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A hypoxic niche regulates glioblastoma stem cells through hypoxia inducible factor 2α

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Glioma growth and progression depend on a specialized subpopulation of tumour cells, termed tumour stem cells. Thus, tumour stem cells represent a critical therapeutic target, but the molecular mechanisms that regulate them are poorly understood. Hypoxia plays a key role in tumour progression and in this study we provide evidence that the hypoxic tumour microenvironment also controls tumour stem cells. We define a detailed molecular signature of tumour stem cell genes, which are overexpressed by tumour cells in vascular and perinecrotic/hypoxic niches. Mechanistically, we show that hypoxia plays a key role in the regulation of the tumour stem cell phenotype through hypoxia-inducible factor 2α and subsequent induction of specific tumour stem cell signature genes, including mastermind-like protein 3 (Notch pathway), nuclear factor of activated T cells 2 (calcineurin pathway) and aspartate beta-hydroxylase domain-containing protein 2. Notably, a number of these genes belong to pathways regulating the stem cell phenotype. Consistently, tumour stem cell signature genes are overexpressed in newly formed gliomas and are associated with worse clinical prognosis. We propose that tumour stem cells are maintained within a hypoxic niche, providing a functional link between the well-established role of hypoxia in stem cell and tumour biology. The identification of molecular regulators of tumour stem cells in the hypoxic niche points to specific signalling mechanisms that may be used to target the glioblastoma stem cell population.

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Keywords: tumour stem cells; side population; glioblastoma; hypoxia; hypoxia inducible factor; hypoxic niche

Abbreviations: ASPHD2 = aspartate beta-hydroxylase domain-containing protein 2; HIF = hypoxia-inducible factor; MAML3 = mastermind-like protein 3; NFATc2 = nuclear factor of activated T cells 2; NFE2L2 = nuclear factor erythroid 2-related factor 2

Introduction

The mortality of patients with glioblastomas, the most frequent brain tumour type, remains high despite multi-modal tumour therapy including surgery, radio- and chemotherapy. Current treatments focus on eliminating the main tumour mass, which may leave unaffected small but crucial populations of tumour cells, which subsequently have the potential to lead to renewed tumour growth (Bao *et al.*, 2006). Indeed, glioblastomas frequently relapse, indicating the need for more targeted therapeutic approaches. A number of studies in recent years have demonstrated that tumours, similarly to normal tissues, are organized in a hierarchical manner (Visvader and Lindeman, 2008). A variety of tumour entities (Lapidot *et al.*, 1994; Al-Hajj *et al.*, 2003; Kim *et al.*, 2005; O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007), and in particular glioblastomas (Hemmati *et al.*, 2003; Galli *et al.*, 2004; Singh *et al.*, 2004; Yuan *et al.*, 2004), are now believed to originate from and be maintained by tumour stem cells. Tumour stem cells are capable of both continuous self renewal and multilineage differentiation and have a strongly enhanced capacity to initiate tumour formation *in vivo*. They also have distinct functional properties that can differ from those of the tumour bulk, including relative quiescence and resistance to chemo- and radiotherapy (Visvader and Lindeman, 2008). This could explain why tumour stem cells may be spared by traditional anti-tumour therapies and be rapidly capable of regenerating the tumour, leading to recurrence.

The isolation of tumour stem cells from brain tumours often relies on sorting for surface markers such as CD133 (Goodell *et al.*, 1996; Singh *et al.*, 2004) or stage-specific embryonic antigen-1 (Read *et al.*, 2009; Son *et al.*, 2009). Such markers, however, vary greatly between samples and are not expressed in all tumours (Son *et al.*, 2009). An alternative approach is the 'side population' technique (Goodell *et al.*, 1996), which is based on the efflux of lipophilic dyes like Hoechst 33342 from tumour stem cells. The side population phenotype is mediated by verapamil sensitive ATP-binding cassette transporter proteins expressed in stem cells (Zhou *et al.*, 2001) and has been used for the isolation of stem cells from both normal tissues (Goodell *et al.*, 1997) and tumours (Kondo *et al.*, 2004; Harris *et al.*, 2008; Bleau *et al.*, 2009).

Although the eradication of tumour stem cells is thought to be a prerequisite for a successful anti-tumour strategy, we still have a very limited understanding of the molecular profiles and the regulatory mechanisms that control tumour stem cell maintenance. Evidence from a number of experimental systems has established a role for reduced oxygen availability (hypoxia) in stem cell maintenance (Keith and Simon, 2007). Hypoxia is a characteristic feature of malignant tumours, which frequently outpace their blood supply, and it is associated with poor patient prognosis. Work over the past decade has shown that the hypoxic microenvironment drives tumour progression by triggering a set of adaptive transcriptional responses that regulate tumour angiogenesis, tumour cell metabolism, motility and survival, and ultimately promote a more aggressive tumour phenotype (Bertout *et al.*, 2008). These cellular responses are primarily controlled by the transcription factor system of the hypoxia-inducible factors (HIFs). HIFs act as heterodimers composed of a shared HIF-β subunit and specific HIF-α subunits (Pouyssegur *et al.*, 2006). At least two mammalian alpha subunits, HIF-1α and HIF-2α, are differentially regulated by cellular oxygen, possess different target specificities and appear to have complementary rather than redundant functions. Interestingly, recent reports have identified new molecular programs by which HIF may regulate cellular differentiation and self renewal, identifying critical stem cell regulators such as Oct4 (Covello *et al.*, 2006), c-myc (Gordan *et al.*, 2007) or Notch (Gustafsson *et al.*, 2005) as direct or indirect HIF targets. Thus, the role of hypoxia and HIF may extend beyond their well established functions in tumour biology to the control of the tumour stem cell phenotype. However, the function of HIFs and their potential downstream targets in tumour stem cell maintenance awaits further clarification.

In this study, we isolated tumour stem cells from human glioblastoma tumours and cell lines using the side population approach and defined a detailed signature of genes differentially expressed in the tumour stem cell population. Using markers from our signature we demonstrate that tumour stem cells are highly enriched in both vascular and necrotic/hypoxic niches within glioblastomas. Moreover, we show that hypoxia controls the expression of several tumour stem cell signature genes, involved in the regulation of stemness, as well as the stem cell properties of glioma cells, through HIF-2α. The clinical relevance of these findings is underscored by the fact that hypoxia regulated tumour stem cell signature genes are overexpressed in newly formed gliomas and are associated with a worse clinical prognosis. In summary, we identify the hypoxic niche as a critical regulator of tumour stem cells pointing to new signalling mechanisms whose disruption can be used to successfully target this crucial subpopulation.

Materials and methods

Glioblastoma cell culture

Primary glioblastoma cell cultures (designated GBM) and samples were obtained from patients undergoing surgery in accordance with a protocol approved by the institutional review board. Established glioblastoma cell lines (G55TL, G142, LN229, U87, U118, U251, U251-A, U343, U373) were kindly provided by M. Westphal and K. Lamszus (Hamburg), M. Nistér (Stockholm) or obtained from American Type Culture Collection. All cell lines were cultured in Dulbecco modified Eagle's medium (Invitrogen, Carlsbad, CA), supplemented with 10% foetal bovine serum (PAN Systems, Aidenbach, Germany). Tumour

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cells undergoing side population analysis were propagated as tumour spheres in serum-free Dulbecco modified Eagle's medium-F12 supplemented with B27 (Gibco), 20 ng/ml basic fibroblast growth factor and 10 ng/ml leukaemia inhibitory factor. In some experiments a combination of epidermal growth factor and basic fibroblast growth factor, or platelet-derived growth factor, basic fibroblast growth factor and leukaemia inhibitory factor was used. Recombinant growth factors were obtained from Chemicon (Temecula, CA) or PeproTech (Hamburg, Germany). Chemicals were purchased from Sigma unless otherwise indicated.

Microarray hybridization

Detailed information on the manufacturing and probe content of the microarrays is available through the ArrayExpress microarray data repository using the accession number A-MEXP-245. All arrays included in the experiment originate from the same print run in order to control for variability between arrays. The 46 128 probes represent 15 088 known genes and an additional 12 151 expressed sequence tags. For detailed information on the microarray analyses, data processing and validation see the online Supplementary material.

Immunostaining

Immunohistochemical and immunofluorescence experiments were carried out essentially as previously described (Beck *et al.*, 2000). The following primary antibodies were used: anti Ki67 (clone Mib-1, Dako, Glostrup, Denmark), CD34 (Dako), CD133/1 (Miltenyi Biotec), Nestin (Pharmingen), βIII tubulin 1 (Promega), doublecortin (Chemicon), glial fibrillary acidic protein (Dako) and βS100 (Swant). Anti-aspartate beta-hydroxylase domain-containing protein 2 (ASPHD2), nuclear factor erythroid 2-related factor 2 (NFE2L2), activator of 90 kDa heat shock protein ATPase homolog 1, glucocorticoid-induced transcript 1 protein and laminin subunit gamma-1 precursor antibodies were obtained from the Swedish Human Proteome Resource project (http://www.proteinatlas.org). Non-specific binding sites were blocked by incubation in 20% normal goat serum. In all immunohistochemical experiments omission of the primary antibody served as the negative control. For immunohistochemical detection the following secondary antibodies were used according to the primary antibody: biotinylated anti-rabbit, anti-mouse, anti-rat or anti-guinea pig (Dianova, Hamburg). The colour reaction was performed with peroxidase conjugated streptavidin (Vectastain Kit ABC, Vector Laboratories, Inc., Burlingame, CA, USA) followed by incubation with diaminobezidine (DAB, Vector Laboratories). The colour reaction was stopped by washing sections in phosphate buffered saline. For immunofluorescence detection the following secondary antibodies were applied: anti-rabbit Alexa 488, anti-mouse Alexa 568, anti-rabbit Alexa 568 and anti-guinea pig Alexa 568 (Molecular Probes). Sections were briefly counterstained in haematoxylin or with 4′,6-diamidino-2-phenylindole, respectively, rinsed in distilled water and mounted in Elvanol. Evaluation of the staining was performed using a light or fluorescence microscope. Quantification of side population signature markers or CD133 expressing tumour cells was performed in at least four high power fields/tumour section in 7–10 glioblastoma biopsies.

Hypoxia and sphere formation assays

For hypoxic incubation cells seeded at 500 cells/well (G55) or 1000 cells/well (primary tumour cultures) in six-well suspension culture plates (6 wells/condition) were grown at 1% oxygen for the indicated time points in a Hypoxic Workstation Invivo$_2$ 500 (Ruskinn Technology, Pencoed, UK). Control cells were incubated in normoxia (21% O_2). For sphere formation assays 10^6 cells were seeded per 10 cm dish, cultivated for 7 days in Dulbecco modified Eagle's medium-F12, B27 supplement, 20 ng/ml basic fibroblast growth factor and epidermal growth factor (tumour sphere medium) at normoxia or hypoxia. The cells were then split in six-well suspension culture plates at a density of 1000 cells/well (primary cell lines) or 500 cells/P-hydroxyethylmethacrylate-coated well (G55) in 2 ml tumour sphere medium; spheres were counted after 7 days.

Overexpression and knockdown

For HIF-1α/2α overexpression G55TL cells stably transfected with pTet Off regulator plasmid (Clontech) were transfected with pTRE2hyg/pur vectors containing as inserts non-hydroxylatable/non-degradable mutants of HIF-1α (HIF-1α mPPN, where Pro^{402}, Pro^{564} and Asn^{803} were mutated to Ala) and HIF-2α (HIF-2α mPPN, where Pro^{405}, Pro^{531} and Asn^{847} were mutated to Ala) with a C-terminal V5-tag, or green fluorescent protein as control. Clones were selected in the presence of 200 μg/ml hygromycin and screened for low transgene background and high transgene induction by western blot analysis. The selected clones were grown in tumour sphere medium.

For knockdown experiments 3.5×10^5 cells in six-well suspension culture plates were transfected overnight with 20 nM small interfering RNAs (Dharmacon) using Oligofectamine (Invitrogen) transfection reagent in Dulbecco modified Eagle's medium-F12/B-27 medium. The medium was exchanged on the next day and the cells were incubated under hypoxia or normoxia for 72 h. Scrambled small interfering RNAs were used as control.

Results

Isolation of glioblastoma stem cells

To characterize the tumour stem cell population of a panel of glioblastoma cell lines we first confirmed that the cells grew under stem cell culture conditions, forming spheres in B-27 supplemented medium (Fig. 1A, Supplementary Fig. S1A). The sphere-forming activity of the cell lines was also observed in medium with defined growth factors (epidermal growth factor/basic fibroblast growth factor; basic fibroblast growth factor/leukaemia inhibitory factor) and was preserved after passaging (Fig. 1B), confirming the self-renewal capacity of the tumour stem cell population. Furthermore, upon addition of serum, the tumour spheres were capable of differentiation (Fig. 1A, Supplementary Fig. S1A) into multiple lineages (Supplementary Fig. S1B).

We next wanted to purify the tumour stem cell population from glioblastoma cell cultures. Since some controversy exists regarding the most appropriate tumour stem cell markers, we chose a functional isolation approach, based on the established property of stem cells to export dyes such as Hoechst 33342. The side population of cells that had excluded the dye was identified by dual wave length emission FACS analysis of nine glioblastoma cell lines (Fig. 1C, Supplementary Fig. S2A). Similarly, acutely dissociated glioblastoma biopsies contained a verapamil-sensitive side population (Fig. 1C). The glioblastoma side population could self-renew

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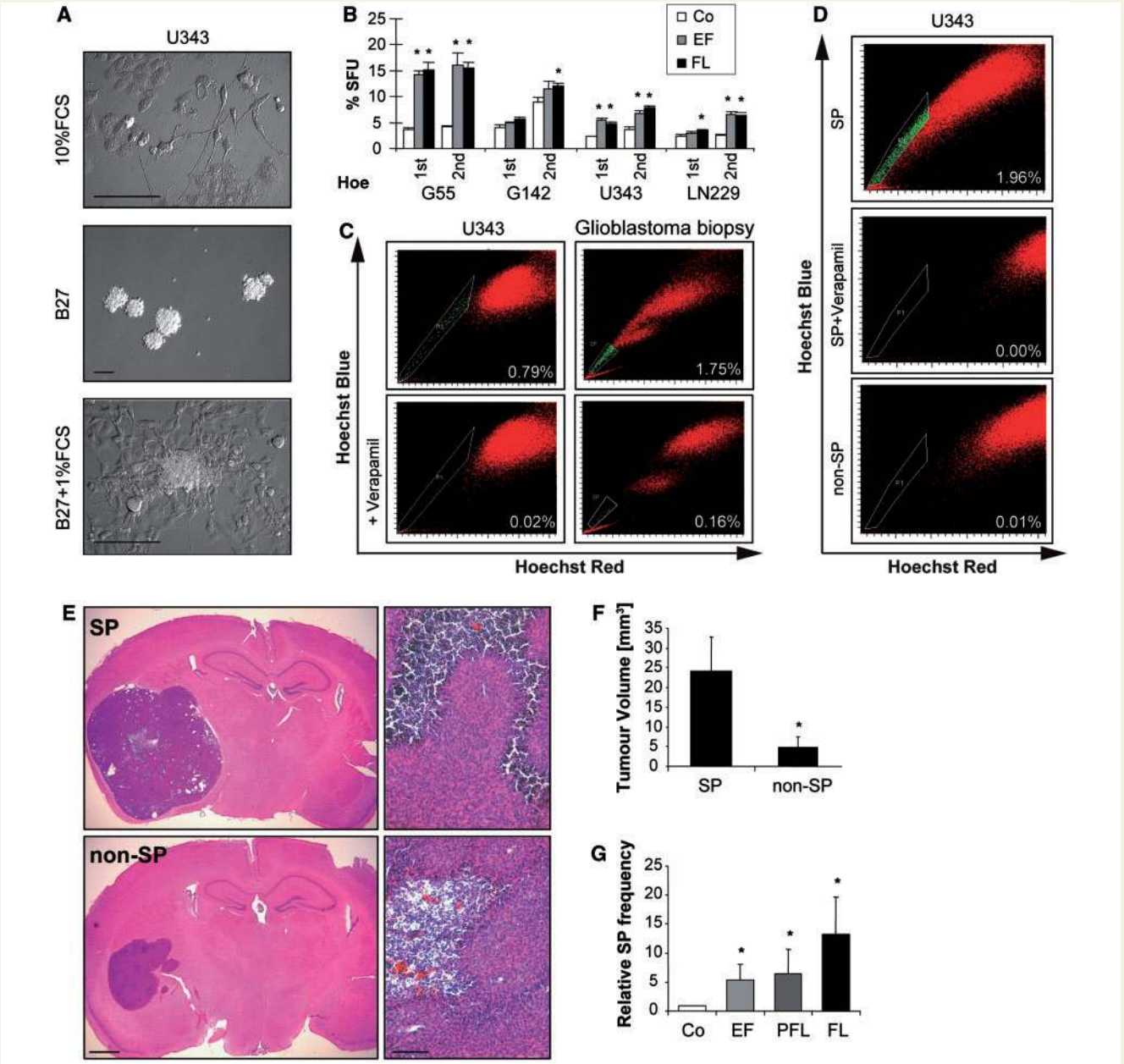

Figure 1 The side population of glioblastoma cell lines and glioblastomas displays tumour stem cell properties. (**A**) Growth characteristics of the glioblastoma cell line U343. U343 cells grow as an adherent layer in medium supplemented with 10% foetal calf serum, form spheres in neural stem cell serum-free medium supplemented with B27 and adhere and spread out in differentiating conditions (addition of 1% FCS), with individual cells forming cell protrusions. Scale bars: 100 μm. (**B**) Glioblastoma cells form primary (1st) and secondary (2nd) spheres when plated at clonal density (250–500 cells/ml), as a measurement for self-renewal ability. Tumour cells retain the ability to form spheres even after withdrawal of growth factors, though at a reduced rate, compared to the standard growth factor combinations epidermal growth factor/basic fibroblast growth factor and basic fibroblast growth factor/leukaemia inhibitory factor (mean ± SEM; $n = 6$; $^*P < 0.05$). (**C**) Following Hoechst 33342 incubation, the majority of tumour cells (U343, human glioblastoma biopsy) show a high fluorescence (right upper quadrant, red) in dual wave length FACS analysis (HoechstRed/HoechstBlue). A small subpopulation exhibits low fluorescence, separating in the tail-like side population (upper panels, left lower quadrant, green) that is blocked by pre-treatment with verapamil (lower panels). (**D**) side population and non-side population were isolated by FACS sorting from U343, cultured separately for two weeks and reassessed for their ability to form side population and non-side population by FACS. Only the side population displays the capacity to re-generate side population and non-side population, whereas the non-side population could not re-populate the side population. (**E**) Intracranial growth of G55 side population and non-side population derived tumours with the histological hallmarks of glioblastomas, including necrosis and pseudopalisading; haematoxylin/eosin staining, scale bars: 1 mm (left panel) and 100 μm (right panel). (**F**) Quantification of the volume of tumours formed by G55 cells demonstrates that the side population forms significantly larger tumours than non-side population (mean ± SEM; $n = 4$; $^*P < 0.05$). (**G**) The side population phenotype is modulated by extracellular signals. Various growth factor combinations increase the size of the side population from G55 cells up to more than tenfold (mean ± SEM; $n = 3$–4; $^*P < 0.05$) compared to control cells cultured without addition of growth factors. EF = epidermal growth factor/basic fibroblast growth factor; FL = basic fibroblast growth factor/leukaemia inhibitory factor; PFL = platelet-derived growth factor/basic fibroblast growth factor/leukaemia inhibitory factor; Co = control; SP = side population; FCS = foetal calf serum.

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and differentiate, since it was able to regenerate both side population and non-side population cells in culture, whereas the non-side population fraction primarily produced non-side population cells (Fig. 1D, Supplementary Fig. S2B).

To address whether the side population has an enhanced capacity for tumour formation, we injected 1000 side population and non-side population glioblastoma cells in the brain of nude mice. The tumours formed by the side population were 5-fold larger than those produced by the non-side population (Fig. 1E and F) and contained extensive necrotic areas (Fig. 1E).

Glioblastoma stem cells are defined by a distinct molecular signature

To explore the signals to which side population cells respond, we cultured G55 tumour cells in the presence of the neural stem cell mitogen basic fibroblast growth factor in combination with epidermal growth factor, leukaemia inhibitory factor or leukaemia inhibitory factor and platelet-derived growth factor (Fig. 1G). All growth factor combinations greatly increased the size of the side population (Fig. 1G), indicating that the side population phenotype is critically modulated by external signals. To characterize the transcriptional changes induced by environmental signals in the side population and non-side population populations at a transcriptome level we undertook a microarray approach to determine their differential gene expression profiles (Fig. 2A, Supplementary Fig. S3). In a total of 49 hybridizations we analysed three cell lines grown with basic fibroblast growth factor/leukaemia inhibitory factor: G55, LN229, U343, as well as G55 cells that were additionally supplemented with platelet-derived growth factor, a known mitogen in glial oncogenesis (Dai *et al.*, 2001) (Fig. 2A, Supplementary Fig. S3). A total of 812 genes were identified as being differentially expressed in at least one of the contrasts (Fig. 2B), 506 of them in the side population (Fig. 2B). Fig. 2C shows the number of probes and genes that were identified as differentially expressed separately for each cell line and growth factor combination. The comparison between the three cell lines revealed a large overlap between the differentially expressed genes (Fig. 2D), suggesting a common molecular side population versus non-side population signature. 73 genes, (Fig. 2D, Supplementary Table S1) were shared by all three cell lines and an additional 139 genes were differentially expressed in at least two of the cell lines (Fig. 2D). Comparison with previously published gene signatures of stem cells revealed that 39 of our side population signature genes were also overexpressed in other stem cell or side population populations (Table S2). Finally, we used the Biocarta, KEGG and Gene Ontology databases (Supplementary Table S3, Supplementary Fig. S4) to place the side population signature genes into a functional context. These analyses revealed a number of pathways and biological processes that were significantly enriched in differentially expressed genes with higher expression in the side population (Supplementary Table S3 and Fig. S4), including the Wnt and tumour growth factor-β signalling pathways that are also known to regulate critically physiological stem cell function.

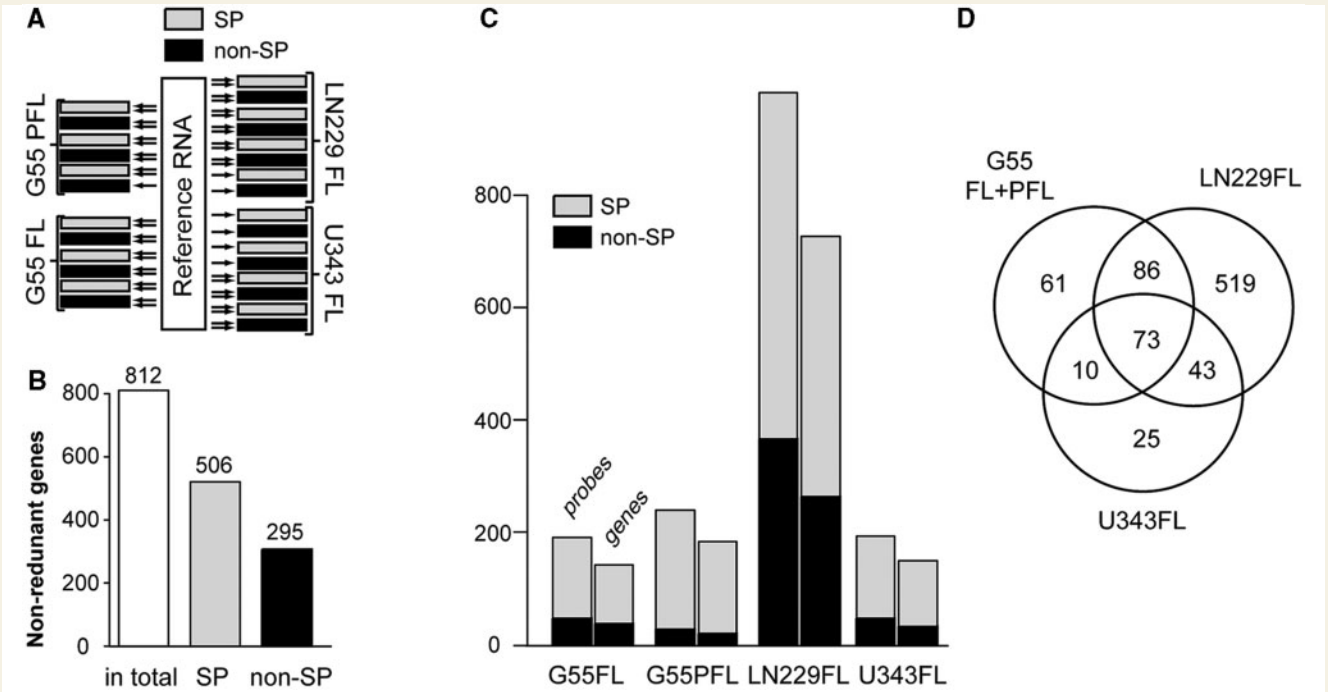

Figure 2 Gene expression profiling of the side population and non-side population. (**A**) Overview of the target preparation approach. (**B**) Global summary of the genes differentially expressed between the side population and non-side population. (**C**) Differentially expressed probes and genes depicted separately for the three glioblastoma cell lines and the two growth factor combinations (FL, PFL), respectively. The majority of differentially expressed genes is overexpressed in the side population. (**D**) Overlap of differentially expressed genes in all three cell lines. For the G55 cell line the data are generated by combining all differentially expressed genes from the two growth factor combinations. The glioblastoma cell lines show a common transcriptional signature of 73 differentially expressed genes. FL = basic fibroblast growth factor/leukaemia inhibitory factor; PFL = platelet-derived growth factor/basic fibroblast growth factor/leukaemia inhibitory factor; SP = side population.

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Side population signature markers reveal an enrichment of tumour stem cells in vascular and perinecrotic/hypoxic niches

Our next aim was to determine whether the side population signature markers we found could be successfully used to identify tumour stem cells in tumours and define the factors that regulate the tumour stem cell phenotype. As a first step, we performed immunohistochemical analysis for five side population signature markers (Supplementary Tables S1–S3) in human glioblastoma biopsies for which validated antibodies were available from the Swedish Human Proteome Resource project. side population signature genes were only expressed in a fraction of tumour cells (e.g. 14.5 ± 2.5% of all tumour cells expressed ASPHD2, $n = 7$). Interestingly, however, the side population signature gene expressing cells were frequently positioned in perivascular or perinecrotic areas as shown for ASPHD2, NFE2L2, laminin subunit gamma-1 precursor (Fig. 3A), or activator of 90 kDa heat shock protein ATPase homolog 1 and glucocorticoid-induced transcript 1 protein (Supplementary Fig. S5), reminiscent of the distinct niches described for physiological stem cells. Importantly, this corresponded to the localization of glioblastoma cells stained for the classical tumour stem cell marker CD133 (Fig. 3A), which was also only detected in a fraction of the tumour cells (6.0 ± 1.6% of all tumour cells, $n = 10$). ASPHD2 positive tumour cells localized next to CD34 positive tumour capillaries and co-expressed glial fibrillary acidic protein and Nestin (Fig. 3B). In addition, side population signature genes were co-expressed, as shown for ASPHD2 and NFE2L2 (Fig. 3B). Moreover, ASPHD2 expressing tumour cells revealed a low proliferation rate, as assessed by Ki67 co-staining (Fig. 3B), in line with the quiescent nature of stem cells. Taken together, these results show that tumour stem cells are located in specific microenvironments (niches) within the tumour. In addition, our findings indicate that the molecular side population signature we found is widely applicable to glioblastomas for the identification and characterization of tumour stem cells.

Hypoxia is an important component of the tumour stem cell niche

Regions of low oxygen concentration (hypoxia) are a characteristic feature of growing tumours, and are frequently found around necrotic areas. Therefore, we next wanted to investigate whether the localization of side population signature gene-expressing cells in the vicinity of necrotic/hypoxic regions could be important for

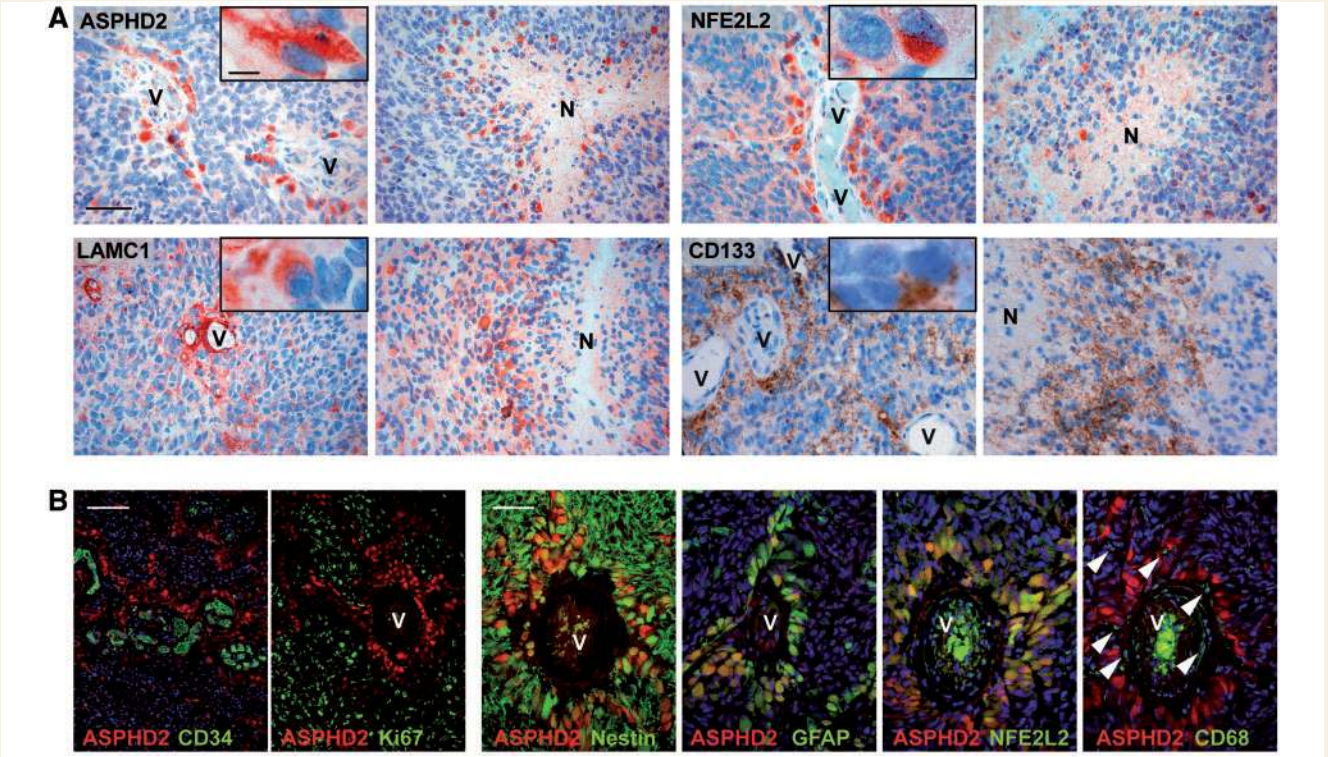

Figure 3 Side population signature genes identify tumour stem cell specific vascular and hypoxic niches in glioblastoma. (**A**) Tumour cells expressing side population signature genes [ASPHD2, NFE2L2, laminin subunit gamma-1 precursor (LAMC1)], as well CD133, are located in perivascular and perinecrotic (hypoxic) areas, as shown by immunohistochemistry. Scale bars: 50 μm and 5 μm (insets). Immunofluorescence staining of ASPHD2 and the endothelial cell marker CD34 reveals the intimate perivascular location of side population signature tumour cells. ASPHD2 positive tumour cells show a low proliferation rate (Ki67), express Nestin and glial fibrillary acidic protein (GFAP) and co-localize with other side population signature genes (NFE2L2), but not with the macrophage marker CD68. Arrowheads indicate CD68 positive macrophages/microglia. V = vessel; N = necrosis. Scale bars: 100 μm and 50 μm, respectively.

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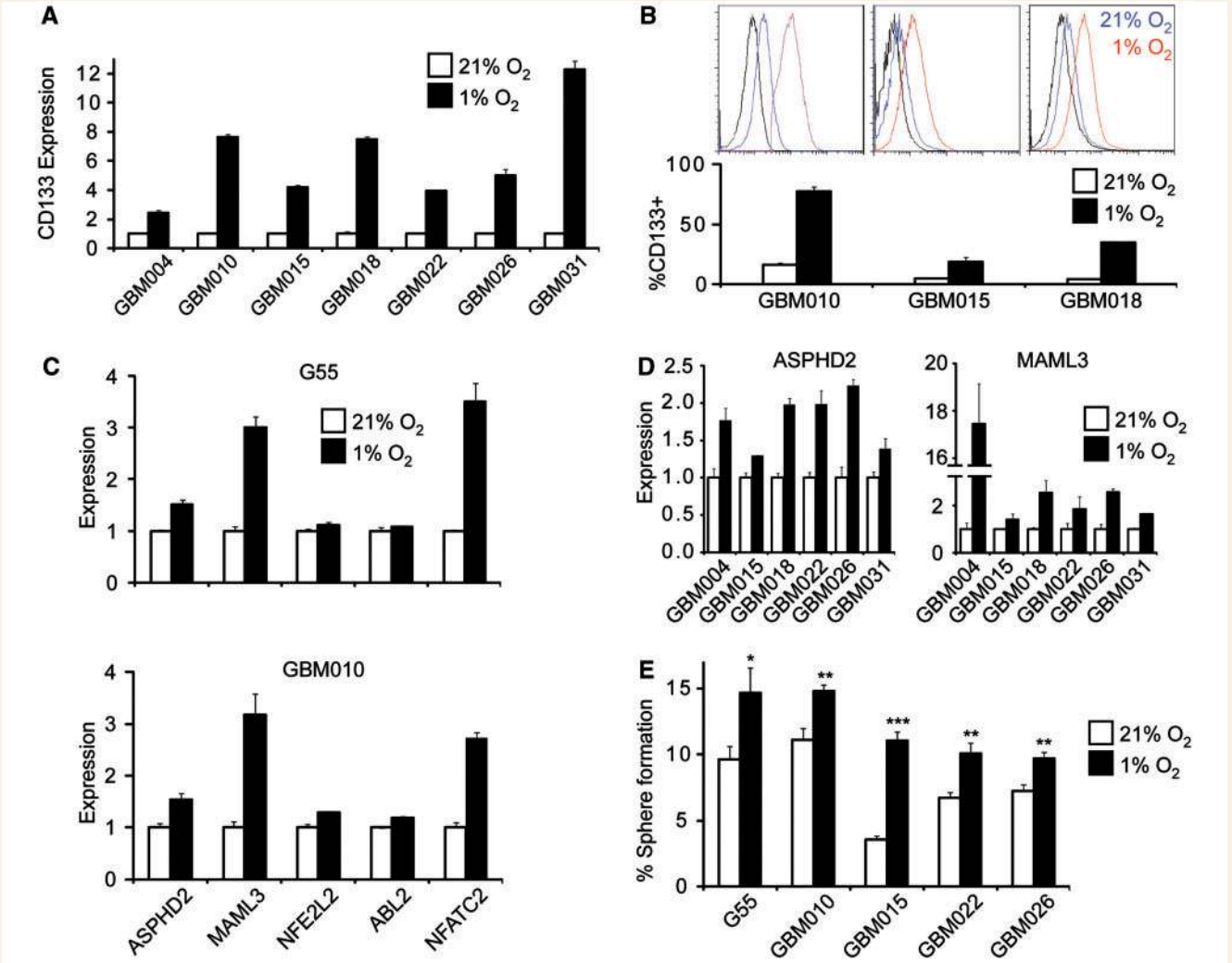

Figure 4 The tumour stem cell phenotype is regulated by hypoxia. (**A**, **B**) Hypoxic incubation (1% oxygen, 96 h) of a panel of primary glioblastoma (GBM) lines increases the expression of CD133, as determined by quantitative PCR (**A**) and the fraction of $CD133^+$cells as determined by FACS analysis (**B**) ($n = 3$). The upper panels in **B** show representative FACS analyses. (**C**) Hypoxia upregulates the expression of several side population markers in G55 and GBM010 cells as determined by quantitative PCR ($n = 3$). (**D**) The enhanced expression of the side population signature genes ASPHD2 and MAML3 under hypoxia was confirmed by quantitative PCR in a panel of additional primary glioblastoma cell lines ($n = 3$). (**E**) Hypoxia enhances the self-renewal capacity of G55 cells and a panel of primary glioblastoma lines, as assessed by their sphere forming capacity (mean ± SEM; $n = 6$; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, relative to normoxic control).

the induction of the tumour stem cell phenotype. First, we analysed the expression of CD133 in a panel of primary glioblastoma lines grown under hypoxia. In all lines, incubation at 1% oxygen led to a significant upregulation of CD133, assessed at the mRNA level (Fig. 4A) and by FACS analysis (Fig. 4B). We tested whether genes from our side population signature were similarly overexpressed following hypoxia. We analysed the expression of five side population signature genes: ASPHD2 and NFE2L2, which were strongly enriched in perivascular and necrotic areas (Fig. 3), as well as mastermind-like protein 3 (MAML3), nuclear factor of activated T cells 2 (NFATc2) and v-abl Abelson murine leukemia viral oncogene homolog 2 (ABL2), which were prominently overexpressed in the side population (Supplementary Table S1). Notably, NFE2L2, NFATc2 and ABL2 were also uncovered in our meta-analysis of previously published stem cell signatures (Supplementary Table S2). Several of the side population genes we identified were found to be significantly upregulated under hypoxia in both G55 cells and in the primary glioblastoma line GBM010 (Fig. 4C). Furthermore, we confirmed the upregulation of ASPHD2 and MAML3 under hypoxia in our panel of primary glioblastoma cells (Fig. 4D). Importantly, the increased expression of side population signature genes was associated with an enhanced capacity to form spheres in both G55 and primary glioblastoma cell lines (Fig. 4E). Together, these data indicate that microenvironmental signals enhance the stem cell properties of glioblastoma cells.

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HIF-2 is a key molecular regulator in the hypoxic niche

Tumour hypoxia activates a complex set of cellular responses, which are differentially regulated by two members of the hypoxia-inducible transcription factors family, HIF-1 and HIF-2. To examine the role that these factors play in the hypoxia-dependent control of the glioblastoma tumour stem cell phenotype we first analysed the *in vivo* expression of HIFs in glioblastoma biopsies. In line with a potential function in the hypoxic tumour stem cell niche, HIF expressing cells were localized to perinecrotic areas (Fig. 5A), similarly to cells expressing side population signature genes (Fig. 3A). The levels of both proteins were strongly increased in primary glioblastoma lines following incubation at 1% oxygen (Fig. 5B). In order to differentiate the specific function of the two factors in the modulation of the tumour stem cell properties we overexpressed HIF-1α and HIF-2α using a Tet-off inducible system that allowed controlled expression at physiological levels, similar to those observed under hypoxia (Fig. 5C). Overexpression of HIF-1α had little or no effect on the levels of side population signature genes, although it robustly upregulated its known target CAIX (Fig. 5D). By contrast, HIF-2α expression led to a striking increase in the levels of all side population markers tested, as well of the established HIF-2 target Oct4 (Fig. 5D), itself a crucial stemness regulator. This suggests that HIF-2 plays a key role in mediating the hypoxia-induced tumour stem cell phenotype. To test this hypothesis directly we knocked down HIF-1α and HIF-2α in the primary GBM010 line and incubated the cells under hypoxic conditions (Fig. 5E). Importantly, knockdown of HIF-2α completely blocked the upregulation of the side population signature genes ASPHD2 and MAML3, and also greatly suppressed the increase of CD133 levels following hypoxia. Silencing of HIF-1α, on the other hand, had no significant effect on CD133 levels and even led to an increase in ASPHD2 and MAML3 levels (Fig. 5E), possibly due to the upregulation of HIF-2α, observed upon HIF-1α knockdown (Supplementary Fig. S6). HIF-2α knockdown also abrogated the hypoxia-mediated increase in the sphere forming capacity of GBM010 cells (Fig. 5F, G). HIF-1α silencing also reduced sphere formation, indicative of a tumour stem cell independent role of HIF-1α in sphere formation. Collectively, our results demonstrate that hypoxia controls the stem cell phenotype of glioblastoma tumour stem cells via HIF-2.

Side population signature gene expression is elevated in primary human gliomas and associates with poor prognosis in primary glioblastoma patients

To test whether the expression of the hypoxia-regulated side population signature genes identified in our study is important for initiating glioma growth, we examined the expression level of five side population signature genes whose expression was induced by HIF-2 and hypoxia (Fig. 4C, D, Fig. 5D) in 115 gliomas of different World Health Organization grades. For all genes we detected higher expression levels in gliomas as compared to adult brain tissue (Fig. 6A). The majority of newly arising malignant gliomas in adults present *de novo* as full-blown aggressive tumours (primary glioblastoma, grade IV). A small fraction of glioblastomas, referred to as secondary glioblastomas, develop through progression from pre-existing lower grade tumours, such as diffuse astrocytoma (grade II) or anaplastic astrocytoma (grade III) (Ohgaki and Kleihues, 2007). All side population signature genes tested were expressed at higher levels in primary glioblastomas compared to secondary glioblastomas (Fig. 6A). In addition, expression of the side population signature genes was higher in diffuse astrocytomas than in anaplastic astrocytomas and secondary glioblastomas (Fig. 6A), suggesting that a high level of expression is associated with *de novo* tumour generation. We also found that several of the side population genes were expressed at higher levels in foetal brain, reaching levels comparable to those detected in diffuse astrocytoma and primary glioblastoma (Fig. 3A), indicating that tumour stem cells may recapitulate mechanisms active during embryonic development.

We next compared our molecular side population signature to a previously published list of prognosis predicting genes in high-grade gliomas (Phillips *et al.*, 2006). This analysis revealed a substantial signature overlap (Fig. 6B). The proliferative and mesenchymal subclass genes that were correlated with shorter survival were frequently overexpressed in the side population signature. At the same time, the proneural genes that were associated with longer patient survival were typically downregulated in the side population, relative to the non-side population (Fig. 6B), providing a strong indication that the side population phenotype confers a more aggressive tumour behaviour. These results suggest that the increased expression of hypoxia-regulated side population signature genes is associated with the initiation of tumour growth and with a worse prognosis. Collectively, our data strongly support the specific and important role of hypoxia regulated side population signature genes in glioma biology.

Discussion

Tumour stem cells are held to be responsible for the initiation, progression and recurrence of gliomas following conventional treatment strategies. Therefore, they are considered critical targets for an effective tumour therapy. An improved understanding of the molecular regulators and profiles of tumour stem cells is crucial for designing treatment strategies aimed at the efficient eradication of this cell subpopulation. In this study, we provide mechanistic and functional evidence that tumour stem cells are, similarly to their physiological counterparts, positioned and controlled in specialized niches.

A hypoxic niche for glioblastoma tumour stem cells

Using different proteins from our side population signature as markers, as well as the classical tumour stem cell marker CD133,

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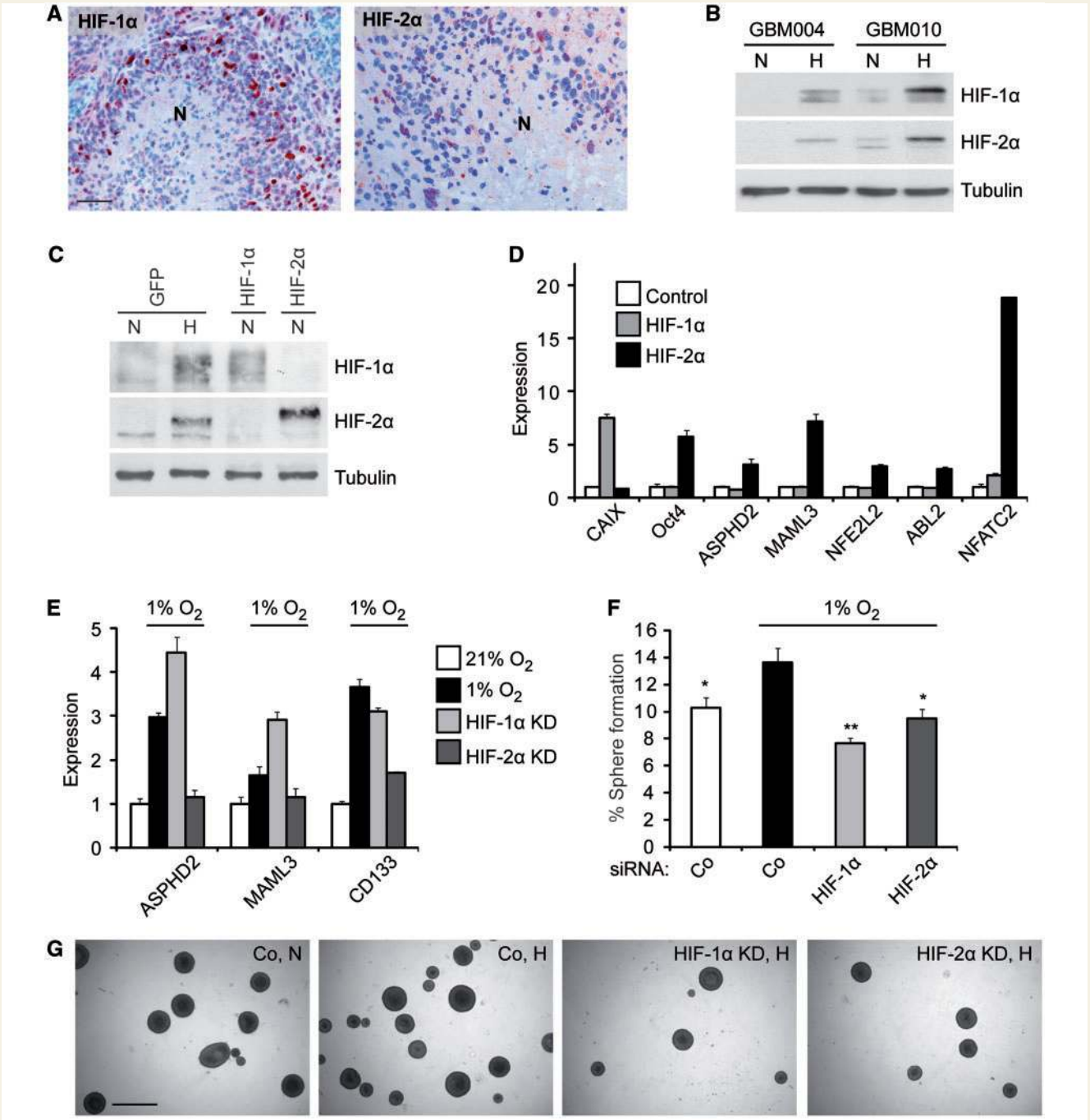

Figure 5 HIF-2 mediates the effects of hypoxia on the tumour stem cell phenotype. (**A**) Immunohistochemical staining for HIF-1α and HIF-2α reveals nuclear expression of both isoforms in a subset of tumour cells around necrotic (N) areas. Scale bar: 50 μm. (**B**) Hypoxia (H; 1% oxygen for 3 days) increases the levels of HIF-1α and HIF-2α proteins in primary glioblastoma lines compared to normoxia (N). Whole cell extracts were analysed by immunoblotting with the indicated antibodies. (**C**) G55 cells transfected with Tet-off inducible, non-hydroxylatable (non-degradable) HIF-1α and HIF-2α constructs express high levels of the respective HIF isoform under normoxia. Whole cell extracts were analysed by immunoblotting with the indicated antibodies. (**D**) Overexpression of HIF-2α, but not HIF-1α, profoundly upregulates the expression of a panel of side population signature genes (ASPHD2, MAML3, NFE2L2, ABL2 and NFATc2) as determined by quantitative PCR (n = 3). CAIX and Oct4 were used as established targets of HIF-1 and HIF-2, respectively. (**E**) Knockdown of HIF-2α, but not HIF-1α abolishes the hypoxia-mediated increase in the expression of CD133 and the side population markers ASPHD2 and MAML3 as quantified by quantitative PCR ($n = 3$). (**F**, **G**) Knockdown of HIFs blocks the hypoxia-mediated increase in the self-renewal (sphere forming) capacity of glioblastoma cells. (mean ± SEM; $n = 6$; $^*P < 0.05$, $^{**}P < 0.01$, relative to hypoxic control. Scale bar in **G**: 1 mm.

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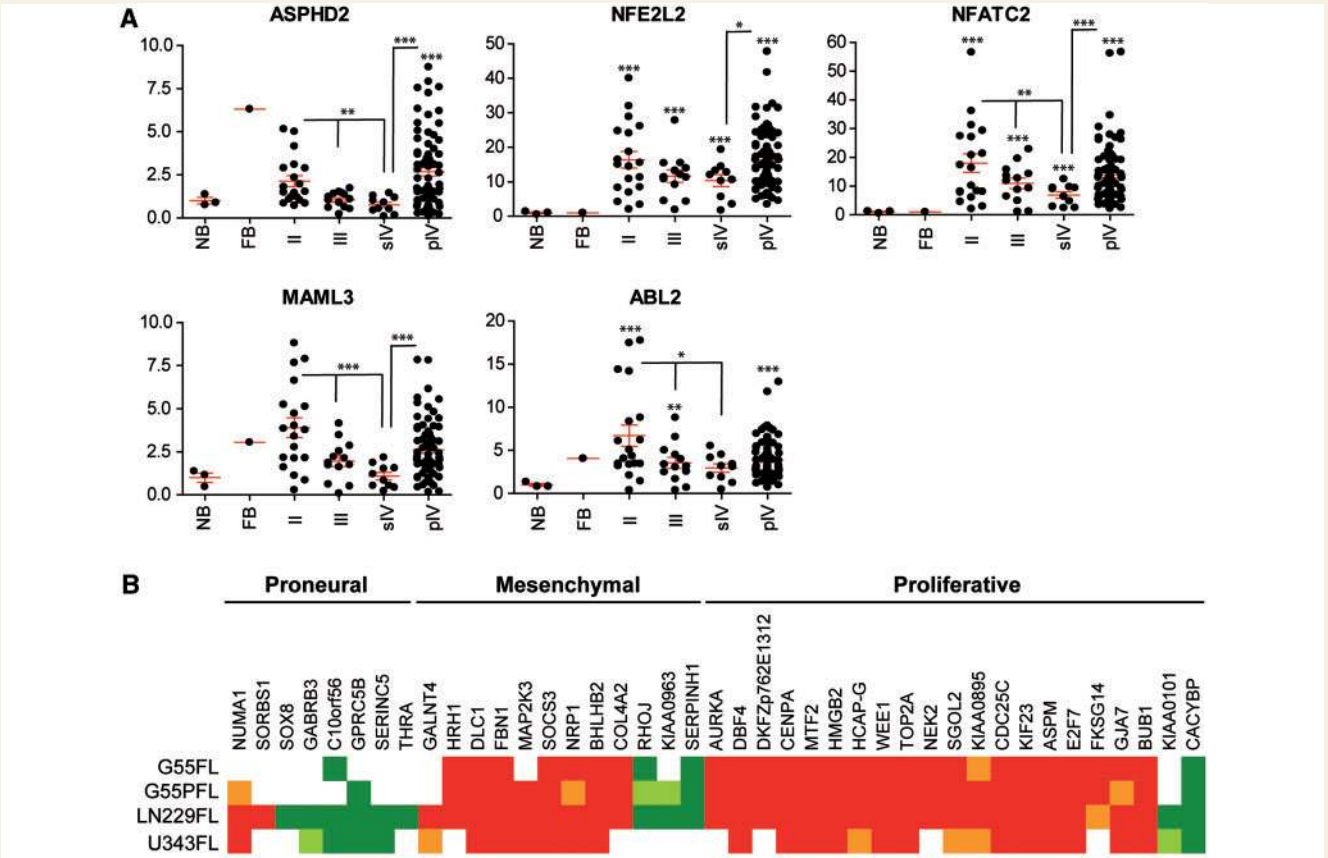

Figure 6 Side population signature gene expression in human gliomas correlates with tumour grade and prognosis. (**A**) Quantitative PCR analysis of side population signature gene expression in normal adult brain (NB), foetal brain (FB), diffuse astrocytomas (WHO grade II), anaplastic astrocytomas (WHO grade III), secondary (s) and primary (p) glioblastomas (WHO grade IV) reveals increased expression of side population signature genes in gliomas relative to normal adult brain. side population signature genes are expressed at significantly higher levels in primary glioblastomas and diffuse astrocytomas (II) than in secondary glioblastomas, indicating that high side population signature gene expression is associated with *de novo* tumour generation. $*P<0.05$, $**P<0.01$, $***P<0.001$ (tumour versus normal adult brain or as indicated). (**B**) Colour-coded comparison of the side population signature with genes previously identified as prognostic for patient survival (Phillips *et al.*, 2006). Genes assigned to the 'mesenchymal' and 'proliferative' subclass that correlated with shorter survival are overexpressed in the side population, shown in orange (*M* value [$\log_2$] >0.1) or red ($M>0.2$), while 'proneural' genes that correlated with longer survival were overexpressed in the non-side population shown by light green ($M<-0.1$) or dark green ($M<-0.2$).

we demonstrate that tumour stem cells are positioned in distinct microenvironments within the tumour, reminiscent of the stem cell niches described for neural stem cells (Mirzadeh *et al.*, 2008; Shen *et al.*, 2008; Tavazoie *et al.*, 2008). Specifically, we show that tumour stem cells are located and controlled within vascular (Fig. 3, Supplementary Fig. S5) and perinecrotic niches. In line with our findings, recent studies have demonstrated that tumour stem cells are maintained and controlled within a perivascular niche (Calabrese *et al.*, 2007; Hambardzumyan *et al.*, 2008). At the same time, a large body of evidence supports the concept that the hypoxic microenvironment within the tumour contributes to cancer progression by activating an adaptive program that promotes tumour angiogenesis, invasion and survival. Intriguingly, stem cells of various tissues are located in hypoxic niches and their function is directly influenced by local oxygen concentrations (Keith and Simon, 2007). We provide direct evidence that oxygen availability regulates the tumour stem cell phenotype in glioblastoma, since hypoxia strongly upregulates not only the tumour stem cell marker CD133, as reported in previous studies (Griguer *et al.*, 2008; Soeda *et al.*, 2009), but also several of our side population signature genes, and functionally enhances the self-renewal capacity in a range of established and primary glioblastoma cells. Vascular proliferation and necrotic areas are two defining hallmarks of glioblastomas. Our findings that environmental signals from these two locations are crucially involved in tumour stem cell maintenance underlines the importance of the niche in controlling tumour stem cells. Together, these findings lend further credence to the hypothesis that tumours promote their growth by the generation of new/ectopic or expansion of pre-existing niches that support the maintenance of tumour stem cells (Clarke and Fuller, 2006). One key area for future studies will be to dissect the relationship between the tumour stem cells found in the hypoxic and in the perivascular niches, and their respective contributions to tumour growth and invasion.

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The effects of hypoxia on the tumour stem cell phenotype are mediated by HIF-2

Hypoxia has complex effects on cellular behaviour that are differentially mediated by members of the HIF family (Bertout *et al.*, 2008). HIFs play a key role in cancer progression by regulating a number of processes, including angiogenesis, proliferation and survival. Our data reveal an additional function of HIFs in controlling the tumour stem cell phenotype and the acquisition or maintenance of stem cell properties. Notably, while HIF-1 expression had no effect on the levels of tumour stem cell genes, HIF-2 induced a striking upregulation of a panel of genes from our side population signature, as well as CD133. Similarly, HIF-2, but not HIF-1 knockdown, abrogated the hypoxia-dependent induction of the tumour stem cell phenotype. These findings are supported by data from previous studies on the role of HIF and hypoxia in tumour cell differentiation. Hypoxia upregulates genes associated with an immature phenotype in neuroblastoma (Jögi *et al.*, 2002). Moreover, the stem cell regulator Oct4 is a specific HIF-2 target (Covello *et al.*, 2006). HIF-2 can also augment the activity of another stem cell gene, c-myc (Gordan *et al.*, 2007). In further support, recent studies have shown that HIF-2 regulates the stem cell properties of tumour stem cells (Li *et al.*, 2009), although the molecular downstream mechanisms of this regulation remained unclear. We now identify a number of potential downstream targets (ASPHD2, MAML3 and NFATc2) that are regulated by HIF-2. Intriguingly, both MAML3, a component of the Notch transcriptional complex, and NFATc2 play critical roles in the control of cellular differentiation and stemness (Chiba, 2006; Horsley *et al.*, 2008) It will therefore be crucial to examine whether the hypoxia regulated side population signature genes are functionally involved in tumour stem cell maintenance and whether some of them may represent potential therapeutic targets. With respect to the specific involvement of HIF-2 in the regulation of tumour stem cells phenotype it is interesting to note that compared to HIF-1α, HIF-2α already accumulates at higher oxygen tensions (Wiesener *et al.*, 1998; Holmquist *et al.*, 2005), which more closely represent the *in vivo* conditions under which tumours arise and grow. At the same time, while HIF-1α gets only transiently upregulated under chronic hypoxia, HIF-2α levels remain elevated under these conditions (Holmquist-Mengelbier *et al.*, 2006). Thus, both the physiological response of HIF-2α to changes in oxygen tension and the target specificities of HIF-2 make this factor a prime candidate to function as a key molecular regulator of the tumour stem cell phenotype in the hypoxic niche.

Transcriptional profiling of tumour stem cells provides insight into the mechanisms of glioma growth and progression

Our detailed microarray analysis of tumour stem cells revealed genes and pathways involved in stem cell regulation (Oct4, Myc, the Notch, tumour growth factor-β, calcineurin/NFAT or Wnt pathways, Supplementary Table S1–3) and potential functional biomarkers to predict patient survival. Interestingly, comparison of our side population signature with genes that had been previously identified as prognostic for the survival of patients with high grade gliomas (Phillips *et al.*, 2006) revealed that genes associated with shorter survival were typically overexpressed, whereas genes associated with longer survival were typically downregulated in our side population signature. Importantly, a number of the side population signature genes we identified were overexpressed in newly formed human tumours, in particular primary glioblastomas, the most common form of glioma in adults. Intriguingly, our data revealed that the expression levels of side population signature genes was lower in secondary glioblastomas, as compared to newly formed diffuse astrocytomas or primary glioblastomas. These findings are consistent with previous observations suggesting a reduction of the tumour stem cell component in secondary glioblastomas (Beier *et al.*, 2007). This may indicate that an elevated level of tumour stem cell gene expression is necessary for initiating tumour growth, but that lower levels are sufficient to support tumour progression. Alternatively, *de novo* tumours may contain a larger fraction of tumour stem cells, which become diluted as the tumour grows. Our data confirm previous reports that primary and secondary glioblastomas, although histologically undistinguishable, constitute different disease subtypes that are characterized by distinct genetic pathway alterations (Ohgaki and Kleihues, 2007; Yan *et al.*, 2009) and possibly also distinct tumour stem cell kinetics.

Potential therapeutic importance of the side population signature genes and their hypoxic dependence for specific targeting of tumour stem cells in gliomas

Although a large body of evidence supports the existence of a tumour stem cell component in various tumour types, their identity and properties, as well as the mechanisms regulating their generation and maintenance are under debate (Gupta *et al.*, 2009; Shackleton *et al.*, 2009). In particular, there is a clear need for a more detailed characterization of the molecular profile of tumour stem cells to aid their identification and isolation, as well as of the microenvironmental cues that control the tumour stem cell phenotype. Our study addresses these issues by defining a molecular signature of tumour stem cells and demonstrating that hypoxia plays an important role in regulating tumour stem cell gene expression and stemness. The characterization of mechanisms that regulate tumour stem cells is crucial for our understanding of how glioblastomas form and expand and may have important implications for future tumour treatment strategies. Effective cancer treatment is likely to require the selective targeting of tumour stem cells as the cell type responsible for tumour regrowth and relapse. Interestingly, combining chemotherapy with verapamil, an inhibitor of the ATP-binding cassette transporters and of the side population, increased the survival of patients with metastatic breast carcinoma (Belpomme *et al.*,

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2000). Our tumour stem cell signature may provide a powerful means for the identification of novel therapeutic targets in glioblastomas and other solid tumours that would specifically compromise tumour stem cell function. Apart from supplying microenvironmental cues for the maintenance of tumour stem cells, the hypoxic niche may shield tumour stem cells from environmental stresses such as chemo- or radiotherapy. Indeed, it has been postulated that stem cells may reside in hypoxic niches to reduce oxidative DNA damage (Moore and Lemischka, 2006). Hypoxia could thus also reduce the effectiveness of radiotherapy, which depends on the generation of reactive oxygen species. In addition, hypoxia/HIF induces the expression of ATP-binding cassette transporters such as multidrug resistance-1 or ATP-binding cassette-G2 that confer multidrug resistance on a variety of cancer cells (Comerford *et al.*, 2002; Krishnamurthy *et al.*, 2004). Thus, targeting the niche to disrupt the instructive and protective signals may prove an effective anti-cancer treatment. In support of the applicability of targeting the hypoxic niche, we show that inhibition of HIF-2α suppresses tumour stem cell maintenance. Indeed, the regulation of the tumour stem cell phenotype and of a number of tumour stem cell signature genes by HIF-2 provides a striking upstream target for the harnessing of glioma stem cells and gives further support for current therapeutic strategies to target the HIF pathway (Poon *et al.*, 2009).

In conclusion, our study has established the control of tumour stem cell maintenance by HIF-2 as an additional, possibly key mechanism through which hypoxia regulates tumour growth and progression. Moreover, the hypoxia-inducible tumour stem cell signature genes defined in our comprehensive transcriptome analysis of these cells represent valuable tools for the identification of tumour stem cells in tumours and for studying the mechanisms of their generation and regulation. Ultimately, they may provide useful means for the targeted eradication of the stem cell population in human glioblastomas.

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Supplementary material

Supplementary material is available at *Brain* online.

References

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003; 100: 3983–8.

Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006; 444: 756–60.

Beck H, Acker T, Wiessner C, Allegrini PR, Plate KH. Expression of angiopoietin-1, angiopoietin-2, and tie receptors after middle cerebral artery occlusion in the rat. Am J Pathol 2000; 157: 1473–83.

Beier D, Hau P, Proescholdt M, Lohmeier A, Wischhusen J, Oefner PJ, et al. CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res 2007; 67: 4010–5.

Belpomme D, Gauthier S, Pujade-Lauraine E, Facchini T, Goudier MJ, Krakowski I, et al. Verapamil increases the survival of patients with anthracycline-resistant metastatic breast carcinoma. Ann Oncol 2000; 11: 1471–6.

Bertout JA, Patel SA, Simon MC. The impact of O_2 availability on human cancer. Nat Rev Cancer 2008; 8: 967–75.

Bleau AM, Hambardzumyan D, Ozawa T, Fomchenko EI, Huse JT, Brennan CW, et al. PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. Cell Stem Cell 2009; 4: 226–35.

Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, et al. A perivascular niche for brain tumor stem cells. Cancer Cell 2007; 11: 69–82.

Chiba S. Notch signaling in stem cell systems. Stem Cells 2006; 24: 2437–47.

Clarke MF, Fuller M. Stem cells and cancer: two faces of eve. Cell 2006; 124: 1111–5.

Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res 2002; 62: 3387–94.

Covello KL, Kehler J, Yu H, Gordan JD, Arsham AM, Hu CJ, et al. HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev 2006; 20: 557–70.

Dai C, Celestino JC, Okada Y, Louis DN, Fuller GN, Holland EC. PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo. Genes Dev 2001; 15: 1913–25.

Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 2004; 64: 7011–21.

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Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996; 183: 1797–806.

Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 1997; 3: 1337–45.

Gordan JD, Bertout JA, Hu CJ, Diehl JA, Simon MC. HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. Cancer Cell 2007; 11: 335–47.

Griguer CE, Oliva CR, Gobin E, Marcorelles P, Benos DJ, Lancaster JR Jr, et al. CD133 is a marker of bioenergetic stress in human glioma. PLoS One 2008; 3: e3655.

Gupta PB, Chaffer CL, Weinberg RA. Cancer stem cells: mirage or reality? Nat Med 2009; 15: 1010–2.

Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, et al. Hypoxia requires notch signaling to maintain the undifferentiated cell state. Dev Cell 2005; 9: 617–28.

Hambardzumyan D, Becher OJ, Rosenblum MK, Pandolfi PP, Manova-Todorova K, Holland EC. PI3K pathway regulates survival of cancer stem cells residing in the perivascular niche following radiation in medulloblastoma in vivo. Genes Dev 2008; 22: 436–48.

Harris MA, Yang H, Low BE, Mukherje J, Guha A, Bronson RT, et al. Cancer stem cells are enriched in the side population cells in a mouse model of glioma. Cancer Res 2008; 68: 10051–9.

Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M, et al. Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci USA 2003; 100: 15178–83.

Holmquist L, Jögi A, Pahlman S. Phenotypic persistence after reoxygenation of hypoxic neuroblastoma cells. Int J Cancer 2005; 116: 218–25.

Holmquist-Mengelbier L, Fredlund E, Lofstedt T, Noguera R, Navarro S, Nilsson H, et al. Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell 2006; 10: 413–23.

Horsley V, Aliprantis AO, Polak L, Glimcher LH, Fuchs E. NFATc1 balances quiescence and proliferation of skin stem cells. Cell 2008; 132: 299–310.

Jögi A, Ora I, Nilsson H, Lindeheim A, Makino Y, Poellinger L, et al. Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proc Natl Acad Sci USA 2002; 99: 7021–6.

Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. Cell 2007; 129: 465–72.

Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 2005; 121: 823–35.

Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci USA 2004; 101: 781–6.

Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem 2004; 279: 24218–25.

Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 1994; 367: 645–8.

Li Z, Bao S, Wu Q, Wang H, Eyler C, Sathornsumetee S, et al. Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. Cancer Cell 2009; 15: 501–13.

Mirzadeh Z, Merkle FT, Soriano-Navarro M, Garcia-Verdugo JM, Alvarez-Buylla A. Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. Cell Stem Cell 2008; 3: 265–78.

Moore KA, Lemischka IR. Stem cells and their niches. Science 2006; 311: 1880–5.

O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007; 445: 106–10.

Ohgaki H, Kleihues P. Genetic pathways to primary and secondary glioblastoma. Am J Pathol 2007; 170: 1445–53.

Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 2006; 9: 157–73.

Poon E, Harris AL, Ashcroft M. Targeting the hypoxia-inducible factor (HIF) pathway in cancer. Expert Rev Mol Med 2009; 11: e26.

Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 2006; 441: 437–43.

Read TA, Fogarty MP, Markant SL, McLendon RE, Wei Z, Ellison DW, et al. Identification of CD15 as a marker for tumor-propagating cells in a mouse model of medulloblastoma. Cancer Cell 2009; 15: 135–47.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007; 445: 111–5.

Shackleton M, Quintana E, Fearon ER, Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell 2009; 138: 822–9.

Shen Q, Wang Y, Kokovay E, Lin G, Chuang SM, Goderie SK, et al. Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. Cell Stem Cell 2008; 3: 289–300.

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature 2004; 432: 396–401.

Soeda A, Park M, Lee D, Mintz A, Androutsellis-Theotokis A, McKay RD, et al. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha. Oncogene 2009; 28: 3949–59.

Son MJ, Woolard K, Nam DH, Lee J, Fine HA. SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma. Cell Stem Cell 2009; 4: 440–52.

Tavazoie M, van der Veken L, Silva-Vargas V, Louissaint M, Colonna L, Zaidi B, et al. A specialized vascular niche for adult neural stem cells. Cell Stem Cell 2008; 3: 279–88.

Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 2008; 8: 755–68.

Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, et al. Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. Blood 1998; 92: 2260–8.

Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, et al. IDH1 and IDH2 mutations in gliomas. N Engl J Med 2009; 360: 765–73.

Yuan X, Curtin J, Xiong Y, Liu G, Waschsmann-Hogiu S, Farkas DL, et al. Isolation of cancer stem cells from adult glioblastoma multiforme. Oncogene 2004; 23: 9392–400.

Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001; 7: 1028–34.

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