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CXCR4 mediates the proliferation of glioblastoma progenitor cells

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Moneeb Ehtesham^{a,b,c,*}, Khubaib Y. Mapara^a, Charles B. Stevenson^a, and Reid C. Thompson^{a,c}

^a Department of Neurological Surgery, Vanderbilt University Medical Center, 21st Avenue & Garland Street, Nashville, TN 37232, USA

^b Department of Cancer Biology, Vanderbilt University Medical Center, 21st Avenue & Garland Street, Nashville, TN 37232, USA

^c The Vanderbilt Ingram Cancer Center, 21st Avenue & Garland Street, Nashville, TN 37232, USA

Abstract

Increasing evidence points to a fundamental role for cancer stem cells (CSC) in the initiation and propagation of many tumors. As such, in the context of glioblastoma multiforme (GBM), the development of treatment strategies specifically targeted towards CSC-like populations may hold significant therapeutic promise. To this end, we now report that the cell surface chemokine receptor, CXCR4, a known mediator of cancer cell proliferation and invasion, is overexpressed in primary glioblastoma progenitor cells versus corresponding differentiated tumor cells. Furthermore, administration of CXCL12, the only known ligand for CXCR4, stimulates a specific and significant proliferative response in progenitors but not differentiated tumor cells. Taken together, these results implicate an important role for the CXCR4 signaling mechanism in glioma CSC biology and point to the therapeutic potential of targeting this pathway in patients with GBM.

Keywords

Brain tumor(s); Glioma(s); Cancer stem cell(s); CXCR4

1. Introduction

Despite significant advances in surgical technique, imaging technology, and adjuvant therapies, the prognosis for patients with glioblastoma multiforme (GBM) remains poor, with a two year survival rate approaching zero [28]. Despite a long-standing recognition of the cellular heterogeneity within these tumors, current therapeutic approaches focus on targeting the entire tumor as a whole. Relatively little attention has been paid to the selective tailoring of treatment approaches towards differing neoplastic cell sub-populations. Indeed, many experimental therapies have been validated, pre-clinically, by confirming their treatment efficacy using predominantly homogenous cell line based *in vitro* and *in vivo* glioma models. Subsequently, when subjected to clinical translation, these therapies have largely failed to make any significant impact. The development of effective treatment approaches for GBM must be

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* Corresponding author. Address: Department of Neurological Surgery, Vanderbilt University Medical Center, 21st Avenue & Garland Street, Nashville, TN 37232, USA. Tel.: +1 615 322 2297; fax: +1 615 343 8104. E-mail address: moneeb.ehtesham@vanderbilt.edu (M. Ehtesham).

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based on a rational understanding of glioma cell biology. In this context, one strategic shift could involve the development of therapies that target selected sub-population(s) of glioma cells known to have a disproportionate contribution towards tumor growth. This would allow for more focused treatment regimens that would aim to eliminate cell populations that are more important for continued tumor growth. This concept is strengthened by recent evidence that has demonstrated the existence, within primary human GBMs, of progenitor cells that may represent focal populations driving tumor initiation and renewal in these neoplasms [17,25, 29,19]. In this study, we now report that the cell surface chemokine receptor, CXCR4, which is known to mediate dissemination, invasion, and proliferation in a wide range of cancers [3, 13], is overexpressed in GBM progenitor cells. In addition, we show that CXCR4's protein ligand, CXCL12, promotes a specific proliferative response in these cells. These findings provide important insight into a key signaling mechanism that may serve as a potent regulator of glioma CSC proliferation and point to the therapeutic potential of targeting the CXCR4 pathway in patients with GBM.

2. Materials and methods

2.1. Tumor specimens and cell culture

All tumor specimens were collected intraoperatively from consented patients undergoing surgical resection of suspected intracranial GBM as approved by the Institutional Review Board at Vanderbilt University Medical Center. Tumor histopathology was confirmed by analysis of H&E stained tissue sections by a qualified neuropathologist. Fresh tumor tissue was physically and enzymatically disassociated (Accumax; Innovative Cell Technologies), subjected to RBC lysis, and seeded in 1:1 DMEM-F12/Neurobasal medium (Invitrogen) supplemented with B-27 (Invitrogen) and 20 ng/ml each of EGF and FGF (Peprotech) at an initial density of $1-1.5 \times 10^6$ viable cells/ml. This yielded neurosphere-like cultures, termed by us as glioma-derived spheres (GDS), which were passaged between 1 and 8 times every 7–14 days by enzymatic disassociation followed by re-seeding in 50% refreshed medium at a density of $2-3 \times 10^5$ viable cells/ml. Differentiated tumor cell cultures were established over a period of 10–20 days by transferring GDS onto laminin coated cultureware containing DM/F-12 medium (Invitrogen) devoid of EGF and FGF and supplemented with 10% fetal bovine serum (Gemini Biosciences).

2.2. Immunohistochemistry

Paraffin-embedded tumor tissue specimens were sectioned using a microtome, mounted on glass slides, deparaffinized, and immunohistochemically probed using standard methodology. The antibodies utilized were anti-Musashi-1 (Chemicon), anti-Sox-2 (Chemicon); and anti-CXCR4 (R&D Systems). Nuclei were stained with DAPI (Vector Labs). Slides were visualized using a Leica DMI6000B fluorescence microscope equipped with a Hamamatsu Orca ER digital camera and Simple PCI (Compix) Digital Imaging software.

2.3. In vivo xenograft generation

Fifty thousand GDS cells were harvested after two passages and implanted intracranially into the right corpus striatum in an athymic nude mouse model (Charles River Labs) using methodology identical to that described by us earlier [11].

2.4. RNA extraction and RT-PCR

Total RNA was isolated from GDS (3 days after the first passage) and differentiated cells using the RNeasy Micro Kit (Qiagen). Five hundred nanograms of RNA from each sample was converted to cDNA by reverse transcriptase, following the supplier's protocol (Superscript III Reverse Transcriptase, Invitrogen). PCR was carried out in a 25 μ L reaction mixture that

contained 2 μ L of 1:10 diluted cDNA as template, specific oligonucleotide primer pairs (Table 1) and Taq polymerase (Sigma). 18s ribosomal RNA was used as internal control for each cDNA sample. The final primer concentration was 200 nM. Cycling conditions were as follows: denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C (30 s), 57 °C (30 s), and 72 °C (30 s), with the reaction terminated by a final 5-min incubation at 72 °C. Expression levels were analyzed on ethidium bromide-stained 2% agarose gels.

2.5. Real-time quantitative PCR

Real-time PCR was performed on an ABI 7300 Real-time PCR System (Applied Biosystems) according to manufacturer's instructions. Equal volumes of cDNA were used for q-PCR using human CXCR4 and 18s ribosomal RNA specific primers (Applied Biosystems). All samples including no-template controls were assayed in triplicate. The relative amount of target transcripts were normalized to the number of human 18s ribosomal RNA transcripts found in the same sample. Human normal brain RNA (BD Biosciences) was used as a calibrator. The relative quantitation of target gene expression was performed with the standard-curve method (Applied Biosystems User Bulletin).

2.6. BrdU labeling and WST-1 proliferation assay

Cells were cultured in medium supplemented with growth factors and CXCL12 (0 ng/mL as control and 10 ng/mL) for 48 h. Following incubation with CXCL12, cells were pulsed with Bromodeoxyuridine (BrdU; BD Pharmingen) at a final concentration of 10 μ M for 6 h. Cells were washed, fixed, permeabilized and denaturation of DNA was carried out using DNase (300 μ g/mL). Subsequently, staining for incorporated BrdU was performed using anti-BrdU-Alexa Fluor 647 (Invitrogen) for 2 h at room temperature. Cells were washed twice and re-suspended in staining buffer (1 $\times$ DPBS + 3% FBS) and analyzed within 2 h with flow cytometry. For WST-1 proliferation assays, as we have previously detailed [10], GDS or corresponding differentiated tumor cell cultures were incubated for 48 h with either 10 or 100 ng/ml of CXCL12 in the presence or absence of 1 μ M AMD3100 (Sigma) following which samples were processed and analyzed colorimetrically as per manufacturer protocol (Boehringer Mannheim).

3. Results

3.1. Human glioblastomas are populated by neural progenitor-like cells

To assess whether primary GBM tumors contained progenitor-like cells, we plated freshly disassociated surgically resected tumor specimens in defined culture conditions that favor the expansion and maintenance of neural progenitor cells. Within a few days of culture initiation, we observed the emergence of non-adherent spherical cellular aggregates morphologically similar to *neurospheres* which is the standard terminology utilized to describe progenitor cell cultures derived from non-neoplastic neural tissue. Given the neoplastic origin of our cultures, we alternately termed our aggregates as glioma-derived spheres (GDS) (Fig. 1A and B). These spheres could be disaggregated using enzymatic methods and serially passaged for six or more generations, with re-formation of spheres observed at each passage. This indicates the presence of cells with self-renewal capacity within GDS. We detected expression of the neural stem cell-related protein nestin (Fig. 1C) as well as CD133 (Fig. 1D), a cell surface protein that has been utilized as a hematopoietic and neural stem cell marker. To further confirm the progenitor-like nature of GDS cells, we re-plated these cultures in serum-supplemented media devoid of EGF and FGF. After 3–5 days, many of the cells became adherent and after two to three weeks, cells with clearly differentiated morphology were visible. We confirmed the phenotypically differentiated status of these cells by detecting expression of Beta-III-tubulin (neuronal marker), GFAP (astrocytic marker), and CNPase (oligodendroglial marker) in immunocytochemically probed sub-populations (Fig. 1E, F, and G, respectively). We also

observed expression of CXCR4 in these cultures (Fig. 1H). To further verify the progenitor-like nature of GDS cells, we performed a more extensive characterization of GDS and differentiated cells derived from GDS using RT-PCR. We observed that GDS cultures demonstrated strong expression of the progenitor cell markers CD133, Bmi-1, Sox-2, and Musashi-1, whereas expression of these antigens was much weaker in corresponding differentiated cells (Fig. 1I). Bmi-1 expression has been reported as being present in almost all brain tumors [27]. Our data are however, consistent, with the characteristic of this marker as a stem cell antigen and our finding of differential expression between GDS and differentiated cultures confirms that we were successfully enriching for a progenitor cell phenotype. These data establish that GDS cultures derived from primary human GBMs contain CXCR4-expressing progenitor-like cells capable of self-renewal and multipotent differentiation.

3.2. Glioblastoma-derived progenitor cells are robustly tumorigenic in vivo

After confirming that GDS represented progenitor cell populations, we wished to confirm if these cells were derived from cancerous components of the tumor. With this aim, we attempted to generate *in vivo* intracranial tumor xenografts using GDS. Utilizing an athymic nude mouse model, 50,000 GDS were inoculated into the basal ganglia and after four weeks, brain tissue was harvested, sectioned, and stained with H&E. We observed the generation of diffuse tumor in the basal ganglia (Fig. 2A), which could be seen extending away from the site of tumor inoculation along white matter tracts. Tumor cells contained large irregular nuclei (Fig. 2B and C) and tumor-normal parenchyma boundaries were not clearly visible and were populated by diffusely invading clusters of tumor cells (Fig. 2B). We verified that tumor cells were of human origin by means of human HLA-specific immunohistochemistry (Fig. 2D and E). Significantly, HLA labeling revealed the robustly invasive nature of GDS-derived tumor cells, as we could detect neoplastic cells migrating into contralateral brain across the corpus callosum (Fig. 2F and G). These data confirm that GDS consist of tumorigenic cells and can generate robust GBM xenografts that replicate the histopathology and invasive capacity of primary human tumors with high fidelity.

3.3. Human glioblastomas demonstrate co-expression of the stem cell markers Sox-2 and Musashi-1 in conjunction with the cell surface chemokine receptor CXCR4

In order to ascertain the location and distribution of cancerous progenitor cells within human GBMs, we used an immunohistochemical approach to probe paraffin-embedded histological sections derived from these same tumors for the stem cell-related antigens Sox-2 and Musashi-1. We observed the presence of both Sox-2 as well as Musashi-1 expressing cells with large pleomorphic nuclei (Fig. 3A) indicating the widespread presence of neoplastic progenitor-like cells within GBM. In particular, Sox-2 appeared to demonstrate nuclear localization within neoplastic cells as opposed to predominantly cytoplasmic expression in adjacent histologically normal (non-neoplastic) tissue (Fig. 3A), a finding consistent with activation-induced nuclear translocation of this transcription factor within GBM. Further confirmation of stem cell antigen expression in human GBMs was obtained using RT-PCR, which demonstrated expression of the additional stem cell markers c-kit, SCF, Atax-in-1 (the human homolog of murine Sca-1), Oct-4, and Fut4 (data not shown). Additionally, we co-probed these specimens for presence of the cell surface chemokine receptor CXCR4, which we have previously identified as a potent mediator of glioma cell dispersal [13]. We observed co-expression of Sox-2 in a significant number of CXCR4 positive cells. To place this in context, we then wished to quantitatively determine whether CXCR4 expression in glioma progenitor cells was significantly higher than in the differentiated tumor. To this end, we utilized quantitative real-time PCR to measure CXCR4 expression in GDS and corresponding differentiated tumor cells. By means of plasmid-based standard-curve analysis, we determined that GDS expressed 2.6 ± 0.42 (average $\pm$ SEM) fold more CXCR4 than corresponding differentiated tumor cells ($P = 0.02$, paired *t*-test) (Fig. 3B). These data indicate the widespread *in situ* presence of cancerous progenitor cells within

GBMs and that these cells overexpress CXCR4, a cell surface chemokine receptor that we and others have identified as a robust mediator of glioma cell proliferation and invasion.

3.4. CXCL12 stimulates glioma progenitor cell proliferation

To place the overexpression of CXCR4 in a functional context, we assayed for the consequences of activating the CXCR4 signaling mechanism in GDS. To this end, we supplemented a GBM-derived *in vitro* GDS culture with recombinant CXCL12, the only known ligand for the CXCR4 receptor. After 48 h, we observed a $2.9(\pm 0.6)$ -fold increase in the number of proliferating cells as assessed by flow cytometry based BrdU incorporation labeling ($P = 0.03$; *t*-test) (data not shown). This strongly indicated that the activity of the CXCR4/CXCL12 axis was contributing to glioma progenitor cell turn-over. These data were consistent with that reported earlier by Salmaggi and colleagues who also demonstrated that supplementation of GDS cultures stimulated a proliferative response [24]. To further verify these results, we supplemented multiple GDS and corresponding differentiated tumor cell cultures with escalating doses of CXCL12 protein either in the presence or absence of AMD3100, a widely validated and clinically utilized specific small molecule antagonist of CXCR4 [23,16,18]. Following analysis of treated cultures with a colorimetric mitochondrial-enzyme activity based proliferation assay, as utilized previously by us for quantitation of neural stem cell proliferative responses [10], we observed a highly significant, dose-dependent, increase in GDS proliferation following supplementation with CXCL12 which could be completely abrogated by concomitant treatment of cultures with AMD3100 (Fig. 3C). Importantly, corresponding differentiated tumor cell cultures did not exhibit this proliferative response to CXCL12 supplementation.

4. Discussion

Rapidly accumulating evidence indicates that many cancers originate from the aberrant proliferation of progenitor cells. The cancer stem cells (CSC) hypothesis dictates that the continued proliferation of a tumor is dependent on a small sub-population of self-renewing and asymmetrically dividing neoplastic stem cells that supply a largely differentiated tumor [2,8]. Originally found in leukemia, CSC, identified on the basis of their self-renewal and ability to form transplantable xenografts, have now been derived from multiple human cancers including, more recently, brain tumors. Galli et al. and Singh and colleagues, among others, have published independent reports documenting their ability to derive progenitor cell cultures from primary human GBMs [17,25]. The rapidly emerging focus on CSC in brain tumors represents a paradigm shift in our understanding of the pathogenesis of these neoplasms. Importantly, the realization that a distinct sub-population of cells contributes disproportionately to the growth and sustenance of gliomas has important implications for the treatment of these tumors. One of the most significant insights from this recent body of work is that therapeutic strategies must be tailored and targeted towards CSC if lasting tumor regression is to be achieved [21]. In this context, we have recently described the sonic hedgehog pathway as a promising target in the setting of Grade II and III glioma progenitor cells [12]. Furthermore, two recent reports have discussed differential radio- and chemosensitivity profiles relating to glioma CSC [4,20]. In the setting of GBM, there remains an urgent need to understand biological signaling mechanisms that are active within CSC in order to develop appropriate CSC-directed treatment approaches.

CXCR4 is a seven-domain G-protein coupled transmembrane receptor initially identified as a co-factor for HIV entry into CD4+ T-cells [15]. Extensive subsequent work has additionally detailed the critical contribution of CXCR4, and its ligand CXCL12, to non-neoplastic progenitor cell proliferation and migration in multiple tissues including the hematopoietic, cardiovascular, and central nervous systems [reviewed in [22]]. In the context of neural stem

cell (NSC) trafficking, we have previously reported the role of CXCR4 in mediating NSC migration *in vitro* and *in vivo* [14]. Over the past five years, numerous reports have also linked CXCR4 activity with key aspects of tumor biology including cell proliferation, dissemination, and neovascularization in various cancers including leukemias, breast cancer, colon carcinoma, melanoma, ovarian cancer, and glioma [reviewed in [7]]. In the specific setting of GBM, we recently described that CXCR4 was highly overexpressed in invasive tumor cells *in vitro* and *in vivo*, and that its activity functionally mediated their infiltrative capacity [13].

With the aim of extending our insight into the role of CXCR4 in GBM biology, we have now demonstrated, using the highly sensitive and precisely quantitative methodology of qRT-PCR that CXCR4 is overexpressed in GBM-derived GDS cultures compared to corresponding differentiated tumor cells. Additionally, we detected co-expression of CXCR4 with progenitor cell markers within cancerous cell populations in histopathological specimens of human GBM. We also provide evidence that addition of CXCL12 ligand stimulated proliferation in GBM GDS but not in differentiated GBM cells. These are significant and novel aspects of our findings, as they provide evidence of an important regulatory role for CXCR4 in glioma CSC biology. The biological relevance of this ligand-activated proliferative response is provided by prior work by our group [13,26] and others [23,1], that has demonstrated significant production of CXCL12 protein within human GBMs particularly by neovascular endothelium which indicates the presence of an abundant *in vivo* source of CXCL12 ligand for activation of CXCR4 signaling in GBM progenitor cells. Several studies have provided strong evidence linking CXCR4 to differentiated GBM tumor cell proliferation [30,6]. In contrast, however, our results, did not demonstrate a proliferative response in differentiated GBM cells following treatment with CXCL12. This discrepancy may well be a result of our exclusive use of freshly established patient-derived cultures as opposed to the long-term propagated and off-the-shelf cell lines described in earlier reports.

Recent studies detailing the establishment of GDS cultures have utilized methodology involving the use of immediate CD133-specific sorting strategies on fresh tumor tissue prior to culture establishment [25,4,5]. This is based on the premise that CD133(+) cells represent the sub-fraction comprising CSC within GBMs, which is supported by evidence that CD133 (–) cells derived from GBMs are not tumorigenic [25,4,5]. We chose to not sort fresh tumor tissue for CD133 prior to GDS culture establishment, and utilized methodology similar to that described by Galli et al. [17]. Our findings that our GDS populations: 1. self-renewed for multiple generations following disassociation into single cells, 2. expressed CD133 as detected both at the message and protein levels, and 3. demonstrated *in vivo* tumorigenicity, indicate that the population of cells we studied contained GBM CSC. Indeed, it should be pointed out that there is currently marked disparity between recent glioma CSC reports with regard to culture methodology with some groups advocating the derivation of CSC from completely differentiated GBM cell cultures [29] whereas others opt for immediate CD133 sorting approaches [25,4,5]. Consistent with the methodology of Galli and colleagues [17], our approach represents an intermediate strategy with immediate culture of fresh tumor tissue in defined serum-devoid stem-cell culture conditions with subsequent confirmation of the expression of CD133 (and other stem cell markers) prior to experimental use. As the field of glioma CSC develops further, there will be an increasing need to establish a consensus on uniform culture methodology to ensure consistency of experimental results.

Given the established biological importance of CXCR4 in gliomas, our findings that this receptor is expressed at higher levels in neoplastic progenitor cells, and that CXCL12 ligand can specifically promote GDS rather than differentiated tumor cell turn-over, provide evidence that CXCR4 may play an important role in the proliferation of glioma CSC. This conclusion is further supported by ours' and others' previous work which has documented the ability of CXCR4 to mediate migration and turn-over in non-neoplastic neural stem cells [9].

Furthermore, while this study focused on the proliferative effects mediated by CXCR4 activation in GBM GDS, given our earlier findings that CXCR4 mediates the *in vivo* migratory capacity of non-tumorous neural stem cells in the brain [14], it is likely that this receptor may play a similar role in the dissemination of glioma CSC. In summary, we provide important evidence implicating a key cell surface chemokine receptor, CXCR4, as a regulator of glioma CSC biology. These findings point to the therapeutic potential of targeting CXCR4 as a means of curtailing CSC turn-over in patients with GBM and underscore the need for further investigation of this pathway in glioma cell biology.

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Abbreviations

GBM	glioblastoma multiforme
GDS	glioma-derived spheres
CSC	cancer stem cells

Fig. 1.

Isolation and characterization of glioma progenitor cells. Fresh primary human GBM explants were disassociated and cultured as described (Section 2) in the absence of serum. Within 3–5 days of culture, we detected the presence of neurosphere-like cellular aggregates termed glioma-derived spheres (GDS) (A and B). These spheres expressed the neural precursor markers nestin (C) and CD133 (D). Furthermore, when re-plated into differentiation inducing conditions, these cells could terminally differentiate into Beta-III tubulin expressing neuron-like cells (E) GFAP positive astrocyte-like cells (F) as well as CNPase expressing oligodendroglial-like cells. (G) These cells also expressed CXCR4 (H; DIC-differential interference contrast image). RT-PCR performed on cDNA generated from these cultures

indicated that GDS cells exhibited markedly stronger expression of stem cell antigens than corresponding differentiated tumor cells (I). These findings indicate that GDS consist of multipotent progenitor cells.

Fig. 2.

Glioma progenitor cells form tumor *in vivo* and recapitulate the histopathology of human GBM with high fidelity. To confirm the tumorigenicity of GDS, 50,000 GDS cells were implanted into the basal ganglia using an athymic nude mouse model. Brain tissue was harvested, sectioned, and analyzed four weeks after implantation. Representative images are depicted. Panel A demonstrates the presence of a diffuse tumor in the basal ganglia (main tumor mass T, demarcated by arrow-heads). Note that the tumor appears to be infiltrating laterally along a white matter tract. Panel B represents a high power image of the tumor-normal tissue boundary from the same section depicted in (A). The tumor T, is clearly visible in the left half of the panel, with large irregular nuclei whereas the right extreme of the panel is occupied by normal parenchyma N. The boundary of tumor-normal parenchyma is not clearly visible and is populated by diffusely invading clusters of tumor cells (arrows). Panel C demonstrates a non-immunohistochemically stained higher power magnification of the tumor demonstrating irregular, hyperchromatic nuclei with visible mitoses (two examples are indicated by arrow-heads). Panel D represents tumor-bearing tissue that has been immunohistochemically stained with an anti-human pan-HLA specific antibody and developed with the peroxidase substrate Nova-Red (red-brown precipitate) in order to definitively identify human tumor cells within mouse brain. Note that infiltrative tumor cells (labeled red-brown) extend far beyond the boundaries of the main tumor mass. Panel E is a higher power magnification of the boxed area in (D). Note the large number of invasive tumor cells that infiltrate parenchyma adjacent to the principal tumor mass, T. The highly invasive nature of these xenografts is further illustrated in Panel F which demonstrates the presence of human-HLA+ migratory tumor cells in the ipsilateral corpus callosum at significant distance from the primary tumor mass (the intraventricular choroid plexus is non-specifically labeled due to endogenous peroxidase activity). Panel G represents a high power magnification of the boxed area in F. These cells appear to be in the form of a migratory stream traversing towards the genu of the corpus callosum.

Fig. 3.

CXCR4 expression and function in glioma progenitor cells. (A) To assess the expression status of CXCR4 in GBM progenitor cells, we immunohistochemically probed histopathologically-verified human GBM tissue sections for the stem cell antigens Musashi-1 and Sox-2 as well as CXCR4. This revealed significant staining for both of these markers in tumor tissue within cells bearing large pleomorphic nuclei, thereby confirming the presence of cancerous progenitor cells in human GBM. Importantly, we also observed expression of Sox-2 within tumor cell nuclei as opposed to predominantly cytoplasmic localization in adjacent non-neoplastic cortical tissue. This may indicate activation induced nuclear translocation of this transcription factor within GBM progenitor cells. CXCR4 co-expression was visible within a

significant portion of Sox-2 expressing tumor cells. (B) To further quantify differences in CXCR4 expression levels between GDS and corresponding differentiated tumor cells, we utilized real-time quantitative PCR to measure expression in cDNA samples generated from these cultures. As depicted in Table 1, we observed an average of 2.6-fold greater CXCR4 expression in GDS cells versus differentiated tumor. (C) To place the overexpression of CXCR4 in GDS in context, we assayed for the functional consequences of CXCR4 activation. GDS cultures were supplemented with recombinant human CXCL12 for 48 h in the presence or absence of AMD3100 (a small molecule antagonist of CXCR4) and then analyzed by means of a WST-1 proliferation assay. We observed a marked proliferative response to CXCL12 in GDS but not in corresponding differentiated tumor cells. This was completely abrogated by AMD3100, indicating the specific role of CXCR4 in mediating this proliferative response.

Table 1

Primer sequences for RT-PCR analysis.

mRNA targets	Oligonucleotides (5' → 3')
Bmi-1	Forward: CTGGTTGCCCATGACAGC Reverse: CAGAAAATGAATGCGAGCCA
Musashi-1	Forward: ACAGCCCAAGATGGTGACTC Reverse: CCACGATGTCCTCACTCTCA
Sox-2	Forward: AACCCCAAGATGCACAACTC Reverse: CGGGGCCGGTATTATAATC
CD 133	Forward: CTGGGGCTGCTGTTTATTATTCTG Reverse: ACGCCTTGTCCTGGTAGTGTTG
18s	Forward: CATTCGAACGTCTGCCCTAT Reverse: GGGCCTGCTTTGAACACTCT