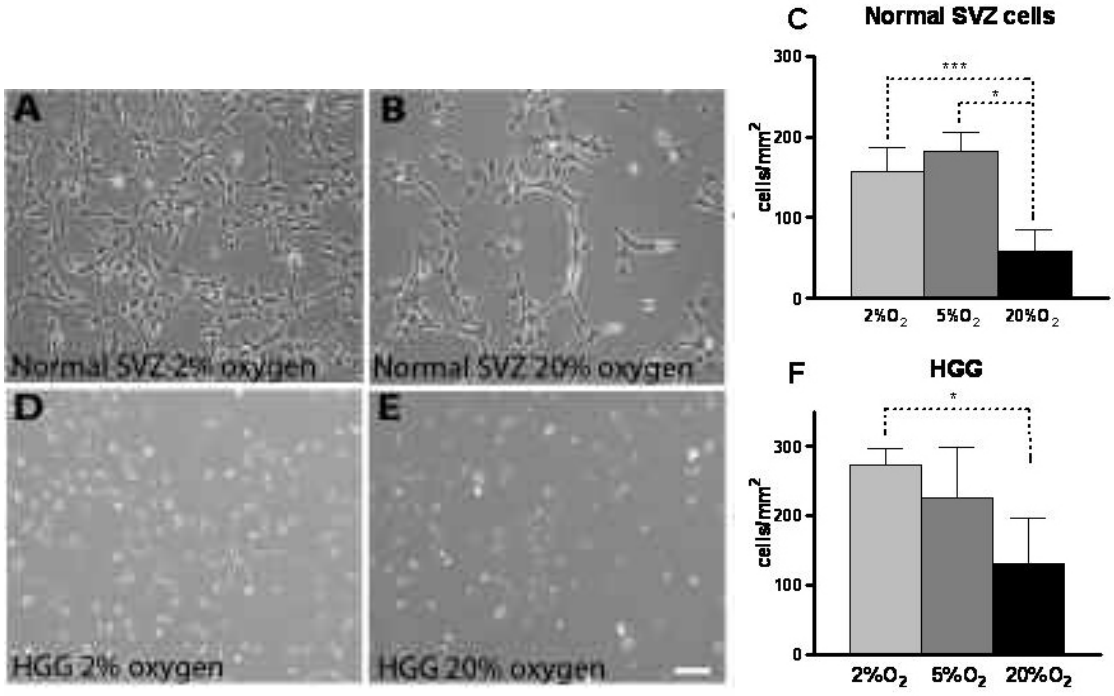


Figure 8. Brain tumor-derived precursors require a lower oxygen tension for optimal expansion than normal precursors. Human high grade glioma (HGG) derived cells (A-B) at passage 3, were plated at medium density (49 cells/mm^2) and expanded 7 days at either 2% (not shown in the picture), 5% or 20% oxygen in presence of mitogens bFGF and EGF (20 ng/ml both). Lowered oxygen tension increases expansion of HGG derived cells cells, alike in normal SVZ derived cells. (C-F) Quantitation after successive passaging and comparing several HGG derived cells, mean ± S.E.M., n= 6 for normal cells n = 7 (for HGG).10X pictures, bar = 0.1 mm.

Higher oxygen tension promotes differentiation in HGG derived cells.

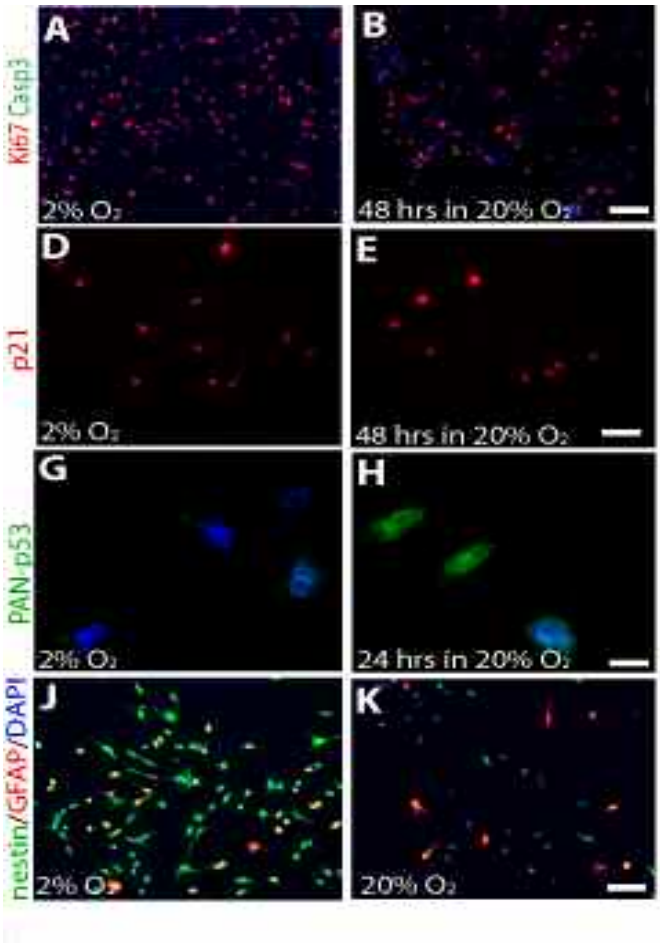
We characterized HGG cells for the expression of several markers, such as nestin (for multipotent precursors) (Tohyama et al. 1992), GFAP (for astrocytes and radial glia) (Casper and McCarthy 2006; deAzevedo et al. 2003), β-III-tubulin (for committed and immature neuronal cells) (Memberg SP, Hall AK 1995) and O4 (for early oligodendrocytes) (Zhang SC et al. 2000). We generally found that HGGs were characterized for the most part by nestin+, GFAP+, nestin/GFAP double positive cells and nestin/β-III-tubulin double positive cells. O4+ oligodendrocytes were only poorly represented (0.9-1% of the total DAPI positive cells, not shown). The percentages of the several subpopulations slightly differed comparing the collected tumor biopsies (table 1), but they were considerably similar considering tumor types. Comparing the percentages of the different subpopulations in HGGs cultured at 2-5% or 20% oxygen tension, we found that at higher oxygen cells were more differentiated, in particular they were characterized by higher level of expression of GFAP+ cells (Fig J-K)). In conclusion, higher oxygen tension generally promotes differentiation of HGG tumor cells toward an astrocytic fate, as previously reported for normal SVZ derived cells.

Higher oxygen tension induces mitotic arrest and activation of BMP signalling pathway in HGG derived cells.

In order to elucidate the molecular mechanisms by which this differentiation occurred, we stained cells by using Ki67, marker for mitotically active cells, phosphorylated forms of p53 and pan-p53 antibody, and $p21^{cip1}$, a cyclin-dependent kinase inhibitor and a direct effector of mitotic arrest. We found that the percentage of Ki67+ cells resulted to be slightly higher in 2% oxygen; $p21^{cip1}$ protein level increased in cells cultured at 20% oxygen, especially after 48 hours of higher oxygen exposure. To test phosphorylation-dependent activation of p53 protein, which is implicated also in cell cycle arrest by mediating activation of p21, we exposed cells that had been grown at 2% oxygen to 20% oxygen tension, for 24 hours and for 48 hours. P53 is phosphorylated at multiple sites in vivo and by several different protein kinases in vitro. We found that raising of oxygen

tension induced particularly phosporylation of Ser20, 37 and 392, and a general increase of pan-p53 was detected.

To see if under higher oxygen tension there was also activation of apoptosis, we analyzed HGG cells using the antibody specific for the proteolitically active cleaved-caspase-3 (which was lowly expressed, around 5.5% of total cells in both oxygen tensions), but we could not detect a significative difference, thus indicating that the decrease of total cell number under 20% oxygen was not related to increase of caspase-3 dependent apoptosis.



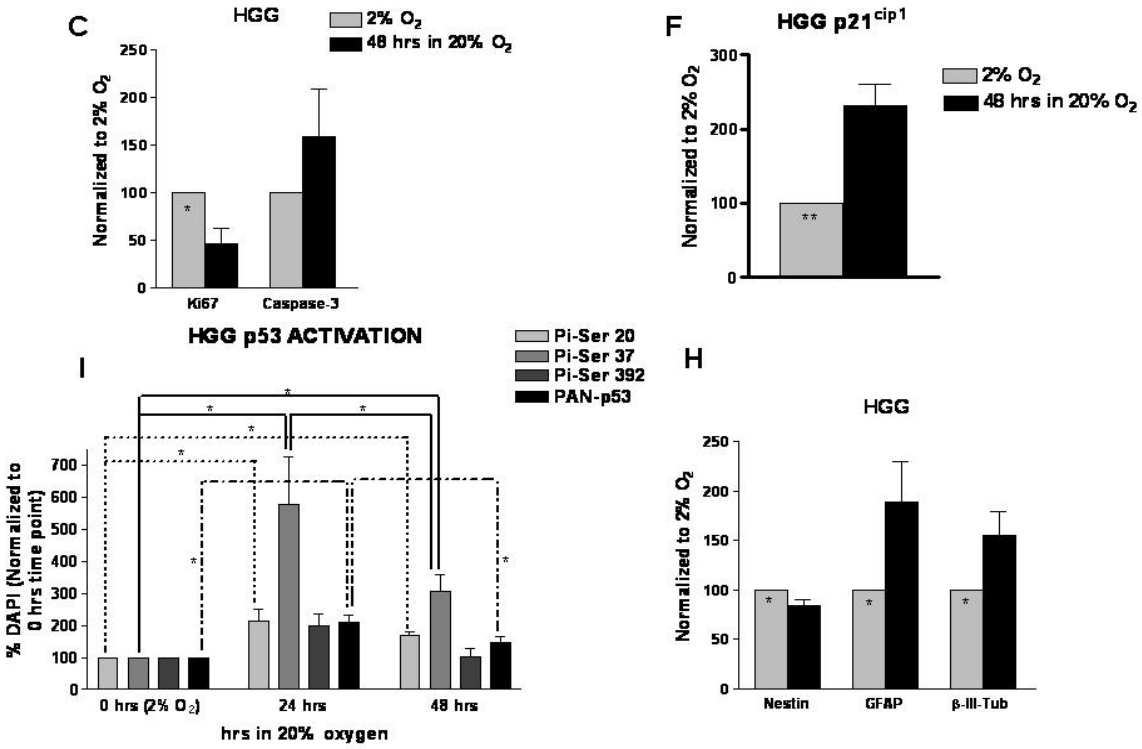


Figure 9. Higher oxygen tension induces p53 activation and p21^{cip1} mitotic arrest in HGG derived cells. Expansion of human HGG derived precursors is enhanced at 2% oxygen, as shown by Ki67 expression. Under 20% oxygen, a slight increase in apoptosis occurs, as measured by activated caspase3, (A-B) and also p21 is more expressed under 20% oxygen (D-E), indicative of a more differentiated cell state. P53 activation, as measured by phosphorylated serine-20,-37, and -392 staining, occurs after 24 hrs of higher oxygen exposure (G-H). Expansion HGG (J-K) nestin+ derived precursors expanded for 5-7 days is enhanced at 2% oxygen. In contrast, 20% oxygen selectively increases β-III-tubulin+ cells and particularly GFAP+ cells. (C-F-I-H) Quantitation after comparing 6 HGGs, mean ± S.E.M., n = 3 for p21, 4 for Ki67, and 4 for Caspase 3. n = 6 for nestin, 3 for GFAP, and 4 for Tuj1. 20X pictures (for A-B-D-E-J-K), bar = 50 μm and 60X pictures (for G-H), bar = 15 μm.

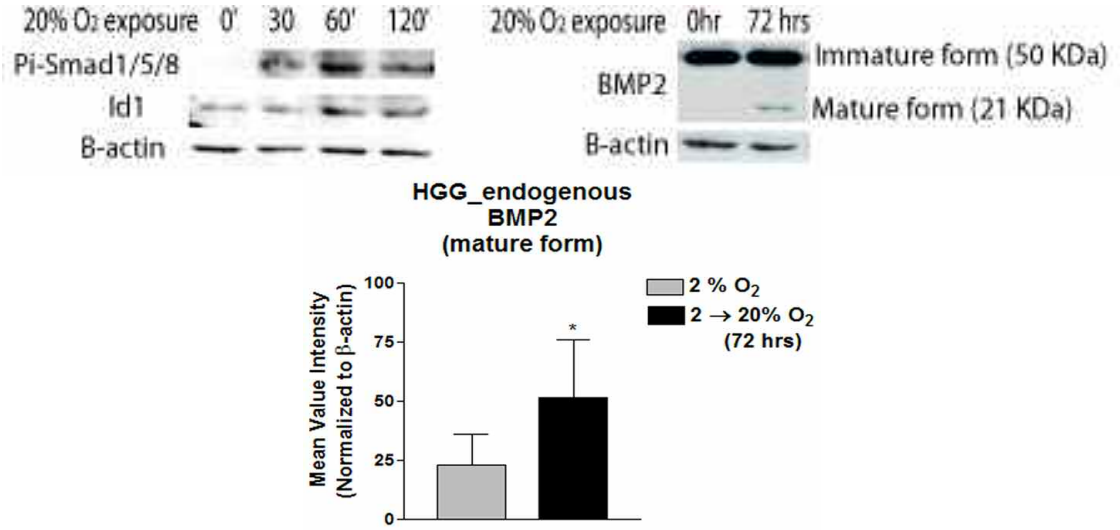
We hypothesized that BMP signaling pathway, known to be important in regulation of astroglial fate (Chen and Panchision, 2007), could be involved in induction of the glial commitment observed after 20% oxygen exposure; thus, we analyzed expression of PiSmad1/5/8 and Id1 at different times of high oxygen exposure. We found that phosphorylation of Smad1/5/8 and expression of Id1 occurred in a time dependent fashion after increase in oxygen tension. Furthermore, we performed gene expression analyses of endogenous BMP2 and BMP receptors (BMPR1A, BMPR1B and BMPRII), using SYBR

green methodology, and we found that all these genes were up-regulated in cells cultured under higher oxygen tension (Table 2).

These results suggest that the mitotic arrest observed in HGGs derived cells after culturing at high oxygen tension is consequence of p53 and p21 ^{cip1} activation, and that the occurring astro-glial commitment is related to activation of the BMP signaling pathway.

	2% oxygen	20% oxygen	P Value
BMP2	1	2.80 (± 0.19)	***
BMPR1A	1	2.64 (±0.522)	*
BMPR1B	1	2.77 (± 0.69)	*
BMPRII	1	1.69 (± 0.18)	*

Table2. RQ-PCR Analysis of BMP2 and BMPR-IA, -IB, -II on HGG samples.



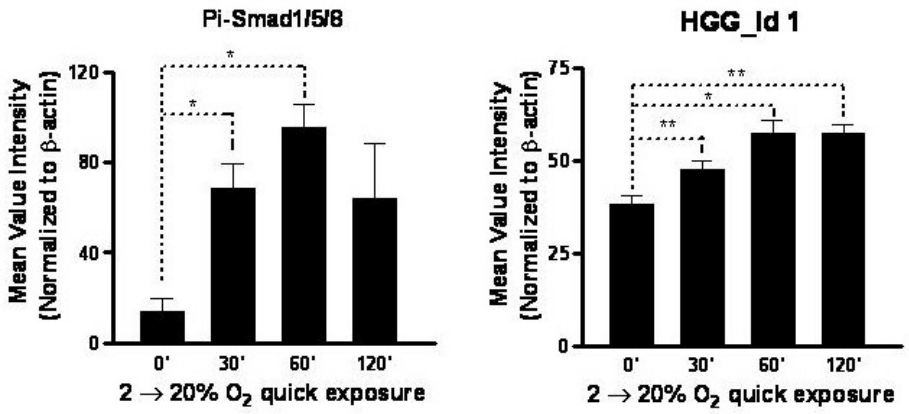


Figure 10. Higher oxygen tension induces activation of BMP signalling pathway in HGG derived cells. Quick high oxygen exposure at different time points promotes phosphorylation of Smad1/5/8 and increases Id1 protein level, indicative of BMP signaling pathway activation. Also endogenous BMP2 level increases after higher oxygen exposure (mature form, while the immature form remains unchanged). Densitometric quantitation by using Scion Software after comparing 2 different HGGs, The mean intensity values have been normalized to β-actin.

Differential Response of tumor cells to in vitro Mitogenic Treatments depending on oxygen tension.

BMP2 inhibits HGG cells but not normal SVZ cells proliferation and this effect is partially inhibited by hypoxia.

We sought to understand if the presence of a hypoxic microenvironment could prevent the gliogenic effects promoted by BMP2 treatment. This is important as the presence in the tumor niche of a hypoxic condition, which seems to maintain HGG in a less committed cell state, may prevent the pro-differentiating effects modulated by physiological soluble factors. Indeed, tumor cells incapability to undergo differentiation is one of the key properties of tumor cells.

BMPs are known to be strong inducers of astroglial fates (Chen and Panchision, 2007) and it has been recently reported that BMP4 (and analogously BMP2) can modulate glial differentiation through increase in GFAP expression in primary cultures of glioblastoma, in particular in the brain tumor inducing population, thus indicating BMPs as potentially useful candidate molecules to promote differentiation of the tumor-initiating cells (Piccirillo et al. 2006). Additionally, as reported in part I, human SVZ derived precursor cells cultured under lowered oxygen tension showed an inhibition of BMP signaling and of

subsequent glial differentiation (i.e. GFAP expression), while a higher oxygen tension promotes BMP signaling, thus suggesting that modulation of the BMP responsiveness is mediated by oxygen in neural precursor cells (Pistollato et al. 2007).

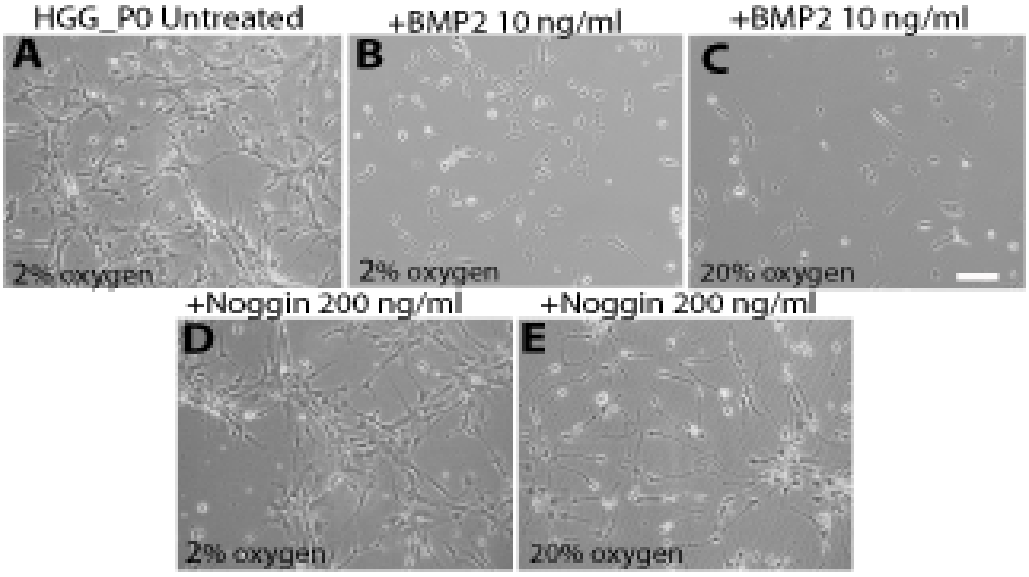
We found that 48 hours treatment in presence of BMP2 (R&D, 10 ng/ml) in HGG derived cells was sufficient to induce a drastic reduction of total cells number under normal environmental 20% oxygen tension, particularly at P0 and at early passage numbers, while not affecting normal cells. Interestingly, this effect was partially inhibited at lower oxygen tension, condition in which the reduction in total cell number even after 3-5 days of BMP2 treatment was really modest, thus suggesting a protective effect mediated by hypoxia toward tumor cells. Noggin (R&D, 100-200 ng/ml), the physiological BMP inhibitor, did not affect tumor cells proliferation, while in normal cells there was increase in total cells number only at 2-5% oxygen (see results in part I). Thus, HGG derived precursors seem to respond more dramatically to BMP2 in vitro treatment depending on the oxygen tension state, while normal SVZ derived cells are not affected by this treatment.

To check the proliferation state of BMP2 treated cells, we analyzed Ki67 expression. While in normal SVZ derived cells, under both oxygen tensions, we could not detect a significative difference after 3-5 days treatment in presence of BMP2 (with an absolute percentage of Ki67 equal to 50-55 on total DAPI for cultures maintained at 2% oxygen, data not shown), conversely, in HGG derived cells, maintained under lower oxygen, BMP2 exposure decreased Ki67 expression by half and this reduction was similar at 20% oxygen. Interestingly, BMP2 inhibition with Noggin, while did not promote any particular effect in cells cultured at 2% oxygen, which are probably already highly proliferating, at 20% oxygen improved cell proliferation to rates comparable to the ones found at lower oxygen; in normal SVZ cells, after noggin treatment, we could find a slight increase in Ki67+ cells only under lower oxygen, no difference at higher oxygen.

Thus, exogenous BMP2 has an effective anti-proliferative action toward HGG cells at lower and particularly at higher oxygen while in normal cells a reduction of the proliferation rate is notable only at higher oxygen tensions; inhibiting BMP2 signaling with Noggin rescues HGG cell proliferation at 20% oxygen, thus suggesting that activation of BMP signaling occurs dependently on oxygen tension increase. As Ki67 is a general marker for cell proliferation (staining all cells in G1, S, G2 or M phase) which does not

permit to discriminate at which phase the inhibitory effects promoted by BMP2 occur, we stained HGG cells with propidium iodide to perform a cell cycle analysis.

The major effect promoted by BMP2 was a G0-G1 phases block, and this block was even stronger under 20% oxygen; in normal SVZ cells there was no significative effect at 2% and under 20% oxygen only a modest G0-G1 block was visible. Thus, BMP2 treatment has a much stronger inhibitory effect on HGG cells than in normal SVZ cells and this proliferative reduction is associated with a block of the cell cycle in G0-G1.



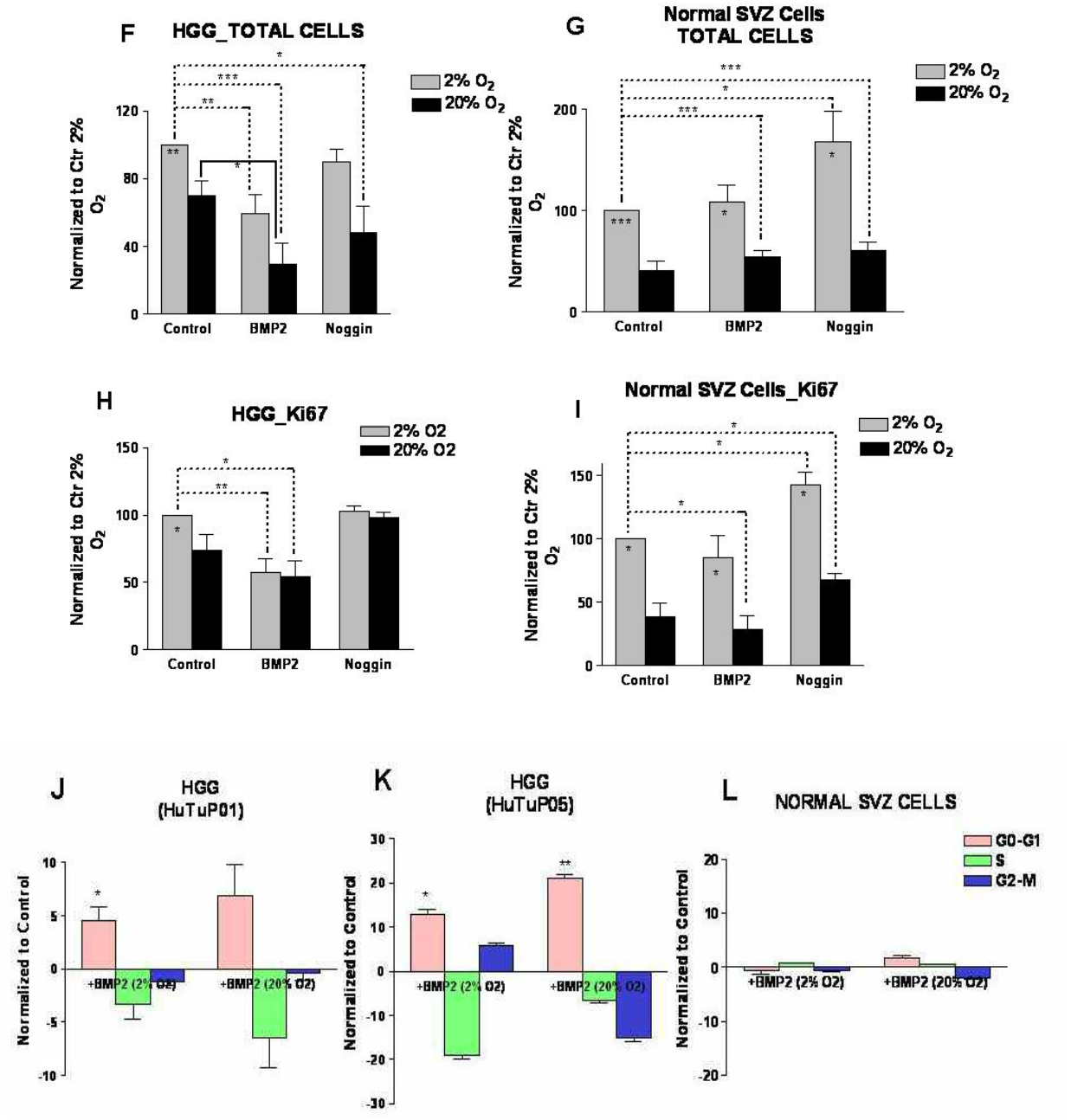


Figure 11. BMP2 inhibits HGG cells but not normal SVZ cells proliferation and this effect is partially inhibited by hypoxia. Human HGG derived cells, previously expanded at 2% oxygen, were replated at medium density (49 cells/mm^2) and expanded for 3-5 days at either 2% or 20% oxygen, and in presence of either BMP2 (R&D, 10 ng/ml) (B-C) or Noggin (R&D, 200 ng/ml) (D-E). HGG derived cells, at P0 and very early passages, were selectively killed by BMP2 treatment and this effect was partially inhibited by culturing the cells under hypoxic condition. Inhibition of BMP2 by noggin does not promote any significative effect at either 2% or 20% oxygen (see F for quantification). Conversely, normal SVZ derived cells, at any passage, were not affected by BMP2 treatment under both oxygen tensions, while noggin treatment promotes expansion only at lower oxygen (pictures not shown, see G for quantitation). (H-I) Analysis of proliferation

by using Ki67 expression. Ki67 in normal SVZ derived cells, under both oxygen tensions, does not difference after 3-5 days treatment in presence of BMP2 (with an absolute percentage of Ki67 equal to 50-55 on total DAPI for cultures maintained at 2-5% oxygen, data not shown); conversely, in HGG derived cells, maintained under lower oxygen, BMP2 exposure decreased Ki67 expression by half and this reduction was similar at 20% oxygen. BMP2 inhibition with Noggin at 20% oxygen improved cell proliferation to rates comparable to the ones found at 2% oxygen; in normal SVZ cells, after noggin treatment, we could find a slight increase in Ki67+ cells only under lower oxygen, no difference at higher oxygen.

Cell cycle analysis with propidium iodide (J-K-L). The major effect promoted by BMP2 in HGG derived cells is a G0-G1 phases block, and this block was even stronger under 20% oxygen; in normal SVZ cells there was no significative effect at 2% and under 20% oxygen. Mean ± S.E.M., for normal SVZ cells, 2% oxygen control/BMP2/Noggin n = 8, 20% oxygen control n =3 /BMP2/Noggin, n = 7. For HGG cells, 2% oxygen control n = 9 /BMP2 n = 11/Noggin n = 16, 20% oxygen control/BMP2/Noggin, n = 6. Ki67 in all the conditions, n = 3-4. For cell cycle analysis, n=2, analyzed two different tumor biospies. 20X pictures, Bar = 50 μm.

BMP2 induces differentiation into astroglia in HGG live remaining cells cultured at 2% oxygen and even more if cultured at 20% oxygen.

To further investigate the effects mediated by BMP2 on HGG subpopulations, we stained tumor cells comparing level of expression of nestin, GFAP and β-III-tubulin. Morphologically the live remaining cells after BMP2 treatment appeared more differentiated and flattened, while they were more elongated and tri-quadri polar in control (untreated) groups and in presence of noggin. The percentage of the flattened cells was higher in cells cultured under 20% oxygen and in presence of BMP2.

We found that in presence of BMP2, HGG cells underwent glial differentiation under both oxygen tensions (2.5 times more GFAP+ cells in 2% oxygen), but particularly at 20% oxygen (4.5 times more GFAP+ cells than 2% oxygen control group), condition in which the percentage of nestin+ cells further decreased.

We performed the same analysis on normal precursors and as reported in part I these cells responded to BMP2 by increasing GFAP expression only when cultured in 20% oxygen (4.5 times more in 20% oxygen compared to 2% oxygen control group).

We also checked if there were effects on the neuronal committed population and we found a slight increase in β-III-tubulin+ cell number after BMP2 treatment and under both oxygen tensions, while the percentage of this subpopulation was comparable to control in presence of noggin (not shown), even though it has been reported that noggin modulates neurogenesis in the normal adult subventricular zone (Lim DA et al 2000).

These results indicate that in normal cells responsiveness to BMP trough gliogenic commitment is repressed at lower oxygen tension, while in HGG tumor cells this effect is present in both oxygen tensions, but it is partially inhibited at 2% oxygen. Furthermore, while in normal cells BMP2 inhibition by noggin was able to modulate neuronal commitment in 2% oxygen cultured cells, in HGG derived cells this treatment did not produce a similar effect, thus suggesting an aberrant ability of the HGG tumor cells to properly respond to the neurogenic effects modulated by noggin.

Analysis of cell surface antigens CD133, which marks human CNS precursors with high clonogenic capacity and multipotency, and CD24, which identifies cells with limited clonogenic capacity in adult human CNS tissue (Uchida et al. 2000) was also performed. CD133 has been recently proposed as a marker also for cancer stem cells, as CD133+ cells isolated from glioblastoma cells were able to recapitulate original tumor formation in nude mice (Singh et al. 2004).

HGG live cells were analyzed for co-expression and intensity of these markers by flow cytometry. We saw that in 2-5% oxygen untreated cells the majority of cells expressed high levels of CD133+ CD24+ cells, in HGG that looked more differentiated and did not present evidence of CD133 only+ cells, subpopulation which very likely represents the most immature and clonogenic one. Other tumor samples, which looked very immature given the high percentage of nestin+ cells, were characterized by a high proportion of CD133 only+ cells which was higher under hypoxic conditions. After BMP2 treatment, a decrease of these two cell types occurred, concomitantly with an increase in the CD133-CD24- cells, considered *bona fide* as the more committed and/or differentiated cells (also considering what observed in part I for normal SVZ cells). In normal SVZ cells a reduction in the CD133+ CD24+ population and a decrease in the CD133-CD24- one was present but not at a significative level.

Importantly, these effects elicited by BMP2 treatment on HGG cells were strongly promoted when cells were cultured at higher oxygen tension.

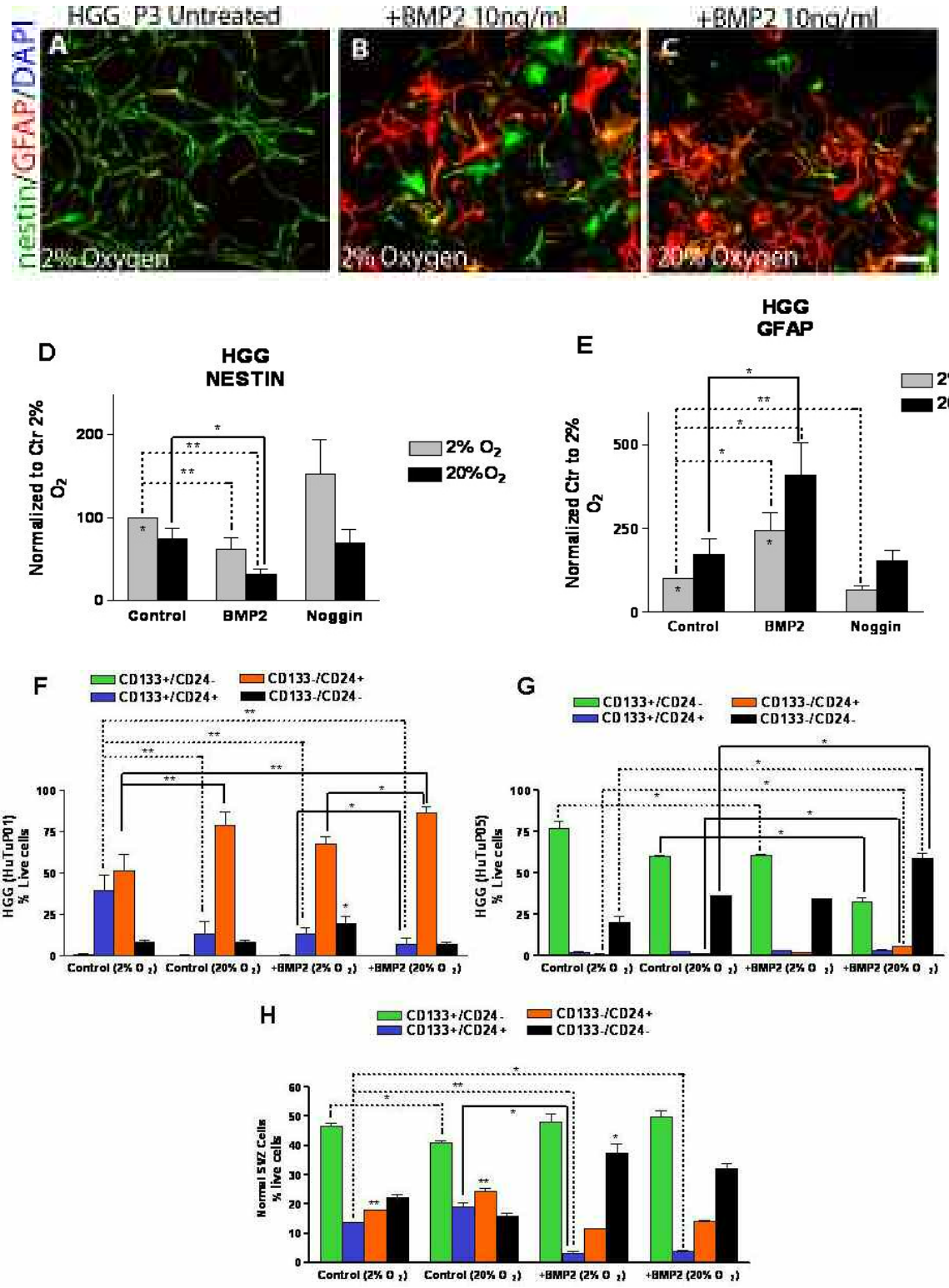

HGG_P3 Untreated
+BMP2 10ng/ml
+BMP2 10ng/ml
nestin/GFAP/DAPI
A
B
C
2% Oxygen
2% Oxygen
20% Oxygen
D
HGG
NESTIN
Normalized to Ctr 2% O2
2% O2
20%O2
Control
BMP2
Noggin
E
HGG
GFAP
Normalized Ctr to 2% O2
2% O2
20% O2
Control
BMP2
Noggin
F
CD133+/CD24-
CD133-/CD24+
CD133+/CD24+
CD133-/CD24-
HGG (HuTuP01) % Live cells
Control (2% O 2)
Control (20% O 2)
+BMP2 (2% O 2)
+BMP2 (20% O 2)
G
CD133+/CD24-
CD133-/CD24+
CD133+/CD24+
CD133-/CD24-
HGG (HuTuP05) % Live cells
Control (2% O 2)
Control (20% O 2)
+BMP2 (2% O 2)
+BMP2 (20% O 2)
H
CD133+/CD24-
CD133-/CD24+
CD133+/CD24+
CD133-/CD24-
Normal SVZ Cells % live cells
Control (2% O 2)
Control (20% O 2)
+BMP2 (2% O 2)
+BMP2 (20% O 2)

Figure 12. BMP2 induces differentiation into astroglia in HGG live remaining cells cultured at 2% oxygen and even more if cultured at 20% oxygen. After few passages in vitro (at P3, P4, P5) of HGG derived cells, the major effect mediated by BMP2 was induction of glial differentiation (B-C), effect that was even stronger in cells cultured at 20% oxygen (C). Noggin maintained cells undifferentiated (i.e. nestin+) when cultured at 2% oxygen (pictures not shown). Quantitation (D-E), mean ± S.E.M., at 2% and 20% oxygen, all the conditions: for nestin/GFAP n = 3-4. Bar = 50 μm. (F-G-H) Flow cytometric analysis of the cell surface markers CD133/CD24. In 2% oxygen, HGG untreated cells expressed high levels of CD133+ CD24+ cells (see tumor HuTuP01) and CD133 only+ cells (see tumor sample HuTuP05), subpopulations which very likely represents the more immature and clonogenic ones. BMP2 treatment decreases these two cell types, concomitantly with an increase in the CD133-CD24- cells, considered *bona fide* the more committed and/or differentiated cells. In normal SVZ cells a reduction in the CD133+ CD24+ population and a decrease in the CD133-CD24- one is present but not at a significative level.

Pi-Smad1/5/8 activation and Id1 expression after BMP2 treatment are reduced by hypoxia.

We hypothesized that oxygen could regulate BMP signalling at multiple steps, including SMAD activation and transcriptional induction of target genes like Id1 (Lopez-Rovira et al., 2001), which is involved in gliogenesis (Nakashima et al., 2001; Vinals et al., 2004; Yanagisawa et al., 2001). We started with cells that had been cultured previously in 2-5% oxygen and only switched to 20% oxygen 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 in more cells and for a longer duration in 20% oxygen than in 2-5% oxygen, similarly to SVZ cells (part I). Also Id1 expression resulted higher in cells that had been cultured at 20% oxygen for 24 hrs, in accordance with the shorter time course of fig 10, and this expression was further promoted by BMP2 treatment, effect not visible at lowered oxygen tension even in presence of BMP2 treatment.

These results indicate that lowered oxygen tension inhibits BMP in vitro stimulation and subsequent glial differentiation of HGG derived cells, while a higher oxygen tension promotes BMP signalling.

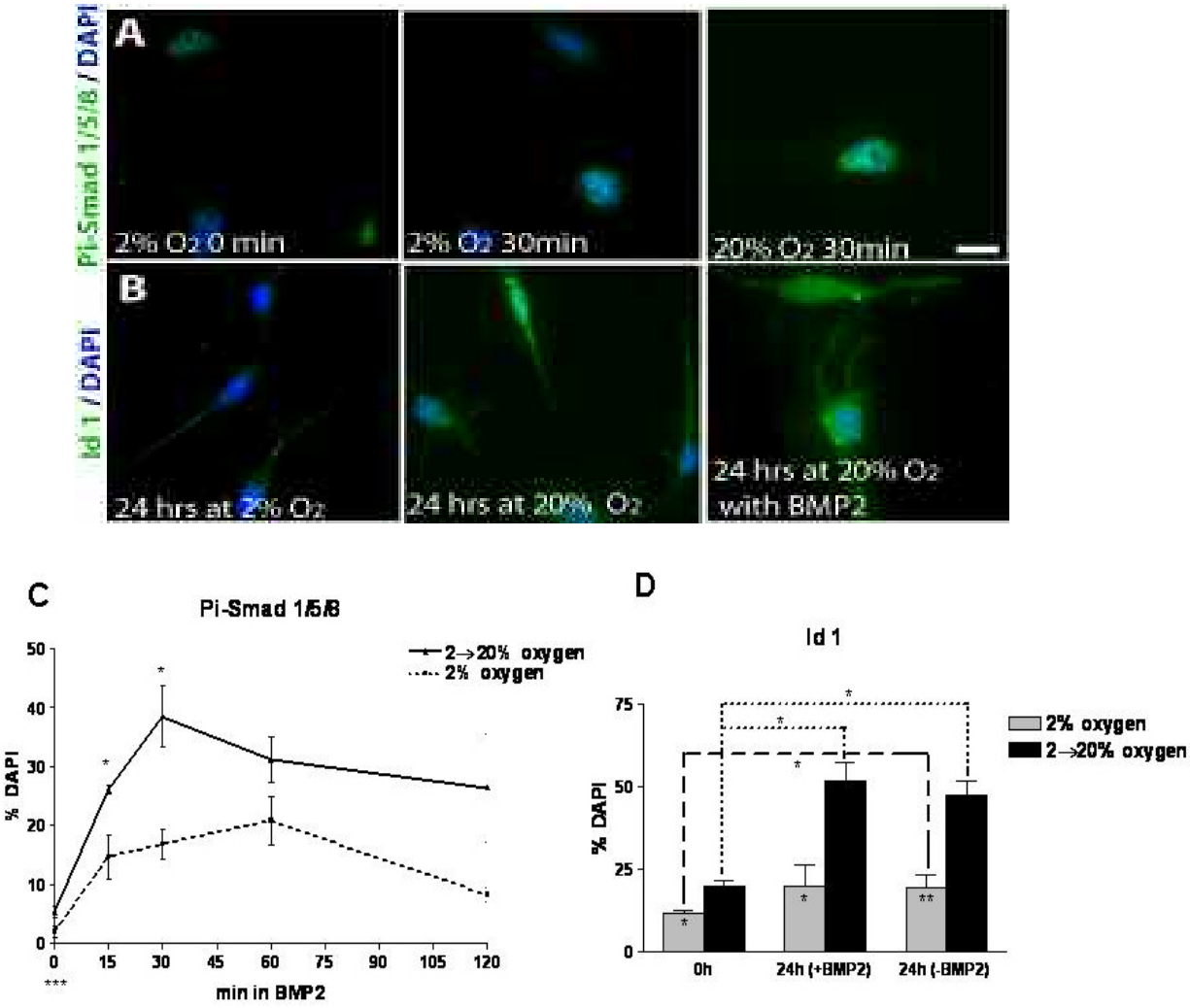


Figure 13. Pi-Smad1/5/8 activation and Id1 expression after BMP2 treatment are reduced by hypoxia. HGG cells had been cultured in 2% oxygen and then switched to 20% oxygen prior to beginning BMP treatment. Phosphorylation of Smads1/5/8, a key step in BMP signal transduction occurs in more cells and for a longer duration in 20% oxygen than in 2-5% oxygen (A). Also Id1 expression resulted higher in cells that had been cultured at 20% oxygen for 24 hrs and this expression was further promoted by BMP2 treatment, effect not visible at lowered oxygen tension even in presence of BMP2 treatment (B). (C-D) Quantitation, experiments have been performed on 2 different tumor samples. 60X pictures, Bar = 15 μm.

BMP2 treatment downregulates Hif1α expression in HGG cells.

The key mediator of hypoxia response is Hif1α, a member of the hypoxia-inducible factor (Hif) family of proteins. Over-expression of Hif1α and its downstream target genes, such as VEGF, GLUT-1, and CAIX, in higher-grade gliomas support the hypothesis that hypoxia may allow the progression of lower-grade tumors to glioblastoma multiforme (Ragel, B. T. et al. 2007).

Our previous findings indicate that a hypoxic environment inhibits BMP signaling pathway in HGG derived cells and, less strongly, in normal SVZ derived cells. We sought to investigate if BMP2 treatment could affect also Hif1α protein expression. Hif1α protein level was in some tumor samples high regardless the oxygen tension tested in culture, while in normal SVZ cells Hif1α protein stability was dependent exclusively on hypoxia and was degraded under 20% oxygen. We found that after BMP2 treatment in both HGG and normal SVZ derived cells Hif1α protein level resulted to be downregulated even under hypoxic condition. In particular a reduction of 30% in the protein level was observed under hypoxic condition after 10 ng/ml BMP2 treatment and of 40% compared to untreated HGG cells cultured at 20% oxygen tension. We also performed gene expression analyses of Hif1α, which was expressed at a comparable level under 2% and 20% oxygen. We found that there was a slight down-regulation of Hif1α mRNA in HGG cells that had been cultured in presence of BMP2 treatment in both the oxygen tensions tested (Table 3).

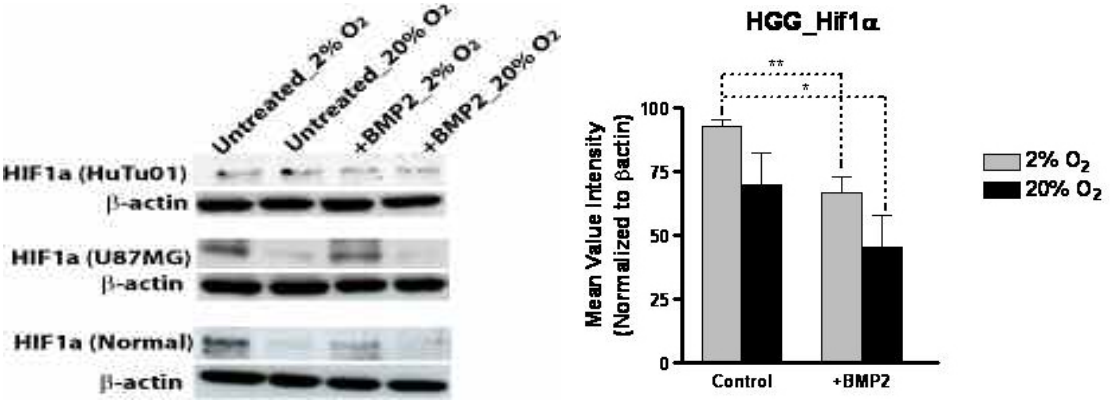


Figure 14. BMP2 treatment downregulates Hif1α expression in HGG cells. Total protein extracts of different HGG derived cells have been collected after 3-5 days in vitro in the following conditions: (1) Untreated cells, at 2% O_2, (2) Untreated cells, at 20% O_2, (3) +BMP2 10ng/ml, at 2% O_2 and (4) +BMP2 10ng/ml, at 20% O_2. Western blot analyses of Hif1α and β-actin have been performed, also comparing normal SVZ cells and U87MG cell line. Hif1α protein level was in some tumor samples high regardless the oxygen tension tested in culture, while in normal SVZ cells and U87MG glioma cell line Hif1α protein stability was

dependent exclusively on hypoxia and was degraded under 20% oxygen. After BMP2 treatment in both HGG and normal SVZ derived cells Hif1α protein level resulted to be downregulated even under hypoxic condition. Densitometric quantitation by using Scion Software after comparing 2 different HGGs, the mean intensity values have been normalized to β-actin.

*p < 0.05, **p < 0.01, ***p < 0.001, paired t-test for all experiments.

Hif1α	**2% oxygen**	**20% oxygen**
Control	1	0,94 (+/- 0,04) (ns)
+ BMP2 10ng/ml	0,88 (+/- 0,04) (*)	0,82 (+/- 0,03) (**)

Table3. RQ-PCR Analysis of Hif1α on HGG samples.

Results Obtained for MDB derived cells

Lowered oxygen tension guarantees expansion of human MDB (medulloblastoma) derived cells.

MDB derived total cell number was significatively reduced by high oxygen tension, in some cases the cells could not survive for more than two consecutive passages. We characterized MDB cells for the expression of several markers, such as nestin (for multipotent precursors) (Tohyama et al. 1992), GFAP (for astrocytes and radial glia) (Casper and McCarthy 2006; deAzevedo et al. 2003), β-III-tubulin (for committed and immature neuronal cells) (Memberg SP, Hall AK 1995). We generally found that brain tumor cells in our cell culture conditions were positive for all these markers, in particular, MDBs were nestin+, β-III-tubulin + and nestin/ β-III-tubulin double positive cells. The proportion of GFAP+ cells in MDBs was very low, in some cases undetectable and generally the few GFAP+ cells were also nestin+. We also found that, alike normal SVZ cells and HGG derived cells, at higher oxygen cells were more differentiated, in particular they were characterized by higher level of expression of β-III-Tubulin in MDBs, at the expense of nestin+ precursor cells. In conclusion, higher oxygen tension generally promotes differentiation of MDB cells toward a neuronal fate.

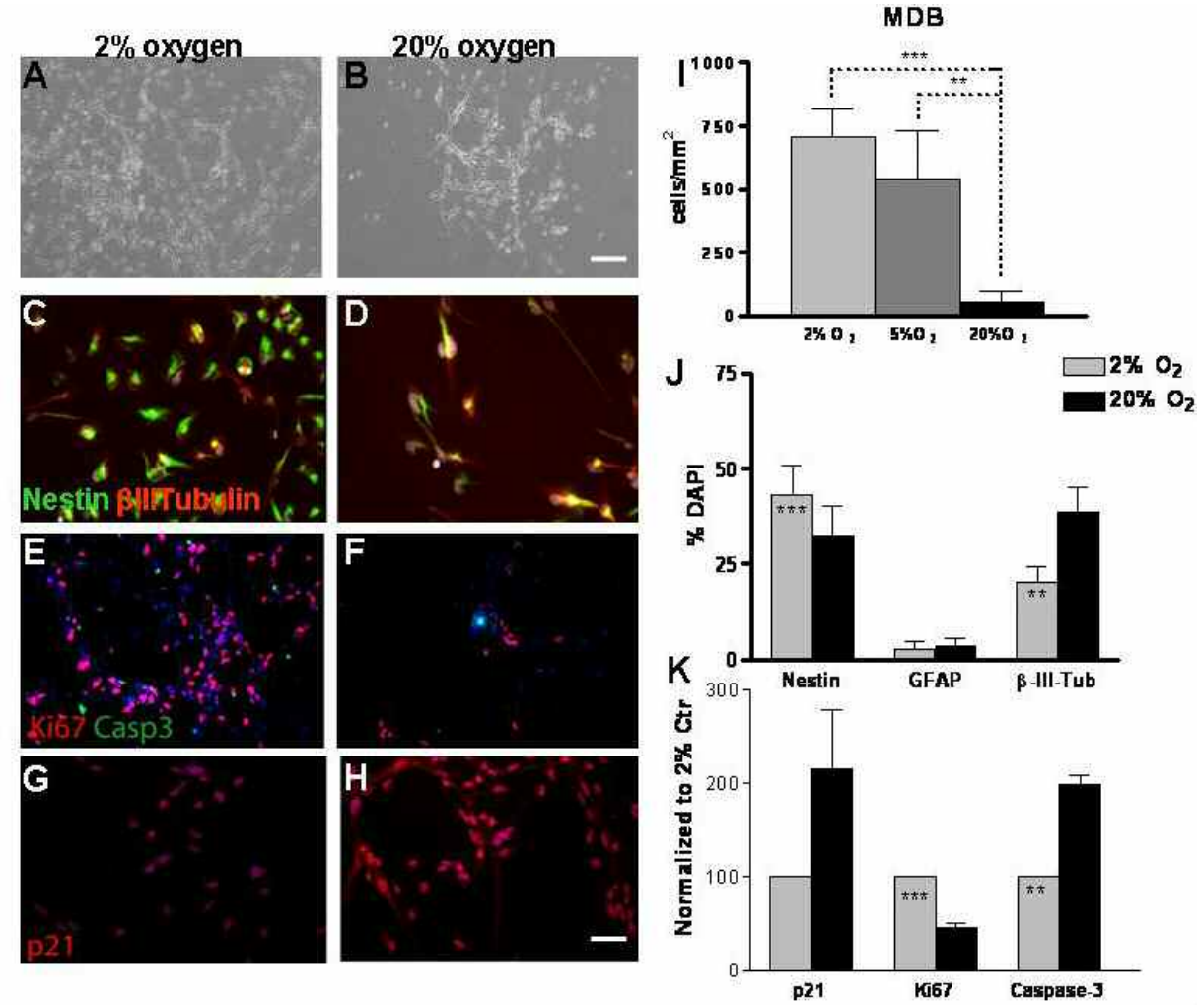


Figure 15. Lowered oxygen tension promotes proliferation of MDB derived nestin+ precursor cells, by preventing mitotic arrest and caspase-3-mediated-apoptosis in MDB. Human medulloblastoma, (MDB) at passage 1 (A-B) derived cells, were plated at medium density (49 cells/mm^2) and expanded 7 days at either 2% or 20% oxygen in presence of mitogens bFGF and EGF (20 ng/ml both). Lowered oxygen tension increases expansion of MDB derived cells. Expansion of human MDB (A-B) derived precursors expanded for 7 days is enhanced at 2% oxygen. Under 20% oxygen, cell death is significantly affected in MDB cells as measured by activated caspase3 staining (E-F) and also p21 is more expressed under 20% oxygen, indicative of a more differentiated state. (G-H) Expansion of human MDB nestin+ derived precursors expanded for 7 days is enhanced at 2% oxygen. In contrast, 20% oxygen selectively increases β-III-tubulin (Tuj-1)+ cells, particularly in MDB cells suggesting glial and neuronal commitments. (I-J-K) Quantitation after successive passaging and comparing several MDB derived cells, mean ± S.E.M., n= 9 (for MDB). 10X pictures (A-B), bar in 10X picture= 0.1 mm. Quantitation after comparing 3 MDB derived cells, mean ± S.E.M., n= 6 for nestin, 4 for GFAP, and 3 for Tuj1. n = 3 for p21, 4 for Ki67, and 3 for Caspase-3, 20X pictures (for C-D-E-F), and 40X pictures (for G-H). Bar in 40X picture = 25 μm.

Higher oxygen tension induces mitotic arrest and caspase-3-mediated-apoptosis in MDB derived cells.

In order to elucidate the mechanisms by which this differentiation and cell death occurred, we stained cells for Ki67 and $p21^{cip1}$and we found that $p21^{cip1}$ level was higher in MDB cells cultured at 20% oxygen, indicative of mitotic arrest. Furthermore, MDB derived cells, cultured under lowered oxygen, expressed more consistently the marker Ki67, indicative of a more pronounced mitotic activity in this condition. We also analyzed the cells using for proteolitically active cleaved-caspase-3, to see if the massive reduction in cell number observed at 20% oxygen was determined by an activation of this apoptotic pathway, and we found that MDB cells expressed a higher percentage of this apoptotic marker at 20% oxygen (see fig 15).

In MDB derived cells, inhibition of BMP2 with noggin seems to increase the already high vulnerability of these tumor cells to high oxygen tension.

We tested the in vitro effects mediated by BMP2 and noggin also on this tumor type. While a previous work (Iantosca MR et al 1999) showed that BMP2 and -4 attenuated apoptosis in a cerebellar MDB cell line (DAOY), it has also been shown that BMP2 mediates retinoid-induced apoptosis in medulloblastoma cell lines through a paracrine effect (Hallahan AR et al 2003). It is important to consider that all these works have been conducted using medulloblastoma cell lines and not primary cultures. In MDB derived cells we found that after BMP2 treatment, conversely to what observed in HGG derived cells, in both 2% and 20% oxygen there was a slight improvement in total cell number, but there was no effect in the proportions of nestin or β-III-tubulin+ cells; interestingly, we found that noggin treatment at 2% oxygen did not improve total cell numbers and nestin+ cells only modestly increased, while β-III-tubulin+ cells reduced. Notably, at high oxygen, condition in which medulloblastoma cells are particularly vulnerable (with a very low viability and an almost complete cell loss after very few passages), noggin in vitro treatment strongly blocked cell proliferation, with a concomitant decrease of nestin+ precursors and Ki67 expression. These effects are consistent with the fact that medulloblastoma derived cells, notoriously difficult to culture (Hatton B.A. et al. 2006), if exposed to anti-differentiating agents, such as noggin, are even more vulnerable than untreated cells to the deleterious effects of high

oxygen. Indeed, noggin has been shown to block extraembryonic cell fate, transiently sustaining Oct4 gene expression, thus resulting in production of neural progenitors (Gerrard L et al. 2005). Conversely, if exposed to pro-survival and pro-differentiating factors (such as BMP2) (Iantosca MR et al. 1999) MDB cells are able to better survive in both oxygen tensions tested.

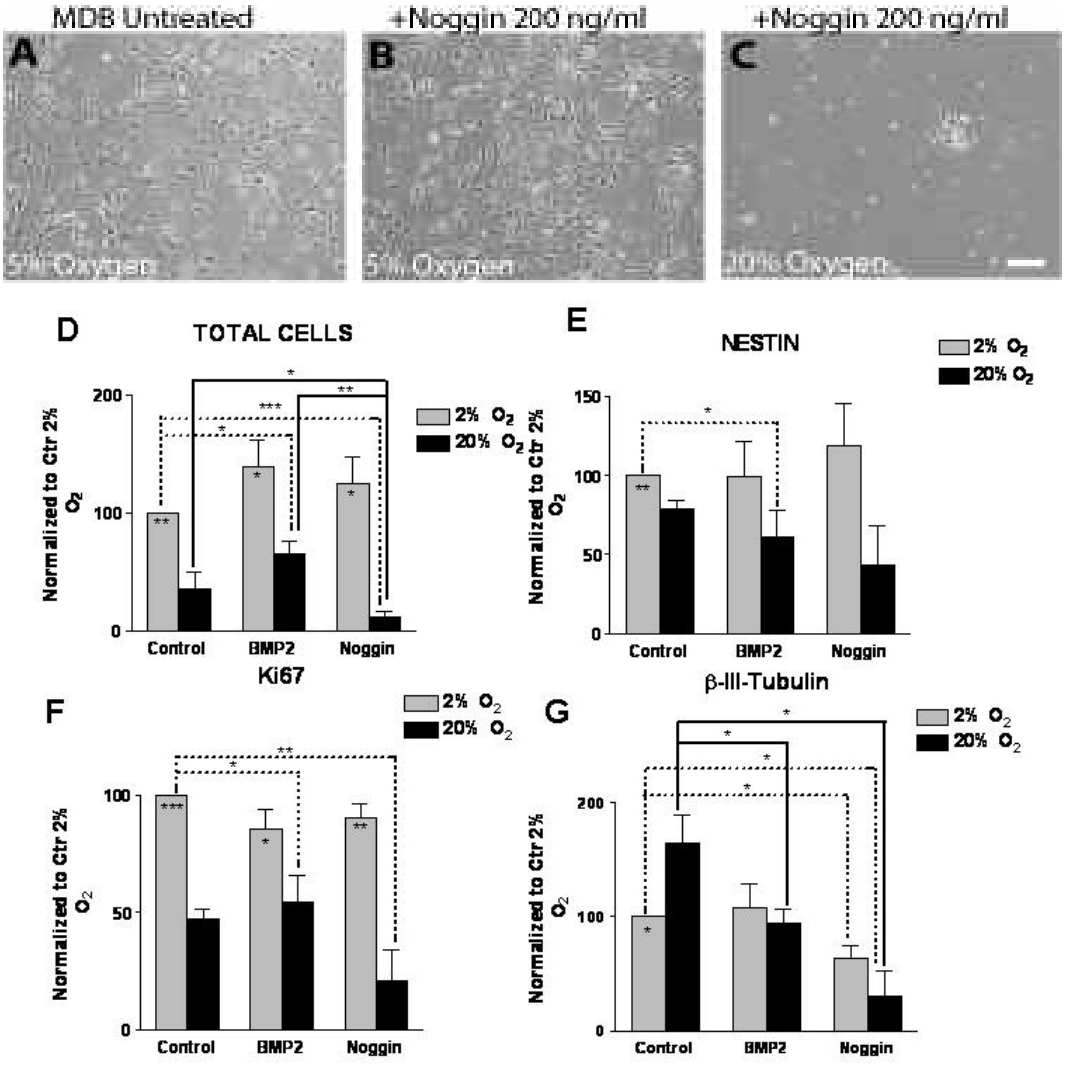


Figure 16. In MDB derived cells, inhibition of BMP2 with noggin seems to increase the already high vulnerability of these tumor cells to high oxygen tension. Human MDB derived cells were plated at medium density (49 cells/mm^2) and expanded for 3-5 days at either 2% (A-B) or 20% oxygen (C), and in presence of either Noggin (R&D, 200 ng/ml (B-C) or BMP2 (R&D, 10 ng/ml) (pictures not shown). MDB cells, cultured at 20% oxygen, after noggin treatment are even more vulnerable than untreated cells to the deleterious effects of high oxygen, as shown by total cell numbers reduction (see D for quantitation) and Ki67 expression (see F for quantitation). Noggin treatment at 2% oxygen did not improve total cell numbers and nestin+ cells only modestly increased, while β-III-tubulin+ cells reduced. After BMP2 treatment, in both 2%

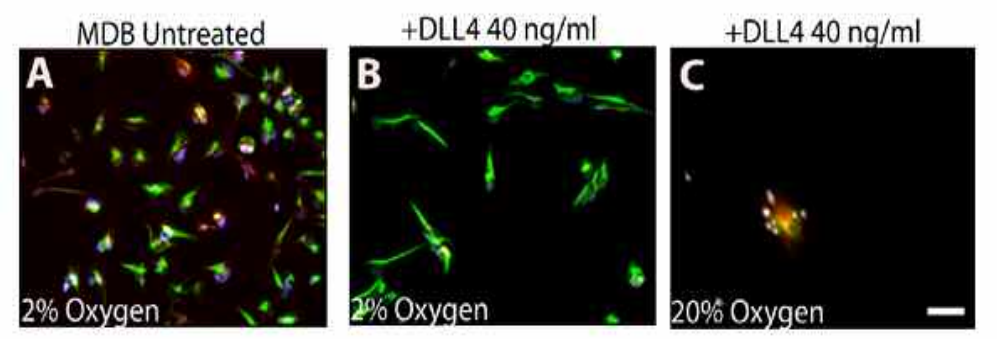
and 20% oxygen there was a slight improvement in total cell number (D), but there was no effect in nestin (E) or β-III-tubulin (G) expression. Quantitation (D-E-F-G), mean ± S.E.M., at 2% and 20% oxygen, all the conditions: for nestin/Tuj-1/Ki67 n = 4, total cells n= 5. Bar = 0.1 mm.

Inhibition of Notch activation by a γ-secretase inhibitor has different effects comparing normal and MDB derived cells and DLL4-mediated notch activation is differentially modulated by oxygen tension.

It has recently been shown that notch pathway inhibition in DAOY cells, by pharmacologic inhibitors of γ-secretase, depletes stem-like cells and blocks engraftment (Fan X et al 2006). Additionally, hypoxia requires notch signalling to maintain the undifferentiated cell state in a mouse myogenic cell line C2C12 and mouse embryonic teratocarcinoma cell line P19 (Gustafsson MV et al. 2005). We treated our MDB cells to see if the proportion of nestin+ precursors was reduced by inhibiting notch signalling and if oxygen played a role also in the modulation of these possible effects. To block notch activation we used γ-secretase inhibitor-X (Calbiochem, cat# 565771), that by binding presenilin blocks notch intracellular domain production (Schroeter EH et al. 2003) (Ikeuchi T and Sisodia SS 2002) (Martys-Zage JL et al. 2000). Interestingly, we found that percentage of nestin+ cells decreased in both 2% and 20% oxygen tensions, and the same effects were seen also by treating normal SVZ derived cells. We found a comparable decrease of total cell numbers in both normal and MDB derived cells after 3 days of in vitro treatment with γ-secretase inhibitor-X, comparing with untreated cells, while Ki67 expression decreased analogously in normal cells and in MDB treated cells, and these effects were seen in both the oxygen tensions tested. Importantly, neuronal committed cells (i.e. β-III-tubulin-only+ cells) percentage increased after notch inhibition particularly in MDB derived cells and, notably, also in MDB cells cultured under lower oxygen, while in normal cells there was increase only in nestin+/ β-III-tubulin+ double positive cells (not shown); additionally, the percentage of nestin only+ cells did decrease in both normal and tumor cells. All these results suggest that blocking notch signalling induces mainly reduction in cell number and strong neuronal commitment in MDB cells and also under lower oxygen tension (tension that normally maintains MDB cells in undifferentiated state) while in normal cells the major effect is inhibition of cell proliferation (i.e. reduction of Ki67+ and nestin+ cells and

decrease of total cell number) that is not followed by increase in neuronal differentiation and that does not seem to be modulated by oxygen. These results perhaps can be explained considering that Notch signalling pathway has been seen to be deregulated in medulloblastoma cells (Hallahan AR et al. 2004) (Dakubo GD et al. 2006), and consequentially, inhibition of this signalling in a normal cell type may drive to block of proliferation, while in tumor cells it could induce neuronal differentiation, but not characterized by a block of cell cycle (i.e. Ki67 reduction), especially under hypoxic conditions.

To test endogenous Notch activation comparing normal and MDB derived cell type, we used DLL4 (R&D), soluble notch ligand, activator of the notch signalling. Notch activation is known to maintain the undifferentiated state, by contributing to human medulloblastoma proliferation and survival (Hallahan AR et al. 2004). We found that DLL4 (40 ng/ml) in SVZ normal derived cells did not have any effect looking at total cell number, Ki67 expression and percentage of nestin+ precursors, it only slightly reduced the proportion of immature committed neurons, and increased the percentage of CD133+ cells (data not shown). Conversely, in medulloblastoma derived cells, DLL4, while in cells cultured at lower oxygen did induce a modest increase in nestin+ precursor percentage, in cells maintained at high oxygen it strongly promoted cell death only after 2 or 3 days of in vitro treatment. Also in this case, analogously to noggin mediated effects, treating MDB derived cells with an anti-differentiating factor, such as DLL4, promotes a stronger sensitization of MDB precursor cells to high oxygen tension deleterious effects.



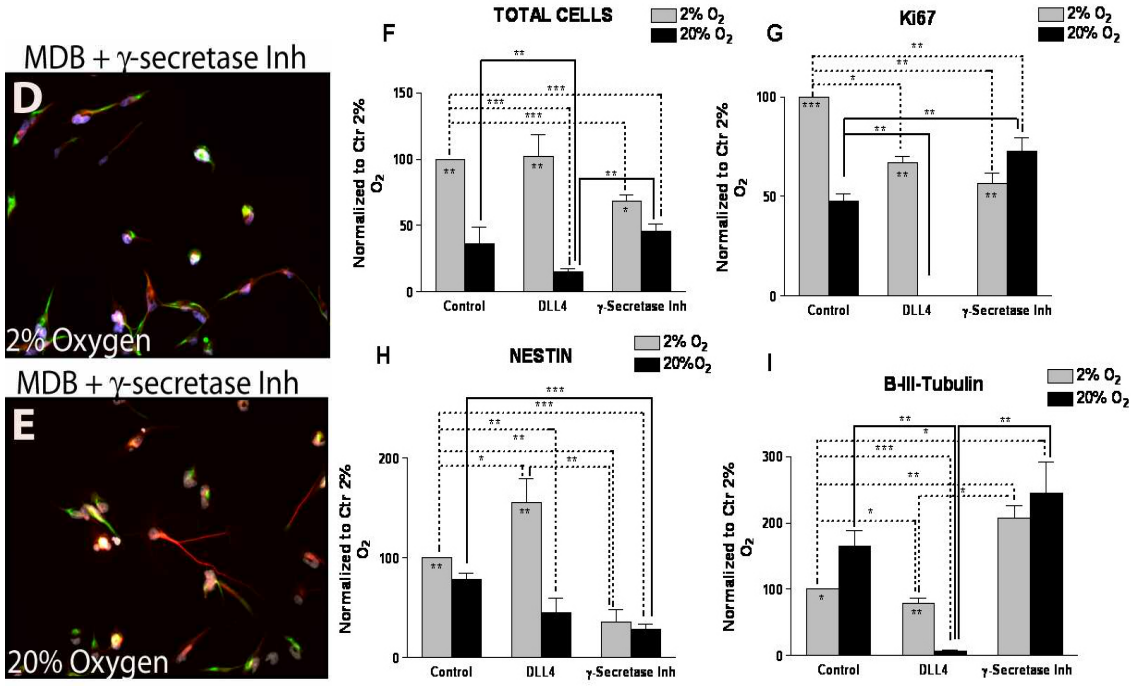


Figure 17. Inhibition of Notch activation by a γ-secretase inhibitor has different effects comparing normal and MDB derived cells and DLL4-mediated notch activation is differentially modulated by oxygen tension. Human MDB derived cells were plated at medium density (49 cells/mm^2) and expanded for 3-5 days at either 2% (A-B-D) or 20% oxygen (C-E), and in presence of either DLL4 (R&D, 40 ng/ml (B-C) or γ-secretase inhibitor-X (Calbiochem, 1μM) (D-E). After DLL4 treatment, nestin+ cells were more expressed at 2% oxygen (see H for quantitation), total cell numbers were unchanged (F) and Ki67 eventually reduced (G); at 20% oxygen, even after 24 hrs, DLL4 treated cells did not survive, and this effect was more dramatic than in untreated cells under the same oxygen tension. Blocking notch activation with γ-secretase inhibitor-X, percentage of nestin+ cells decreased in both 2% and 20% oxygen tensions, and the same effects were seen also by treating normal SVZ derived cells (pictures not shown), Ki67 expression decreased analogously in normal cells and in MDB treated cells, and these effects were seen in both the oxygen tensions tested (see G for quantitation). β-III-tubulin-only+ cells percentage increased after notch inhibition particularly in MDB derived cells and, importantly, also in MDB cells cultured under lower oxygen, while in normal cells there was increase only in nestin+/ β-III-tubulin+ double positive cells (not shown); also nestin+ cells did decrease in both normal and MDB cells (H). Quantitation (F-G-H-I), mean ± S.E.M., at 2% and 20% oxygen, all the conditions: for nestin/Tuj-1/Ki67 n = 4, total cells n= 5. Bar = 50 μm. Nestin (green)/ Tuj-1 (red).

In vivo experiments:

Brain tumor derived cells grafting into the motor cortex of NOD-SCID mice.

Validation of tumor-initiating (cancer stem cell) sub-population. Preliminary data support CD133+ cells as the tumor-initiating population. Thus we validated this data also for the brain tumor derived cells we collected, with the additional effort to perpetuate the original tumor cells characteristics across serial transplantation by providing a more

physiologically compatible cellular environment. First of all we confirmed that CD133+ cells were the one responsible for brain tumor heterogeneity recapitulation, (Singh, S. K. et al. 2004), in particular giving rise to nestin^{+} and GFAP^{+} glial cells for GBM, and a consistent amount of nestin^{+} ßIIItubulin^{+} cells in MDB.

Interestingly, we also found that MDB derived cells, which were normally poorly growing in vitro, were able to acquire a proliferative capacity considerably higher after in vivo transplantation, with a strong increase in nestin and Ki67+ cells expression when compared to their in original vitro cell cultures. This could be due to the presence of growth factors (such as noggin and notch ligands) and environmental conditions (such as the presence of a physiological hypoxic microenvironment in the mouse brain), which are critical promoters of in vivo proliferation of these cells and that are missing once transferred the tumor derived cells in vitro.

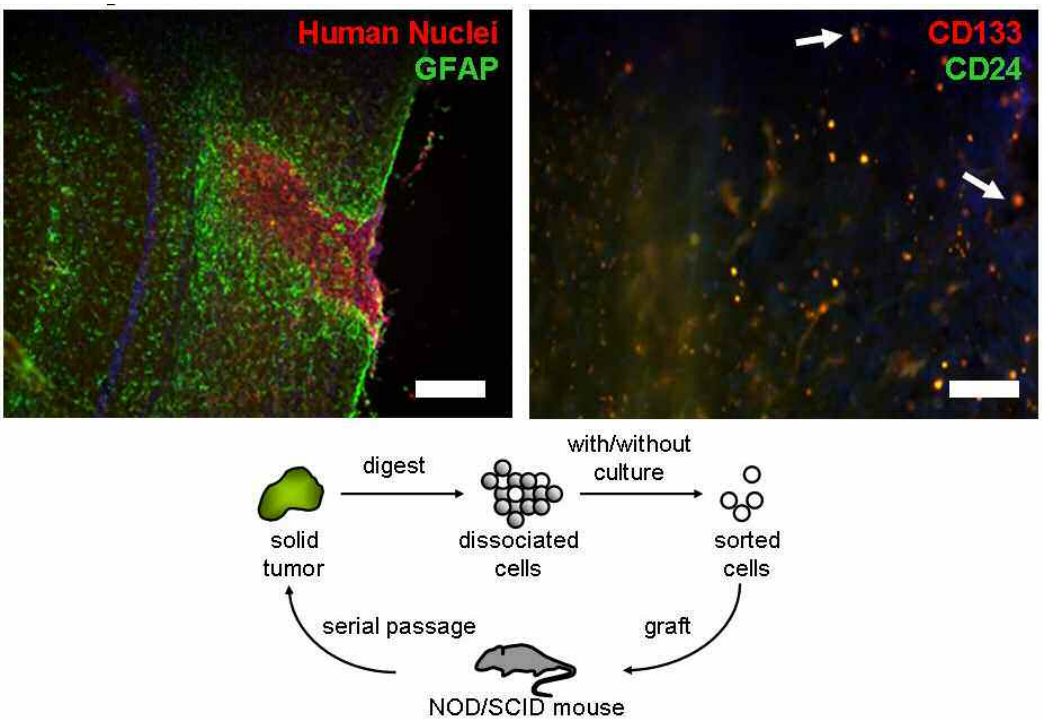


Figure 18. Grafting and expansion of CD133+ cells in NOD/SCID mice. Grafting of sorted CD133+ cells from high grade glioma into NOD/SCID mouse produces a human tumor (measured by Human Nuclear antigen) that generates large numbers of GFAP+ glia (left) but maintains a population of CD133+CD24- cells (right, arrows). Bar = 400 µm (right), 100 µm (left). Below, model of in vivo grafting in NOD/SCID mice.

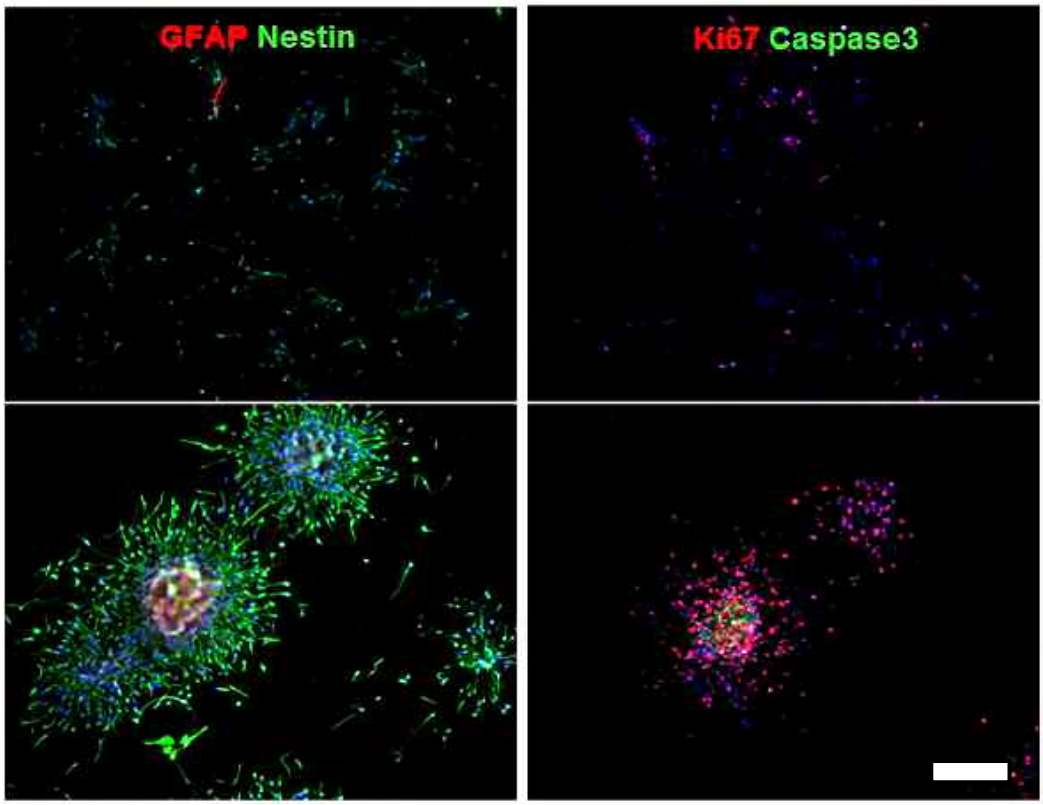


Figure 19. **Immunocytochemical analyses of brain tumor transplanted cells after recovery.** MDB tumor derived unsorted cells were recovered 4 weeks post grafting and analyzed for expression of nestin and Ki67 markers. The proportion of cells positive for both these markers strongly increased when compared to the orginal tumor cells at passage 1 in vitro. Average of 3 independent experiments. 10X pictures, Bar = 100 μm

DISCUSSION

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. In this study we showed for the first time that multi-lineage competence of human CNS precursors and the selective differentiation of oligodendrocytes is 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 found 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 is enhanced in lowered oxygen.

In this study we also showed 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 (T3) (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 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. Indeed, we found that the number of $O4^+$ cells is not decreased by acute exposure to 20% oxygen but in that condition oligodendrocyte

precursors increase their expression of $p21^{cip1}$, indicating that raising oxygen tension depletes oligodendrocyte progenitors primarily through terminal differentiation.

Surprisingly, we also found 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 Smad1/5/8 activation, Id1 expression and gliogenesis at 20% but not 5% oxygen. These 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 found that in CNS precursors oxygen acts in a novel manner to directly repress Smad activation (fig 4C). Our future studies, and the results obtained in part II of this study are going toward that direction, 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 showed 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).

Furthermore, in the last years a big effort has been devoted to understand tumor microenvironment characteristics, the tumor niche, and to elucidate tumor inducing cells characteristics that distinguish them from their normal counterpart, also looking at possible disrupted responses of the tumor cell population to signaling molecules involved in the regulation of cell proliferation, cell fate and apoptosis. In particular, it has been reported that hypoxia plays a key role in normal homeostasis of stem cells and in the initiation, development and aggressiveness of gliomas, developing the concept of a tumor special microenvironment surrounding the glioma, the neo-niche concept, in which hypoxia could

be crucial to recruit and deregulate different stem cells for gliogenesis process (Diabira, S. and Morandi, X. 2007). Indeed, it is known that lowered oxygen tensions positively correlate with tumor aggressiveness (Azuma Y et al. 2003) (Helczynska K et al. 2003) (Jogi A et al 2002) and over-activity of Hypoxia Inducible Factor-1α (HIF-1α) (Smith K et al. 2005) is implicated in tumor progression. The correlation between hypoxia and tumor aggressiveness has been causally linked to increased genomic instability (Koshiji M et al. 2005), but it is also related to increased survival of proliferating cells by suppression of p53 and its associated cell growth control (Zhang L, Hill RP. 2004). Importantly, hypoxia has been shown to promote de-differentiation of neuroblastoma cells (Jogi A et al. 2002) (Jogi A et al. 2003) (Jogi A et al. 2004), suggesting that it may reinforce an environment for aggressive tumor growth. It may also prevent a pre-existing stem cell population from differentiating, which is important in light of increasing evidence that cancer is initiated by dysfunctional stem cells (Al-Hajj M et al. 2003) (Bonnet D et al. 1997) (Singh SK et al. 2004).

Based on these evidences it is reasonable to think either that paracrine cell signaling could be disrupted in this tumor neo-niche or that tumor cell response to mitogenic stimuli could be not correctly modulated (i.e. insensitivity or oversensitivity of tumor cells to extracellular factors). In this regard, it seems that hypoxia modulates some cell signaling pathways. In particular it has recently been reported that hypoxia is an inducer of Notch signaling and hypoxia-induced Notch signalling may determine endothelial identity (Diez H et al. 2007). A recent publication by Gustafsson and colleagues demonstrated that hypoxia blocks cell differentiation through the regulation of Notch signalling in myogenic and neural precursor cells. This study showed that hypoxia-inducible factor (Hif)-1α interacts and acts in synergy with the Notch intracellular domain (NIC) and subsequently activates transcription of Notch targets. All these works strongly indicate that oxygen tension is a crucial modulator of normal and tumor cell response to mitogens stimuli, but this has not been shown in brain tumor specimens' derived cells.

In the experiments described in part II we showed that brain tumor cells, specifically HGG derived cells, expand better at lowered oxygen, while at 20% oxygen expansion of more differentiated GFAP+ cells, at the expense of nestin+ precursors, increases; furthermore

mitotic arrest through p53 and p21 activation and caspase-3 mediated apoptosis are induced.

We obtained similar results also for MDB derived cells, which resulted to be much sensitive to a non physiological oxygen tension (i.e. 20%), with a strong induction of neuronal differentiation (i.e. β-III-tubulin+ cells) activation of caspase-3 mediated apoptotic pathway.

Moreover, 20% oxygen exposure activates BMP signaling pathway also in HGG derived cells (alike normal SVZ cells, part I), as shown by Smad 1/5/8 phosphorylation and Id1 expression, after quick high oxygen exposure, and also endogenous BMP2 and BMP receptors (BMPR-IA,-IB and –II) mRNA expression result to be up-regulated under 20% oxygen. These results correlate BMP signaling pathway activation, and the consequential astroglial commitment, to high oxygen tension.

Recently other works showed a correlation between hypoxia and BMP signaling. For example, in pulmonary endothelial cells BMPR signaling is altered during the pathogenesis of pulmonary hypertension (PH), such as hypoxia-induced PH (Takahashi K et al. 2007). There is also report that hypoxia suppresses the expression of Id1, downstream target of the BMPR2 pathway, in human pulmonary artery smooth muscle cells (HPASMC) (Wu X et al. 2006). In part I, we showed in normal human SVZ derived precursor cells a novel repressive effect of low oxygen on BMP signaling, through inhibition of Smad 1/5/8 phosphorylation and Id1 activation (Pistollato F. et al. 2007).

The importance of investigating the pro-differentiating effects mediated by BMPs on glial tumors has been reported by Piccirillo et al. who showed that bone morphogenetic proteins (BMPs), amongst which BMP4, and analogously BMP2, trigger a significant reduction in the stem-like, tumour-initiating precursors of human glioblastomas (GBMs). While in a previous work (Iantosca MR et al. 1999) it has been shown that bone morphogenetic proteins-2 and -4 attenuated apoptosis in a cerebellar MDB cell line (DAOY), it has also been reported that BMP-2 mediates retinoid-induced apoptosis in medulloblastoma cell lines through a paracrine effect (Hallahan AR et al. 2003). It is important to consider that all these works have been conducted using brain tumor cell lines and not primary cultures, notoriously difficult to expand under conventional in vitro conditions, and importantly the potential effects mediated by different oxygen tensions on primary brain tumor cells growth

in vitro had not been previously described. In this regard, we sought to investigate BMP, particularly BMP2, and Noggin (BMP2 inhibitor) effects on tumor cells also considering oxygen tension as a variable. What we found is that BMP2 in vitro exposure induced cell death and glial differentiation only on HGG derived cells, and not normal cells, in both the oxygen tensions tested, but these effects were partially inhibited by lowered oxygen.

The results observed by treating with BMP2 or Noggin medulloblastoma derived cells indicate that BMP2 does seem to promote a slight increase in total cell number especially at 20% oxygen, unlike what observed by Hallahan et al. where BMP2 promoted retinoid-induced apoptosis in medulloblastoma cells (where they used a very high concentration of BMP2, 100 ng/ml), but analogously to Iantosca et al. work in which they found an attenuation of apoptosis after 10-20 ng/ml BMP2 treatment in MDB cell lines. Interestingly, Noggin, while not improving nestin+ cells number at lower oxygen, seemed to increase cell senescence/cell death at high oxygen tension, as if, by blocking a pro-differentiating signal (i.e. BMP pathway) cells may be more vulnerable to the detrimental effects of high oxygen exposure.

In conclusion, oxygen tension seems to modulate tumor cells proliferation and cell response to extracellular factors such as BMP2.

In MDB derived cells we also found that activation of notch signaling through DLL4 (notch ligand) only at 2% oxygen promoted proliferation and expansion of nestin+ cells, while increasing MDB cell death at 20% oxygen. These effects can be explained by considering that in these tumor cell type there is a higher endogenous Notch activation (Raffel C et al. 1997) (Fan X et al 2004) (Hallahan AR et al 2004), (Fan X et al 2006); indeed, exogenous in vitro stimulation of notch with DLL4 does not promote any further effect rather than an improvement in nestin+ cells maintained at lowered oxygen, but the same treatment at 20% oxygen, by maintaining the undifferentiated cell state through activation of Notch pathway, increased the already high vulnerability of MDB cells to oxygen tension, similarly to what observed with Noggin treatment. Interestingly, Notch inhibition with a γ-secretase inhibitor, while decreasing nestin+ cells by improving commitment toward a neuronal fate (i.e. higher proportion of β-III-tubulin+ cells), preserved 20% oxygen cultured cells from cell death, effect probably due to expansion of more differentiated cells with this treatment.

Additionally, overexpression of Hif-1α and its downstream target genes, such as VEGF and GLUT-1 in higher-grade gliomas support the hypothesis that hypoxia may allow the progression of lower-grade tumors to glioblastoma multiforme (Ragel et al. 2007). High grade gliomas and glioblastoma in particular are highly migrating tumors and this characteristic may explain the reason why we could find high level of Hif1α protein expression in the tumor samples we analyzed, even regardless the presence of hypoxic condition, as Hif1α promotes metastatic seeding and increases invasive and migrating properties of tumor cells into the extracellular matrix (Elstner et al. 2007).

In this study we found that Hif1α protein level and mRNA are down-regulated by BMP2 treatment in both the oxygen tensions tested. In conclusion, we suggest that a hypoxic microenvironment in the brain tumor niche maintains stem-ness also by down-regulating BMP signaling and its pro-differentiating effects. We also conclude that BMP molecules may be used as pro-differentiating factors, in accordance with Piccirillo et al., especially given their ability to downregulate Hif1α, crucial mediator of hypoxia response in the solid tumor niche.

In a recent publication (Fujiwara et al. 2007) introduction of Hif1α-targeted small interfering RNA (Hif1α siRNA) into glioma cell lines resulted in downregulation of Hif1α expression, and significantly suppression of glioma cell migration in vitro. Furthermore, it has also been reported that inhibition of Hif1α decreases VEGF secretion and tumor growth in malignant gliomas (Jensen et al. 2006).

Thus, targeting Hif1α molecule may be a novel therapeutic strategy for malignant gliomas, and our novel finding of a Hif1α downregulation dependent on BMP2 in vitro stimulation may open new perspective clinical applications for malignant glioma treatment.

We still need to further elucidate if also in MDB derived cells, there is an analogous BMP2 dependent Hif1α downregulation and also if γ-secretase inhibitor-X can potentially elicit similar effects. Additionally, we will further investigate if a physical molecular interaction between Hif1α and any of the BMP signaling pathway molecules (such as Smad proteins 1, 5 or 8) occurs.

Finally, our in vivo data confirmed the theory of a hierarchic origin of brain tumors. Indeed, recent evidence indicates that tumors consistently arise from a specific subset of cells defined as cancer stem cells and it has been shown that $CD133^{hi}$ cells can be considered *bona fide* the cancer stem cell subpopulation in glioblastoma and medulloblastoma as they have the highest frequency of initiating new tumors when grafted in NOD/SCID mice (Singh SK et al. 2004). Our in vivo data confirmed the CD133+ population as the cancer stem cells subpopulation responsible for brain tumor recapitulation. Notably, cells that had been grafted and then recovered from the murine brain for continuous serial passaging, exhibited an increase in proliferating cancer precursors (i.e. nestin+Ki67+ cells) and this may be explained considering that the presence in the murine brain of determined soluble factors (such as noggin and notch-delta ligand) and of a hypoxic niche could regulate brain tumor cells proliferation and maintain a stem cells pool in the tumor. Indeed, when cells derived from a tumor biopsy are transferred in vitro the only way to keep the cells for continuous passaging is providing such parameters.

In conclusion, oxygen tension seems to modulate non-tumor and tumor cells behaviour and cell response to extracellular factors such as BMP2 and Notch activating molecules (i.e. DLL4).

Thus, it will be important, in light of recent proofs, to consider the pivotal role of oxygen and its elicited effects on tumor cells growth in order to improve current cancer therapies, especially considering that the outcome for children with primary CNS tumors is still poor and for most tumors has been unchanged over the past decade.

MATERIALS AND 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 2-5% oxygen culturing, cells were incubated in a customized, computer-controlled system (Biospherix, Ltd., Syracuse, NY). This 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; for 2% oxygen 2% O_2, 5% CO_2, 93% N_2 balance) and eliminates artifacts introduced by periodic oxygen reperfusion. 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 (Axovert 10, Zeiss), with a mounted digital camera (QColor3, Olympus), for cell visualization and recording (Fig. 1C).

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 seventh day using Cell Dissociation Buffer (Invitrogen). In some experiments, cultures were supplemented with erythropoietin (10 ng/ml, R&D Systems, Minneapolis, MN), ascorbic acid (200 μM,

Sigma, St.Louis, MO), B27 without retinoic acid (2% v/v, Invitrogen, Carlsbad, CA), 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 7-10 d 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, Carlsbad, CA), 10 ng/ml neurotrophin-3 and 10 ng/ml ciliary neurotrophic factor (both from R&D Systems, Minneapolis, MN) 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.

For the second part of the study, normal SVZ derived cells were supplemented with Bone morphogenetic protein 2 (BMP2, 10 ng/ml, R&D Systems), Noggin (200 ng/ml, R&D Systems), DLL4 (40 ng/ml R&D Systems) or γ-secretase inhibitor-X (2 μM, Calbiochem).

After 3-5 days expansion in presence of BMP2, Noggin, DLL4 or γ-secretase inhibitor-X, cells were fixed in cold 4% paraformaldehyde for 15 min, rinsed and stored prior to analysis. Alternatively, total RNA extraction (using Tryzol methodology) or total protein extractions (using a specific lyses buffer) were performed.

Isolation and Expansion of Human Tumor Cells

Human pediatric tumor tissue was acquired with parental consent using an approved protocol in accordance with Institutional Review Board guidelines. Three medulloblastoma (MDB) and nine high grade gliomas (HGG) have been considered as suitable for the purposes of the study. Tumor tissues were gently minced with a scalpel, then incubated in 1X Hank's Buffered Saline Solution (HBSS, Ca^{2+}/Mg^{2+}-free, plus Hepes and 1.55 g/L glucose, without bicarbonate, pH 7.2, Invitrogen, Carlsbad, CA) containing 200 U/ml DNase I (Roche), 1 mM $MgCl_2$ and 200 μg/ml Liberase-1 (0.62 WU/ml collagenase and 66.7 U/ml dispase, Roche, Indianapolis, IN) for 1 hr at 37°C. Samples were spun at 200 x g for 5 min, resuspended in fresh HBSS/DNase/$MgCl_2$ without enzyme, and triturated with 3 rounds of 8 passes through an ART 1000E pipet tip. After each round of trituration, the tissue was allowed to settle 5 min and the top 800 μl of suspended cells were transferred

into a new tube to avoid further disturbance. The combined cell suspensions for each group were spun down again and resuspended in medium appropriate for further use. 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 20 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 seventh day or when confluent using TrypLE (Invitrogen). In some experiments, cultures were supplemented with Bone morphogenetic protein 2 (BMP2, 10 ng/ml, R&D Systems), Noggin (200 ng/ml, R&D Systems), DLL4 (40 ng/ml R&D Systems) or γ-secretase inhibitor-X (2 μM, Calbiochem).

After 3-5 days expansion in presence of BMP2, Noggin, DLL4 or γ-secretase inhibitor-X, cells were fixed in cold 4% paraformaldehyde for 15 min, rinsed and stored prior to analysis. Alternatively, total RNA extraction (using Tryzol methodology) or total protein extractions (using a specific lyses buffer) were performed.

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, St. Louis, MO), polysialated neural cell adhesion molecule (PSA-NCAM, mouse, 1:400, Chemcon), β-III-tubulin (rabbit, 1:2000, Covance), galactocerebroside-C (GalC, mouse, 1:300, Chemicon), PDGFRα (rabbit, 1:100, Santa Cruz), $p21^{cip1}$ (mouse, 1:800, LabVision), phospho-p53 Sampler Kit (as directed, Cell Signaling), Phospho-Smad1/5/8 (rabbit, as directed, Cells Signalling) or Id1 (rabbit, 1:50, Santa Cruz). After incubation, cells were washed and incubated with species-specific fluorescent secondary antibodies (Alexa dyes, Invitrogen, Carlsbad, CA). 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, or VICO microscope, Nikon) and images compiled for

figures using Illustrator 7 (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 2%, 5% or 20% oxygen, cells were passaged and resuspended in flow cytometry buffer, consisting of 1X 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 x 10^6 cells/ml buffer; analysis was performed with 25 µl aliquots containing 1 x 10^5 cells. For surface marker analysis, I used antibodies against human CD133 (clone AC133/2-PE, as directed, Miltenyi) and human CD24 (FITC-conjugated mouse IgG_{2a}, BD Biosciences, or PC5-conjugated mouse IgG1, Immunotech, a Coulter company). Cells were incubated at 4^{o}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., 2007), or by using a Cytomics FC 500 flow cytometer (Beckman Coulter). Fluorescent intensities for cells in the population were point-plotted on 2-axis graphs or histogram using CellQuest software (BD Biosciences), or Expo32 software (Beckman Coulter). For cell cycle analysis by using propidium iodide at least 1 x 10^6 cells per group were resuspended in PBS 1X and fixed in cold ethanol 70%, then stored at -20 ^{o}C for at least 2 hrs prior analysis. Cells were washed in PBS 1X and preincubated for 30 min in 1 ml of PI/Triton X-100/RNase A (containing 0.1% Triton X-100 in PBS 1X, 2 mg RNAse A, and 200 µl of 1mg/ml propidium iodide). After incubation cells were analyzed.

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., 2007). 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.

Western blot and densitometric analyses

After culturing cells under lower or atmospheric oxygen tension total protein extracts were rapidly collected in order to minimize Hif1α protein degradation. As Hif1α turnover is rapid, with a progressive decrease in protein levels occurring after only 4-16 min of re-oxygenation (Jewell UR. et al. 2001), I performed total protein extraction by scrapering the cells from the culture dishes and cellular protein extracts were isolated by using a specific protein lyses buffer, containing TPER Reagent (Pierce), 300 mM NaCl, 1 mM orthovanadate, 200 mM PEFABLOC (AEBSF) (Roche), 1 μg/mL Aprotinin (Sigma), 5 μg/mL Pepstatin A (Sigma), 1 μg/mL Leupeptin (Sigma). This buffer is particularly indicated to preserve protein phosphorylated residues. Equal amounts of protein (10–20 μg) were resolved using a SDS-PAGE gels (at different percentage of acrylamide depending on the protein analyzed) and transferred to PVDF Hybond-p membrane (GE Healthcare).

Membranes were blocked with ECL Advance Blocking (Amersham Pharmacia, 2%) overnight, under rotation at 4°C. Membranes were then incubated with primary antibodies monoclonal anti-Hif1α (BD, Murine, 1:250), or polyclonal Id1 (Santa Cruz, Rabbit, 1:1000), polyclonal Pi-Smad1/5/8 (Cell signaling, Rabbit, 1:1000) or monoclonal β-actin (Sigma, Murine, 1:10.000) for 2 hrs. Membranes were next incubated with peroxidase-labeled goat anti-rabbit IgG (Sigma, 1:100.000) or peroxidase-labeled goat anti-murine IgG (Sigma, 1:100.000) for 60 min. All membranes were visualized using ECL Advance (GE Healthcare) and exposed to Hyperfilm MP (GE Healthcare). Densitometric analysis of the films was performed using Scion Image densitometer software.

Real-Time PCR analyses.

RNA from HGG cells were isolated by the Tryzol method (GIBCO BRL) and 1 μg of total RNA reverse-transcribed using SuperScript RNAse H- Reverse Transcriptase (Life Technologies). Quantitative RT-PCR reactions were run in duplicate using Brilliant® SYBR® Green QPCR Core Reagent Kit (Stratagene, La Jolla, CA). Fluorescent emission was recorded in real-time (Sequence Detection System 7900HT, Applied Biosystems, Foster City, CA, USA). Gene expression profiling was completed using the comparative Ct method of relative quantification. Relative RNA quantities were normalized to GAPDH as

endogenous control and 2% oxygen untreated (control group) has been considered as the calibrating condition.

Primers Sequences.

For real time-PCR, the following primers were used:

BMP2

Forward: ATGTGGACGCTCTTTCAATGG

Reverse: ACGCTAGAAGACAGCGGGTC

BMPR1A

Forward: TAAAGGTGACAGTACACAGGAACA

Reverse: TCTATGATGGCAAAGCAATGTCC

BMPR1B

Forward: TACAAGCCTGCCATAAGTGAAGAAGC

Reverse: ATCATCGTGAAACAATATCCGTCTG

BMPR2

Forward: 5'-GCTAAAATTTGGCAGCAAGC-3'

Reverse: 5'-CTTGGGCCCTATGTGTCACT-3'

Hif1α

Forward: 5'- CGTTCCTTCGATCAGTTGTC -3'

Reverse: 5'-TCAGTGGTGGCAGTGGTAGT -3'

GAPDH

Forward: 5'-ACGGATTGGTCGTATTGGG-3'

Reverse: 5'-CAGAGTTAAAAGCAGCCCTGGT-3'

The specificity of the primers was confirmed for every PCR run by dissociation curve analysis. RT-PCR amplification conditions consisted of 50 cycles with primers annealing at 61°C.

Statistical analysis

Graphs and statistical analyses were prepared using Prism 3.03 (Graph Pad) or Excel. All values are 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 2-5% oxygen counterpart; an asterisk over a bracket indicates a significant difference with another variable as indicated.

In Vivo Grafting of brain tumor derived cells and CD133+ isolated brain tumor stem cells.

Recapitulation of tumor has been obtained by orthotopic grafting in Motor Cortex using NOD/SCID mice. These mice exhibit nearly complete immune deficiency and have been used successfully in other studies involving stem and tumor cell grafting (Al-Hajj, M. et al. 2003). Stereotaxic Injections of 1-5 $x10^5$ cells tumor derived cells and CD133+ isolated tumor cells have been performed. A period of incubation of about 4-12 weeks was necessary before intracardiac perfusion w/PFA 4% and brain immunohistochemistry or brain tumor resection for continuous serial passaging.

Cells for grafting were taken (a) from acutely dissociated tumors, (b) from acutely dissociated tumor cells after sorting by flow cytometry, or (c) from in vitro expanded cells following sorting by flow cytometry. For serial passaging, contaminating mouse cells were selectively removed using an antibody against mouse histocompatibility class I (H2kd, BD Pharmingen). Eight week old NOD/SCID mice were anesthetized by i.p. injection of ketamine/xylazine (300 mg ketamine combined with 20 mg xylazine in a 4 ml volume of HBSS vehicle; 0.2 ml per 20 g mouse) as previously described (Al-Hajj, M. et al. 2003). For brain grafts, dissociated tumor cells were stereotaxically grafted into the brain parenchyma (motor cortex). For this procedure, a mid-line scalp incision was made and the coronal suture exposed. A burr hole was made 1.5 mm to the right of midline and 0.5 mm anterior to the coronal suture. Using a Hamilton syringe, 2 µl of the cell suspension was injected to a depth of 2.5 mm and at a rate of 1 µl/minute. Bone wax was applied on the injection site and the skin closed with Nexaban Liquid (a surgical glue). After injection, mice were i.p injected with a recovery solution (Yohimbine (0.02 mg/ml final, 0.2 ml per 20 g mouse), allowed to recover on a warm heating pad and then returned to the cage. The procedure was performed using stereotaxic coordinates to ensure reproducible site of grafting.

As a negative control, we performed normal SVZ derived cells grafting. When the tumor cell-grafted mouse generated a new tumor, the mouse was sacrificed by CO2 inhalation followed by cervical dislocation and the tumor mass, generally clearly visible was dissected out from the mouse brain and dissociated for immunocytochemical analyses. Alternatively, cardiac perfusion of saline solution followed by 15 min of 4% paraformaldehyde perfusion was performed and the whole mouse grafted brain was recovered, overnight fixed at 4C in 4% paraformaldehyde, then frozen in OCT liquid and immunohistochemically analyzed. Human grafted cells were distinguished from murine brain cells by using an antibody anti human nuclei (Murine, Chemicon).

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