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ORIGINAL ARTICLE

Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1α

A Soeda[1], M Park[1], D Lee[1], A Mintz[2], A Androutsellis-Theotokis[3], RD McKay[3], J Engh[2], T Iwama[4], T Kunisada[5], AB Kassam[1,2], IF Pollack[1,2] and DM Park[1,2,6]

[1]Brain Tumor Program, University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA; [2]Department of Neurological Surgery, University of Pittsburgh, Pittsburgh, PA, USA; [3]Laboratory of Molecular Biology, National Institutes of Health, Bethesda, MD, USA; [4]Department of Neurosurgery, Gifu University School of Medicine, Gifu, Japan; [5]Department of Tissue and Organ Development Regeneration and Advanced Medical Science, Gifu University School of Medicine, Gifu, Japan and [6]Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA, USA

Hypoxia contributes to the progression of a variety of cancers by activating adaptive transcriptional programs that promote cell survival, motility and tumor angiogenesis. Although the importance of hypoxia and subsequent hypoxia-inducible factor-1α (HIF-1α) activation in tumor angiogenesis is well known, their role in the regulation of glioma-derived stem cells is unclear. In this study, we show that hypoxia (1% oxygen) promotes the self-renewal capacity of CD133-positive human glioma-derived cancer stem cells (CSCs). Propagation of the glioma-derived CSCs in a hypoxic environment also led to the expansion of cells bearing CXCR4 (CD184), CD44^{low} and A2B5 surface markers. The enhanced self-renewal activity of the CD133-positive CSCs in hypoxia was preceded by upregulation of HIF-1α. Knockdown of HIF-1α abrogated the hypoxia-mediated CD133-positive CSC expansion. Inhibition of the phosphatidylinositol 3-kinase (PI3K)-Akt or ERK1/2 pathway reduced the hypoxia-driven CD133 expansion, suggesting that these signaling cascades may modulate the hypoxic response. Finally, CSCs propagated at hypoxia robustly retained the undifferentiated phenotype, whereas CSCs cultured at normoxia did not. These results suggest that response to hypoxia by CSCs involves the activation of HIF-1α to enhance the self-renewal activity of CD133-positive cells and to inhibit the induction of CSC differentiation. This study illustrates the importance of the tumor microenvironment in determining cellular behavior.
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Correspondence: Dr DM Park, University of Pittsburgh Cancer Institute, UPMC Cancer Pavilion, 5th Floor, 5150 Centre Avenue, Pittsburgh, PA 15232, USA.
E-mail: parkdm@upmc.edu
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Introduction

Gliomas, the most common primary brain tumor, are believed to be initiated and maintained by cancer stem cells (CSCs), a population of cells capable of extensive self-renewal, differentiation into multiple lineages and recapitulation of the original tumor in immunodeficient mice (Galli *et al.*, 2004; Singh *et al.*, 2004). In that CSCs seem to have a central role in tumor initiation and recurrence, it has been suggested that definitive cancer treatment needs to target this population of cells (Reya *et al.*, 2001; Pardal *et al.*, 2003; Bao *et al.*, 2006a; Vescovi *et al.*, 2006; Park *et al.*, 2007). Similar to other cancers, microenvironmental interactions are believed to be critical in the pathogenesis and progression of gliomas (Calabrese *et al.*, 2007). As gliomagenesis and subsequent growth of the tumor may, in part, result from an aberrant interaction between the cancer cells and the tumor niche, a better understanding of the microenvironmental cues may lead to the identification of new therapeutic targets (Pouyssegur *et al.*, 2006; Keith and Simon, 2007; Calabrese *et al.*, 2007). Elucidating the tumorigenic role of the microenvironment may be as important as understanding the mechanisms involved in cellular transformation. Similar to the normal neural stem cell (NSC) niche in the subventricular zone, the CSC microenvironment of the glioma may provide the strictly regulated signals necessary to maintain the undifferentiated state of the CSCs, thereby preserving their proliferative and multipotential capacities (Pouyssegur *et al.*, 2006; Keith and Simon, 2007).

The proliferative phase leading to tumor expansion is thought to be governed by microenvironmental cues, including the regulation and availability of oxygen and nutrients (Keith and Simon, 2007). Although hypoxia may be an inevitable outcome of the rapidly growing tumor outstripping its vascular supply, it confers certain advantages on tumor cells (Pouyssegur *et al.*, 2006). For example, hypoxia renders tumor cells with greater resistance to anticancer therapy such as radiation. Therefore, factors that regulate the hypoxic state represent potential targets for anticancer therapy. Although the self-renewal capacity and the undifferentiated state of NSCs

are known to be enhanced by hypoxia (Gustafsson *et al.*, 2005; Chen *et al.*, 2007), such an effect on CSCs is unclear. One well-characterized mechanism of the cellular response to reduced oxygen availability involves the enhanced expression of the hypoxia-inducible factor-1α (HIF-1α) (Semenza and Wang, 1992; Maxwell *et al.*, 1997; Carmeliet *et al.*, 1998). HIF-1α has also been shown to be induced by numerous stimuli other than hypoxia, including insulin, insulin-like growth factors, epidermal growth factor (EGF) and heavy metals (Richard *et al.*, 1999; Zhong *et al.*, 2000; Fukuda *et al.*, 2002). In addition, the mammalian target of rapamycin (mTOR), a downstream effector of the PI3K pathway, is involved in hypoxic signal transduction and has been implicated in the regulation of HIF-1α stabilization, accumulation and activation (Hudson *et al.*, 2002).

We have shown earlier that EGF signaling promotes sphere formation and enhances the self-renewal capacity of CSCs, including the CD133-positive sub-population, a putative marker of glioma CSCs (Soeda *et al.*, 2008). In this study, we examined the role of hypoxia and the subsequent activation of HIF-1α in the self-renewal of human glioma-derived CSCs. The effect of hypoxia on CSCs was characterized by surface stem/progenitor and other marker expression including CD133, CD44, A2B5, CD24 and CXCR4 (CD184). We also investigated the regulation of HIF-1α by PI3K-Akt-mTOR and ERK/MAPK pathways in the glioma-derived CSCs. Furthermore, we characterized the differentiation state and the multipotential capacities of CSCs under normoxic and hypoxic conditions. Our data suggest hypoxia promotes self-renewal and proliferation of human glioma-derived CSCs, in part, by activation of HIF-1α, and that normoxia induces differentiation and restricts self-renewal of CSCs.

Results

Hypoxia enhances CD133+ CSC self-renewal and proliferation

All experiments were performed with previously established malignant-glioma-derived CSC lines from freshly resected surgical specimens under a protocol approved by the local Institutional Review Board (Oka *et al.*, 2007; Soeda *et al.*, 2008). These CSC lines express markers of the undifferentiated phenotype, show self-renewal and are multipotent. These cells express the neural stem/progenitor markers such as nestin, musashi-1 and CD133 in the presence of EGF in serum-free medium. Transplantation of 100–1000 CD133-positive cells into the brains of immunodeficient mice recapitulates the original tumor (Oka *et al.*, 2007). These cells also express markers of neuronal, astrocytic and oligodendroglial lineages in serum containing differentiation medium (Supplementary Figure 1). The self-renewal capacities of these cells were determined using a modified sphere-forming assay (Soeda *et al.*, 2008). At hypoxic condition (1% oxygen), the sphere-forming abilities of the dissociated single cells were strikingly increased compared with normoxia (Figure 1a). Analysis of the CD133+/CD133− ratio showed that hypoxia preferentially expanded the CD133+ CSCs in a time-dependent manner (Figures 1b–d).

Hypoxia promotes expression of HIF-1α and vascular endothelial growth factor

In that HIF-1α is an important molecule in the response of cells to hypoxia, we hypothesized that hypoxia may promote the self-renewal of the CD133-positive CSCs by stabilizing HIF-1α expression. To test this hypothesis, we cultured tumor spheres in hypoxia and determined HIF-1α expression by western blot (Figure 2a). At 1% oxygen condition, HIF-1α was observed within 2 h. Interestingly, there was no difference in HIF-2α expression. As vascular endothelial growth factor (VEGF) is an important paracrine growth factor involved in angiogenic response to HIF-1α, we determined the VEGF levels at normoxia and hypoxia. After exposure to hypoxia for 72 h, the VEGF levels were nearly threefold higher in hypoxia. Interestingly, CSCs derived from grade III glioma (X03) produced less VEGF compared with grade IV-derived CSCs (X01 and X02).

We used a transient knockdown approach to further investigate the role of HIF-1α, HIF-2α, prolyl hydroxylase domain-containing protein-2 (PHD2) and Notch1 in promoting the sphere-forming capacity of glioma CSCs. Transfection of CSCs propagated in hypoxia with HIF-1α small interfering RNA (siRNA) abrogated the expression of HIF-1α and decreased the CD133-positive sub-population (Figure 2c). This also resulted in inhibition of sphere formation (Figure 2c). HIF-1α depletion experiment performed at normoxia had no effect on CSC sphere-forming capacity (data not shown). PHD2 depletion at normoxia resulted in low expression of HIF-1α (Figure 2d). However, this did not lead to a significant difference in sphere formation. Depletion of HIF-2α and Notch1 also showed no difference in the sphere-forming ability (Figures 2e and f). These data suggest that for the X01, X02 and X03 CSC lines, hypoxia is a potent inducer of HIF-1α expression, and that HIF-1α has a critical role in hypoxia-mediated self-renewal.

Hypoxia-induced expression of HIF-1α is augmented by the EGFR signaling pathway

Epidermal growth factor receptor (EGFR) signaling is important to the survival and growth of glioma cells. We have shown earlier that soluble EGF enhances the self-renewal capacity of CD133-positive CSCs in a dose-dependent manner. To determine the combined effect of hypoxia and EGFR signaling, recombinant EGF was added to CSCs propagated at normoxia and hypoxia. The addition of EGF to CSCs in normoxia had no effect on HIF-1α. However, EGF was capable of further amplifying the hypoxia-induced expression of HIF-1α, suggesting that EGFR signaling may have the potential to augment the hypoxia-mediated CSC self-renewal

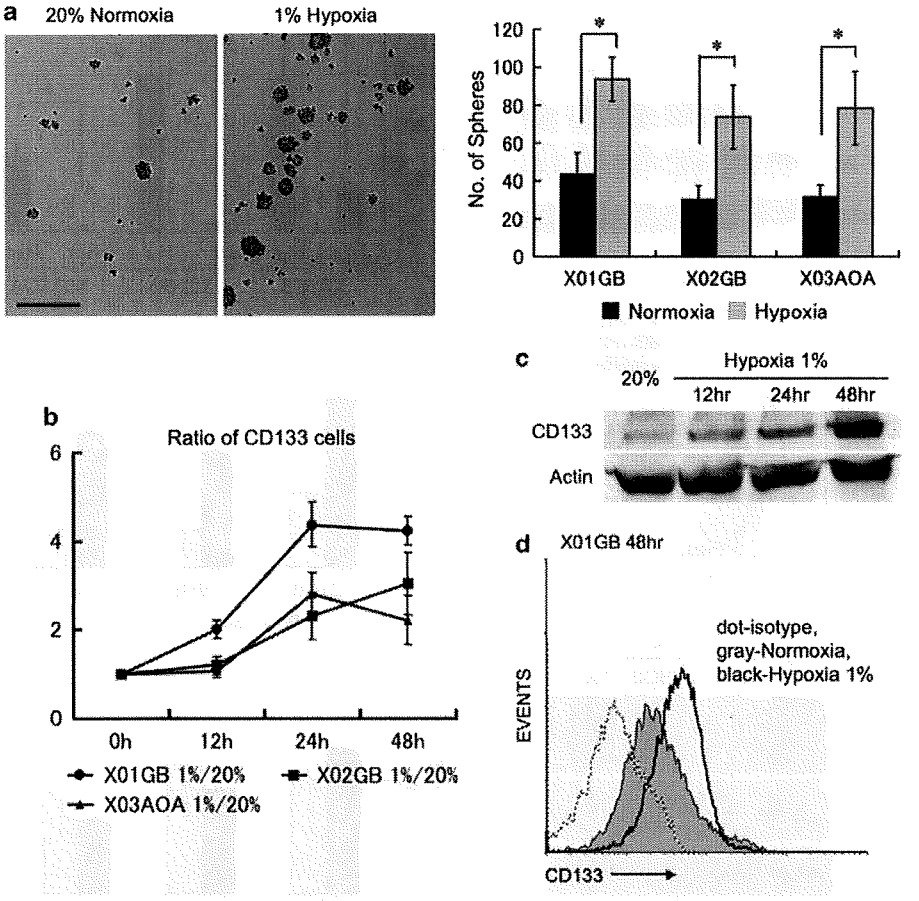

Figure 1 Hypoxia promotes self-renewal of glioma cancer stem cells (CSCs). (**a**) Sphere-forming assay shows that the self-renewal capacity of CSCs is increased when incubated at 1% oxygen (hypoxia) compared with 20% oxygen. CSCs cultured in serum-free medium supplemented with epidermal growth factor (20 ng/ml) were incubated for 5 days. On the left, the picture of cells in a representative well was taken with phase contrast microscopy. On the right, the *X* axis of the graph indicates different CSC lines, and the *Y* axis indicates the number of spheres present. Using a 24-well plate format, one thousand cells were seeded per plate in 2 ml medium, and the number of spheres was counted after 5 days. The results shown in the graph are mean ± s.d. from three experiments. $^*P<0.05$, bar = 100 μm. (**b**) The relative number of CD133-positive cells increases in response to hypoxia. The graph shows the relative expansion of CD133-positive cells as determined by a ratio of CD133-positive cells to CD133-negative cells at hypoxia versus normoxia at different time points. The increasing ratio implies a specific expansion of CD133-positive cells rather than a nonspecific expansion because of the overall increase in total cell number. (**c**) Representative immunoblot for X01 CSC line shows increasing expression of CD133 total protein level in a time-dependent manner under hypoxia. (**d**) Representative fluorescence-activated cell sorting histogram for the X01CSC line shows an expansion of CD133-positive cells in hypoxia.

capacity (Figure 3a). Yet, in the absence of exogenous EGF, hypoxia alone was able to expand the CD133-positive CSCs (Figure 3b). These observations suggest the presence of cross talk between the hypoxic response and EGFR signaling, but the latter is not required for hypoxia-induced expansion of CD133-positive CSCs.

PI3K/Akt/mTOR and ERK pathways interact with hypoxia signaling

Hypoxia is capable of stimulating key signaling pathways activated in stem cells and cancer (Keith and Simon, 2007). In the absence of exogenous EGF, hypoxia alone was sufficient to activate the PI3K and MAPK pathways in the CSCs by augmenting phosphorylation of Akt, ERK and p70S6-kinase (Figure 4a). To further characterize the cross talk between the growth factor and hypoxia signaling pathways in the glioma-derived CSCs, cells cultured at hypoxia were exposed to various inhibitors: PI3K inhibitor (LY294002), ERK inhibitor (PD98059) and mTOR inhibitor (rapamycin). Cells were pretreated with the inhibitors for 2 h before placement in the 1% oxygen environment. Hypoxic induction of HIF-1α was attenuated by these inhibitors (Figure 4b). Finally, we investigated the effect of such inhibitors on the hypoxia-induced CD133-positive CSC expansion. Inhibition of the PI3K and ERK pathways independently abrogated the HIF-1α-mediated expansion of CD133-positive CSCs (Figure 4c). These data suggest that inhibition of the growth factor signaling pathway may attenuate hypoxia-induced expansion of CD133-positive CSCs.

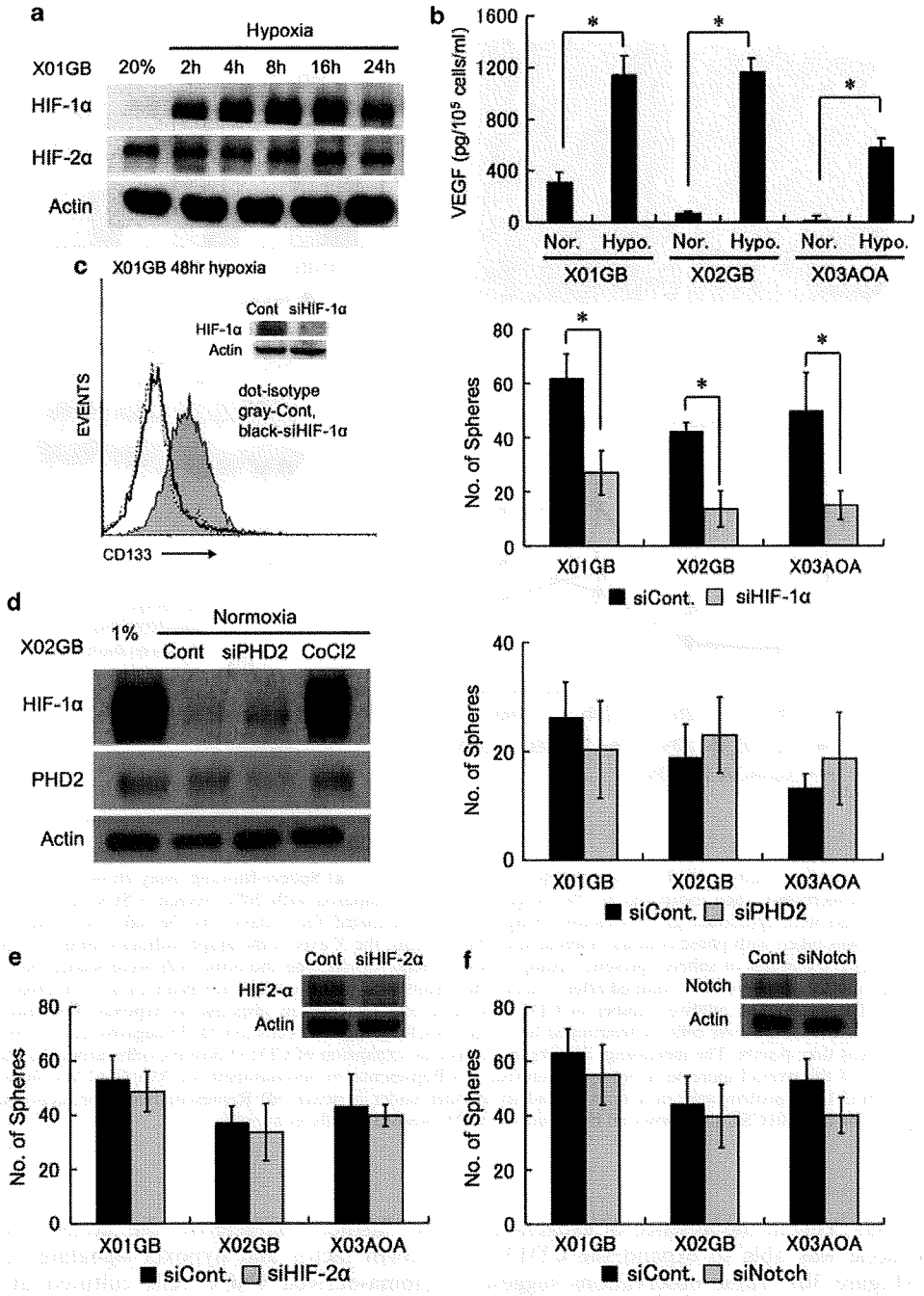

Figure 2 Hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expressions under hypoxic condition. (**a**) HIF-1α expression level is seen at hypoxia in a time-dependent manner. HIF-2α expression appears stable and unaffected by oxygen tension in the X01 cancer stem cell (CSC) line. (**b**) Hypoxia promotes VEGF production by CSCs. The results shown in the graph are mean ± s.d. from three experiments. $*P<0.05$. (**c**) Treatment of CSCs with HIF-1α small interfering RNA (siRNA) abrogates the induction of HIF-1α protein in response to hypoxia and decreases CD133 expression. The adjacent graph on the right shows that the depletion of HIF-1α results in a striking reduction in the sphere-forming capacity. Data are from representative experiments repeated three times. (**d**) Depletion of prolyl hydroxylase domain-containing protein-2 (PHD2) induces expression of HIF-1α at normoxia, but does not show enhanced sphere formation. PHD2 inhibition with siPHD2 at room oxygen tension results in low expression of HIF-1α. However, there is no significant difference in sphere formation. (**e** and **f**) Depletion of neither HIF-2α nor Notch1 affects the sphere-forming capacity.

Propagation at hypoxia expands the CD133/CXCR4/CD44^{low}/A2B5/CD24-positive cells

To further characterize the phenotype of the CSC subpopulation showing expansion at reduced oxygen, we performed fluorescence-activated cell sorting analysis of CSCs with a series of surface markers associated with stem/progenitor and cancer cells. The effect of hypoxia on the differential expressions of CD133, CXCR4

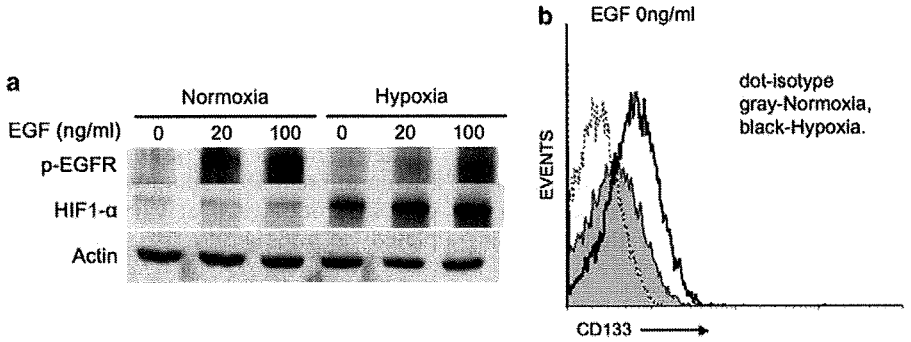

Figure 3 Hypoxia-inducible factor-1α (HIF-1α) induction by hypoxia is amplified by epidermal growth factor receptor (EGFR) signaling, but is not required. (**a**) HIF-1α proteins were analysed by western blotting experiments with different concentrations of EGF (0, 20, 100 ng/ml) at normoxia and hypoxia. (**b**) Fluorescence-activated cell sorting analysis shows that hypoxia is capable of expanding the CD133-positive cancer stem cell (X01 line) in the absence of exogenous EGF ligand. Data are from representative experiments repeated three times.

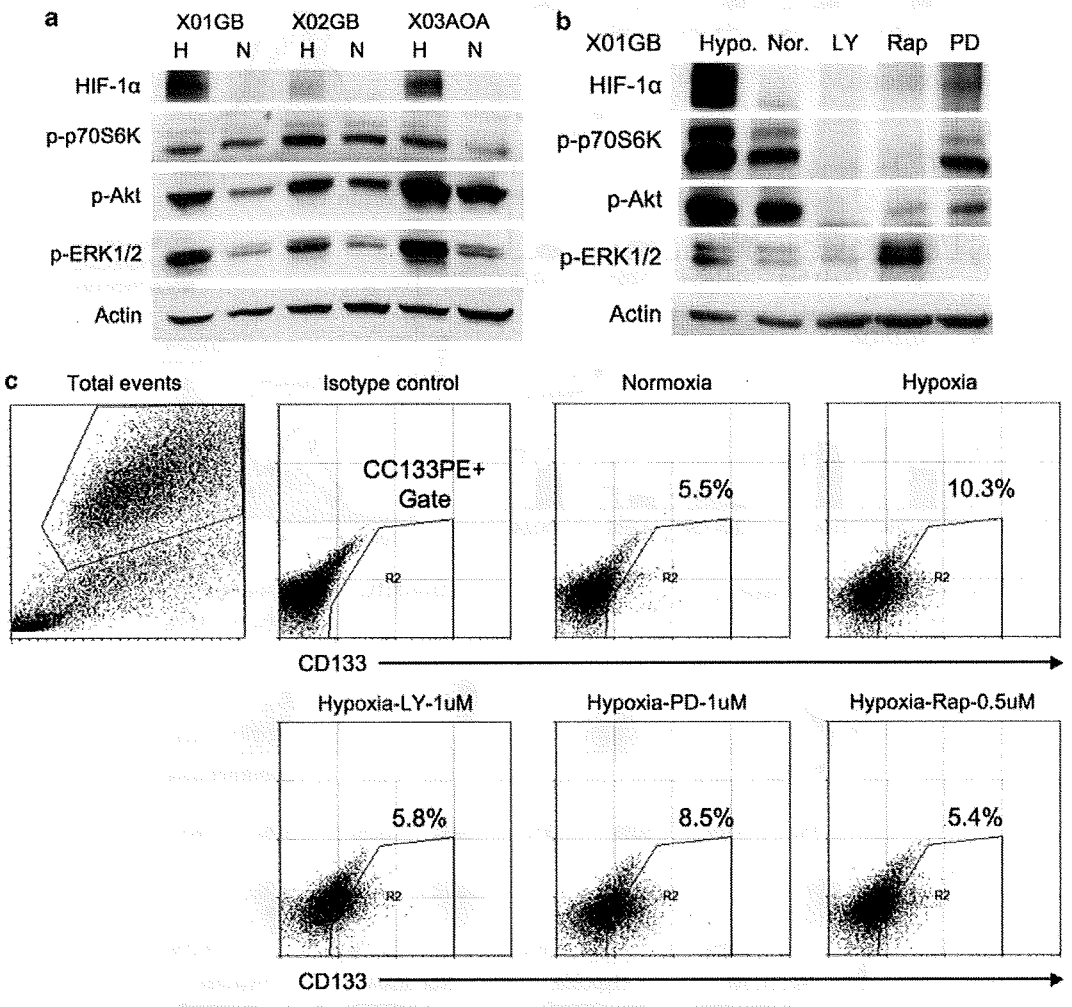

Figure 4 Hypoxic cellular response interacts with growth factor signaling pathway. (**a**) In the absence of the exogenous EGF ligand, hypoxia enhances the phosphorylation of p70S6K, Akt and ERK. (**b**) Hypoxic induction of hypoxia-inducible factor-1α (HIF-1α) is attenuated by growth factor signaling pathway inhibitors. Cancer stem cells (CSCs) were pretreated with LY294002-10 μM (LY), PD98059-20 μM (PD) and rapamycin-5 μM (Rap) for 2 h before 2 h of incubation in a hypoxic environment. Growth factor signaling inhibitors suppress HIF-1α expression under hypoxia. Data are from X01 CSC line representative experiment repeated at least three times. (**c**) Inhibition of the PI3K and ERK pathways independently suppresses the hypoxia-mediated expansion of CD133-positive CSCs. The total number of CD133-positive CSCs at 20% oxygen, 1% oxygen, and with exposure to LY294002 (LY; 1 μM), PD98059 (PD; 1 μM) and Rapamycin (Rap; 0.5 μM) was determined by fluorescence-activated cell sorting dot plot analysis. Data are from X03 CSC line representative experiment repeated at least three times.

(CD184), CD44, A2B5 and CD24 was examined. Propagation at hypoxia led to an increase in the number of cells bearing CXCR4, CD44^{low}, A2B5 and CD24, with a reduction in CD44^{high}-expressing cells (Figures 5a–c). Fluorescence-activated cell sorting dot plot analyses show an overall reduction in the proportion of CD44-positive cells and an increase in the CD133- and CXCR4-positive cells with exposure to hypoxia (Figure 5d). These results show that hypoxia preferentially expands the CD133/CXCR4/CD44^{low}/A2B5/CD24-positive sub-population of CSCs.

Hypoxia inhibits CSC differentiation

To determine the role of hypoxia in the maintenance of the undifferentiated state, we compared the phenotype of CSCs propagated at varying oxygen tensions. Cells plated at a density of 1000/cm^2 under different

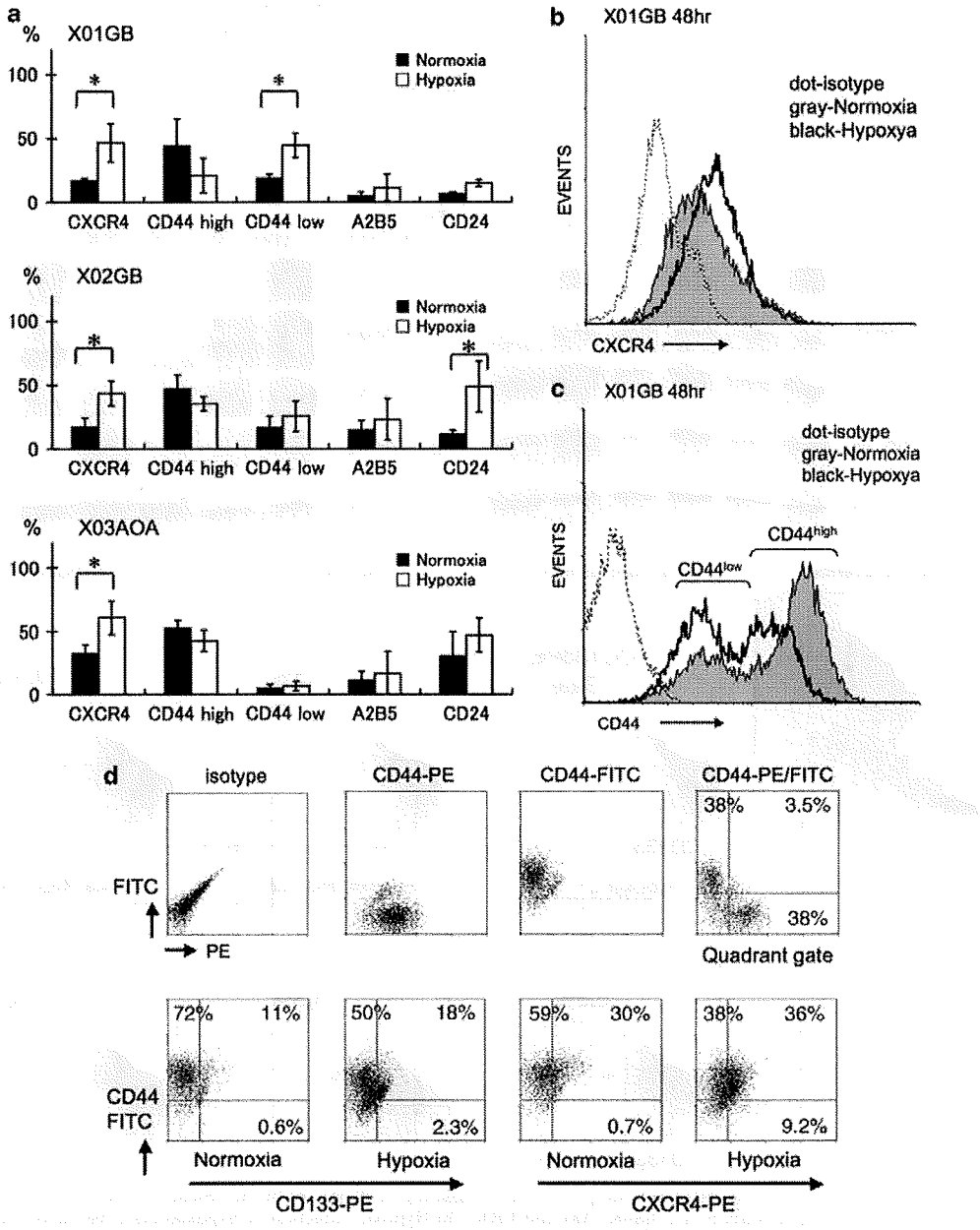

Figure 5 Hypoxia expands the CD133-positive cells enriched for CXCR4, CD44^{low}, A2B5 and CD24 surface markers. (**a**) Cancer stem cell (CSC) lines show a statistically significant expansion of CXCR4 under hypoxia. The X01 lines showed significant expansion of CD44^{low}, and X02GB showed a statistically significant expansion of CD24. (**b**) Representative fluorescence-activated cell sorting (FACS) histogram for X02GB CSC shows expansion of CXCR4-positive cells in hypoxia. (**c**) Representative FACS histogram for X01GB CSC shows expansion of CD44^{low} in hypoxia. (**d**) Representative FACS dot plot analysis for X03AOA CSC shows that hypoxia results in increase in the number of CXCR4 and CD133. Cells exposed to a hypoxic environment for 48 h were fixed and analysed by FACS. Data are from representative experiments repeated at least three times.

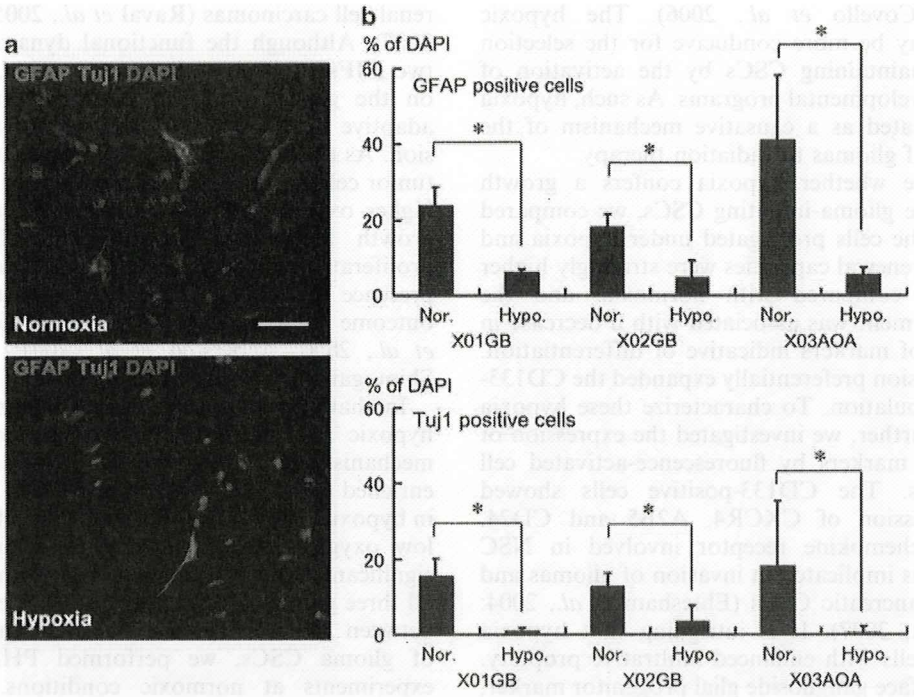

Figure 6 Hypoxia maintains undifferentiated cancer stem cells (CSCs). (**a**) Normoxia induces differentiation of glioma CSCs. Representative colonies were stained with glial fibrillary acidic protein (GFAP) (astrocytic marker) and Tuj1 (neuronal marker) under 20 and 1% oxygen tensions with a medium supplemented with 10% serum. Bar = 50 μm. (**b**) The number of cells expressing lineage-restricted markers was strikingly reduced in cells propagated at hypoxia compared with normoxia. The results shown in the graph are means ± s.d. from three experiments. $*P<0.05$.

conditions were analysed by indirect immunofluorescence microscopy using two lineage-restricted markers: glial fibrillary acidic protein (astrocytic) and Tuj-1 (neuronal). A greater number of cells were present (almost two-fold) at hypoxia than at normoxia. In addition, the number of cells expressing the differentiation markers was significantly reduced in cells propagated at hypoxia compared with CSCs cultured at normoxia (Figures 6a–b). These data indicate that hypoxia promotes proliferation of CSCs and maintains their undifferentiated phenotype.

Discussion

Determining the growth-promoting mechanisms of the tumor microenvironment will enhance our understanding of cancer biology and may identify new therapeutic approaches. In this study, we investigated the role of hypoxia on glioma CSC maintenance and growth, and sought to identify a potential mechanism. We found that hypoxia confers a growth advantage to CSCs by promoting self-renewal of the CD133-positive cells through an HIF-1α-dependent mechanism and further contributes to tumor pathogenesis by maintaining the CSCs in the undifferentiated state.

Growth of CSCs in hypoxia

Neural stem cells are regulated by extracellular microenvironmental cues to maintain the self-renewal capacity and the undifferentiated state (Wurmser *et al.*, 2004). Recent studies implicate oxygen tension as a key regulator of the NSC metabolic state, survival and fate (Gustafsson *et al.*, 2005; Chen *et al.*, 2007). That tumors are hypoxic is well known, but the reasons are less clear. Most solid tumors, including gliomas, are well vascularized and their growth depends on vascular formation (Louis *et al.*, 2007). This is the fundamental principle behind the antiangiogenesis strategy in cancer therapy (Folkman, 1971). Tumor development is characterized by an initial phase of rapid expansion, followed by a period of slowed growth as the proliferating tumor cells outstrip the local supply of oxygen and nutrients (Louis *et al.*, 2007). Although many tumor cells respond to this metabolic stress through induction of the apoptotic mechanism, some cells may be able to cope with this stress through alterations in cellular metabolism (Li *et al.*, 2004). These cells may subsequently stimulate neovascularization and contribute to further growth of the tumor (Zagzag *et al.*, 2000; Bao *et al.*, 2006b; Oka *et al.*, 2009). Hypoxia also contributes to tumor growth by changing the metabolic state of the cells, resulting in the production of macromolecules needed for cellular proliferation (DeBerardinis *et al.*, 2008). In the hypoxic environment, tumor cells rely more on glycolysis for energy production without complete oxidation of the intermediates. Another tumorigenic property of hypoxia is the activation of specific pathways and transcription factors that control stem cells, such as Notch and Oct4 (Gustafsson

et al., 2005; Covello *et al.*, 2006). The hypoxic environment may be more conducive for the selection of the tumor-maintaining CSCs by the activation of specific early developmental programs. As such, hypoxia has been implicated as a causative mechanism of the poor response of gliomas to radiation therapy.

To investigate whether hypoxia confers a growth advantage to the glioma-initiating CSCs, we compared the growth of the cells propagated under hypoxia and normoxia. Self-renewal capacities were strikingly higher under hypoxia compared with normoxia, and the hypoxic environment was associated with a decrease in the expression of markers indicative of differentiation. Low oxygen tension preferentially expanded the CD133-positive sub-population. To characterize these hypoxia response cells further, we investigated the expression of various surface markers by fluorescence-activated cell sorting analysis. The CD133-positive cells showed increased expression of CXCR4, A2B5 and CD24. CXCR4 is a chemokine receptor involved in NSC migration, and is implicated in invasion of gliomas and metastasis of pancreatic CSCs (Ehtesham *et al.*, 2004; Hermann *et al.*, 2007). It is intriguing that hypoxia might expand cells with enhanced infiltrative property. A2B5, a cell surface ganglioside glial progenitor marker, is a putative CSC marker also associated with tumor recapitulation in xenografts (Ogden *et al.*, 2008). Finally, high levels of CD24 expression have been used to identify transit-amplifying cells as well as differentiated neurons, and CD24 is required for the terminal differentiation of neuronal progenitors (Nieoullon *et al.*, 2005; Panchision *et al.*, 2007). Hypoxia confers a growth advantage to the glioma CSCs by amplifying a distinct sub-population of undifferentiated CD133-positive cells enriched for CXCR4, A2B5 and CD24 surface markers.

HIF-1α-mediated mechanism of growth

Both normal and tumor cells respond to a decrease in ambient oxygen tension by increasing the expression of HIF molecules. HIFs, a member of the basic-helix-loop-helix family of transcription factors, are heterodimers of an α- and β-subunit (Wang and Semenza, 1993; Wang *et al.*, 1995). Although the β-subunit is constitutively expressed, the α-subunit is stabilized only at low oxygen tension. At normoxia, the α-subunit undergoes prolyl hydroxylation and is targeted for degradation by the von Hippel–Lindau tumor suppressor protein (Iliopoulos *et al.*, 1996; Semenza, 2003). The hypoxic microenvironment of tumors results in changes in metabolism, angiogenesis and survival of the cells that are orchestrated by HIF-1α, and, depending on tissue specificity, also by HIF-2α (Keith and Simon, 2007). Both HIF-1α and HIF-2α stimulate the expression of genes involved with angiogenesis, whereas the former also activates glycolytic enzyme gene transcription for adenosine triphosphate synthesis in an oxygen-independent manner (Keith and Simon, 2007). Another difference between the two HIFs is that HIF-2α is capable of activating genes encoding transforming growth factor-α and cyclin D1, and enhancing the c-Myc function in renal cell carcinomas (Raval *et al.*, 2005; Gordan *et al.*, 2007). Although the functional dynamics between the two HIFs are likely to be tissue-specific, tumor cells rely on the gene expression programs directed by these adaptive transcription factors for growth and progression. As the tumor tissue is heterogeneous with hypoxic tumor cells in close proximity to tumor cells exposed to higher oxygen tension, the increased secretion of pro-growth factors by the hypoxic cells may induce proliferative activity of the neighboring cells. The presence of HIFs has been associated with poor outcome in a variety of different neoplasms (Birner *et al.*, 2000; Aebersold *et al.*, 2001; Semenza, 2003; Shimogai *et al.*, 2008; Li *et al.*, 2009).

In that HIF stabilization is a major effector of the hypoxic response, we hypothesized an HIF-mediated mechanism for the expansion of CD133-positive cells enriched for CXCR4, A2B5 and CD24 surface markers in hypoxia. HIF-1α was detectable in cells propagated at low oxygen tension and HIF-1α depletion led to a significant reduction in the sphere-forming capacity of all three glioma CSC lines. To further establish the link between HIF-1α expression and enhanced self-renewal of glioma CSCs, we performed PHD2 knockdown experiments at normoxic conditions. Although the depletion of PHD2 led to low-level expression of HIF-1α at normoxia, there was no effect on self-renewal of CSCs as assessed by sphere formation. As the HIF-1α expression level at 1% oxygen is significantly greater than that seen with PHD2 depletion, we speculate that with the CSC lines we have studied, the PHD2 knockdown approach alone may be insufficient in inducing adequate HIF-1α to promote self-renewal activity. Combined depletion of both PHD2 and factor-inhibiting HIF-1 may be necessary to retain sufficient HIF-1α at normoxia.

Interestingly, knockdown of HIF-1α using siRNA showed an incomplete reduction of CD133-positive cells. Even with the complete inactivation of HIF-1α as seen by the immunoblot, a subtle expansion of CD133-positive cells was seen. This suggests the presence of another mechanism for hypoxia-induced CD133-positive CSC expansion distinct from HIF-1α. As earlier work with renal cell carcinoma showed that HIF-2α regulates transforming growth factor-α, cyclin D1 and c-Myc, we considered the possibility that both factors may be contributing to the hypoxia-induced expansion of CD133-positive CSCs (Raval *et al.*, 2005; Gordan *et al.*, 2007). However, we did not detect changes in the level of HIF-2α, in CSCs cultured at different oxygen tensions. Furthermore, HIF-2α depletion studies failed to show changes in the self-renewal capacity of the CSC. Although this does not definitively exclude an HIF-2α response in the glioma CSC lines we have studied, it does illustrate the importance of determining regional tissue tumor-specific mechanisms. Also, it was reported earlier that Notch signaling may be necessary for hypoxia-mediated stem cell maintenance (Gustafsson *et al.*, 2005). Notch1-specific depletion did not decrease the sphere formation capacity of the three CSC lines studied here.

Growth factor and hypoxic signaling
Convergence of signaling pathways driven by growth factors and hypoxia has been well described (Pouyssegur *et al.*, 2006). Activation of growth factor receptors leads to the augmentation of HIF expression, which subsequently amplifies expression of VEGF. Even more rigid coupling is seen in hematopoietic stem cells; these cells are unable to express HIF-1α mRNA in the absence of growth factor receptor activation, and growth factor-dependent HIF-1α is involved in the determination of intracellular glucose fate (Lum *et al.*, 2007). Such observations suggest that HIFs cooperate with growth factor signaling in the governance of cellular metabolism.

We observed a self-renewing mitogenic effect of reduced oxygen on glioma CSCs. To investigate the presence and significance of the cross talk between hypoxic signaling and growth factor signaling in glioma CSCs, we interrogated the PI3K and MAPK signaling mechanisms. As reported earlier by other groups, we found that hypoxia was capable of enhancing the activation of growth factor signaling pathways (Alvarez-Tejado *et al.*, 2001; Xu *et al.*, 2004). Furthermore, blocking these pathways resulted in the attenuation of hypoxic induction of HIF-1α by glioma CSCs. It was shown earlier that PI3K pathway activation was neither required nor sufficient by itself for HIF-1α-dependent gene transcription (Arsham *et al.*, 2002). Our experience with glioma-derived CSCs suggests that, indeed, the PI3K pathway activation is not required for HIF-1α induction, but growth factor signaling may amplify such induction under hypoxic conditions. Such observations suggest that specific cellular response to hypoxia is tissue or cell-type specific. Strict dependence of glioma cells on growth factor signaling pathways may link the hypoxia and these pathways more intimately in gliomas.

Our findings indicate that hypoxia contributes to glioma tumor growth by enhanced self-renewal activity and maintenance of the undifferentiated state of a subset of the CSC populations. It further suggests a context-dependent regulation of the tumor-initiating CSC phenotype. Interestingly, growth factor signaling pathways only partially overlap with hypoxia-mediated signaling response. This suggests the importance of fully characterizing the hypoxia-signaling mechanisms in glioma-derived CSCs, because targeting both the hypoxia-growth factor pathway and the hypoxia-specific signaling cascade may provide improved therapeutic opportunities for the treatment of malignant gliomas.

Materials and methods

Tissue specimen
Three CSC lines were established from acutely resected human tumor tissues. The X01 line was derived from a woman with a glioblastoma multiforme. X02 line originated from a man with glioblastoma multiforme. X03 was derived from a woman with anaplastic oligoastrocytoma.

Cell culture
Tumor-sphere cultures were performed as described earlier, with some modifications, in medium containing Dulbecco's modified Eagle's medium-F12 (Gibco-Invitrogen, La Jolla, CA, USA), penicillin G, streptomycin sulfate, B-27 (Gibco-Invitrogen), and recombinant human EGF (20 ng/ml; R&D Systems, Minneapolis, MN, USA) (Singh *et al.*, 2004; Oka *et al.*, 2007; Soeda *et al.*, 2008). Cells were cultured in HERAcell incubators (Thermo Electronic Corporation, Asheville, NC, USA) at 37 °C, 95% relative humidity, and 5% CO_2 with 20% oxygen or 1% oxygen conditions.

Fluorescence-activated cell sorting analysis
To characterize the effects of hypoxia on the CD133-positive CSC sub-population propagated as spheres, $1x10^6$ cells were placed in a proliferation medium containing EGF. After 12–72 h, cells were evaluated on a Coulter EPICS cytometer (Beckman Coulter, Fullerton, CA, USA). To further characterize the effect of hypoxia on CSCs, each sample was labeled with phycoerythrin-conjugated anti-human CXCR4, CD44, CD24 (BD Biosciences, San Jose, CA, USA), phycoerythrin-conjugated CD133/1 (AC133) (Miltenyi Biotec, Auburn, CA, USA), or A2B5 (Miltenyi Biotec) with phycoerythrin-secondary antibody (BD Biosciences) according to the manufacturer's recommendation. Appropriate compensation and isotype controls were used. All experiments were performed in triplicates.

Western blotting
Western blot analyses were performed as described earlier (Soeda *et al.*, 2008). The following antibodies were used: CD133 (Cell Signaling Technology, Beverly, MA, USA), HIF-1α (BD Biosciences), HIF2-α (Novus Biologicals, Littleton, CO, USA), actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-EGFR (P-Tyr1068, Cell Signaling Technology), Akt (Cell Signaling Technology), ERK1/2 (Cell Signaling Technology), phospho-ERK1/2 (P-Thr202/Tyr204, Cell Signaling Technology), phospho-p70S6 kinase (Thr-389, Cell Signaling Technology), PHD2 (Cell Signaling Technology), and Notch1 (Cell Signaling Technology). Briefly, tumor spheres were lysed in a buffer consisting of 20 mM Tris-HCl (pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). After brief sonication, lysates were clarified by centrifugation at $12\,000 \times g$ for 10 min at 4 °C, and protein content in the supernatant was measured according to the Bradford method. Aliquots (30–50 μg of protein per lane) of total protein were separated by 7.5–15% SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose transfer membranes (0.2 μm; Amersham Biosciences, Buckinghamshire, UK). Each membrane was blocked with 5% non-fat dry milk in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.01% Tween-20) for 1 h at room temperature, followed by incubation with the appropriate primary antibodies overnight at 4° C. After extensive washing with TBS-T, each membrane was further incubated with horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibodies (1:1000) for 1 h at room temperature in TBS-T containing 5% non-fat dry milk. Detection was performed using an enhanced chemiluminescence reagent (Amersham Biosciences), according to the manufacturer's protocol.

Enzyme-linked immunosorbent assay
Vascular endothelial growth factor protein levels were determined by enzyme-linked immunosorbent assay performed with Quantikine immunoassay for human VEGF (R&D systems) according to the manufacturer's instructions. CSCs

(10^6) were dissociated into single cells and transferred to T75 Falcon culture flasks with suspension medium containing EGF at different oxygen tensions. After 72 h incubations, supernatants were used immediately or frozen at −20° C until they were processed. All experiments were performed in triplicates.

Indirect immunofluorescence microscopy

Immunocytochemistry of CSCs was performed as described (Oka *et al*., 2007; Park *et al*., 2007). Primary antibodies used were as follows: anti-β-III-tubulin (Tuj1; mouse mAb, 1:200; Chemicon, Temecula, CA, USA) and anti-glial fibrillary acidic protein (GFAP; rabbit pAb, 1:500; DAKO, Glostrup, Denmark). Alexa Fluor 488 and 555 secondary antibodies were used (1:1000; Molecular Probes, Eugene, OR, USA). Cells were counterstained with 4′,6-diamidino-2-phenylindole. The following hardware was used: Zeiss Axiovert 200 microscope (Carl Zeiss, Gottingen, Germany), Plan-Neofluar ×20 and ×40 objectives, AxioCam MrM CCD camera. Axiovision software was used for image acquisition (Carl Zeiss).

RNA interference

Hypoxia-inducible factor-1α, HIF-2α, PHD2 and Notch1 siRNA oligonucleotides were obtained commercially (Santa Cruz Biotechnology). A previously designed siRNA directed against green fluorescent protein was used as a control. The day before transfection, 5×10^6 CSCs were dissociated into single cells and transferred to T25 Falcon culture flasks with suspension medium containing EGF. Cells were then transfected with siRNA using a nucleofecting electroporator according to the manufacturer's protocol (Amaxa Inc., Gaithersburg, MD, USA). After 24 h, the medium was replaced, and cells were harvested for additional experiments.

Statistical analysis

Differences in the various surface maker expressions between normoxia and hypoxia were evaluated with Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)