Evidence for label-retaining tumour-initiating cells in human glioblastoma

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Individual tumour cells display diverse functional behaviours in terms of proliferation rate, cell-cell interactions, metastatic potential and sensitivity to therapy. Moreover, sequencing studies have demonstrated surprising levels of genetic diversity between individual patient tumours of the same type. Tumour heterogeneity presents a significant therapeutic challenge as diverse cell types within a tumour can respond differently to therapies, and inter-patient heterogeneity may prevent the development of general treatments for cancer. One strategy that may help overcome tumour heterogeneity is the identification of tumour sub-populations that drive specific disease pathologies for the development of therapies targeting these clinically relevant sub-populations. Here, we have identified a dye-retaining brain tumour population that displays all the hallmarks of a tumour-initiating sub-population. Using a limiting dilution transplantation assay in immunocompromised mice, label-retaining brain tumour cells display elevated tumour-initiation properties relative to the bulk population. Importantly, tumours generated from these label-retaining cells exhibit all the pathological features of the primary disease. Together, these findings confirm dye-retaining brain tumour cells exhibit tumour-initiation ability and are therefore viable targets for the development of therapeutics targeting this sub-population.

Keywords: brain tumour; cancer stem cells; glioblastoma; label-retaining cells; tumour-initiating cells

Abbreviations: ABC = adenosine triphosphate-binding cassette; CFSE = carboxyfluorescein diacetate succinimidylester; MCM = mini chromosome maintenance; NOD/SCID = non-obese diabetic/severe combined immunodeficient
Introduction

Human glioblastoma is the most malignant and common primary adult brain tumour, with a median survival time of 9–15 months, despite aggressive therapy (Vescovi et al., 2006). This grim prognosis is due to therapy-resistant glioblastoma tumour cells that initiate tumour regrowth after discontinuing therapy. The identification and characterization of cell populations with specific properties that initiate brain tumour recurrence is essential for the development of effective therapeutics. Distinct sub-populations of tumour-initiating cells have been identified in leukaemia (Lapidot et al., 1994) and in many solid tumours including Ewing’s sarcoma (Suva et al., 2009), breast (Al-Hajj et al., 2003), prostate (Collins et al., 2005), lung (Eramo et al., 2008), colon (Ricci-Vitiani et al., 2007), liver (Suet sugu et al., 2006), pancreas (Li et al., 2007), ovarian (Bapat et al., 2005) and brain cancer (Ignatova et al., 2002; Galli et al., 2004; Singh et al., 2004; Bao et al., 2006), opening up the possibility of characterizing this tumour cell population for the development of targeted therapies. For human glioblastoma, tumour-initiating cells have been identified and isolated based on the expression of several neural stem cell surface markers such as CD133 (Singh et al., 2003, 2004), CD15 (Son et al., 2009) and A2B5 (Ogden et al., 2008; Tchoghandjian et al., 2010). Tumour-initiating cells have also been identified based on the functional criteria such as aldehyde dehydrogenase activity (Bar et al., 2007; Kast and Belda-Iniesta, 2009; Rasper et al., 2010) and the ability to exclude Hoechst 33342 dye (defined as ‘side population’), reflecting elevated expression of adenosine triphosphate-binding cassette (ABC) transporters, such as breast cancer resistance protein (ABCG2) (Kondo et al., 2004; Patrawala et al., 2005; Bleau et al., 2009). Recently, several groups have also utilized the propensity of label retention to identify tumour-initiating cells from solid tumours such as in breast (Krishnamurthy et al., 2008; Pece et al., 2010), skin (Roesh et al., 2010) and pancreatic (Dembinski and Krauss, 2009) cancer. To determine if a similar population of cells also exists within human glioblastoma, we exploited the properties of the prodrug carboxyfluorescein diacetate succinimidyl ester (CFSE), which is converted by cellular esterase activity into a fluorescent compound covalently bound to proteins and retained within the cells (Lyon, 2000). CFSE dye enables quantification of cell proliferation, as it is equally divided between daughter cells after division. Here, we describe the isolation and characterization of an infrequently cycling (i.e. CFSE retaining), tumour-initiating sub-population in human glioblastoma cells that may represent a target to improve response to therapy.

Our study provides further evidence supporting the notion that functional and phenotypic features can be used to identify cells that initiate and drive tumour growth. These findings confirm that functional intra-tumour heterogeneity exists within glioblastoma cell populations, and that identification of the cells driving tumour initiation may be important for understanding tumour dynamics and developing effective treatments.

Materials and methods

Tumour sample, primary culturing and propagation

All brain tumour samples used in this study were collected from patients undergoing surgical treatment and were obtained following informed consent and Institutional Review Board approval. Biopsies were classified by neuropathologists as glioblastoma or grade III glioma according to WHO guidelines (Louis et al., 2007). After surgical removal, the tissue was washed and mechanically dissociated before being placed in an enzymatic cocktail containing trypsin/ethylenediaminetetraacetic acid (0.05%) for 10 min at 37°C, followed by filtration through a 40-μm filter. Dead cells were quantified using trypan blue labelling and the cells were then transferred (at a density of 50 000 viable cells per ml) into neurosphere assay growth conditions (Deleyrolle and Reynolds, 2009). This serum-free culture system containing epidermal growth factor (EGF, 20 ng/ml, R&D) and basic fibroblast growth factor (bFGF, 10 ng/ml, R&D) and enables isolation and expansion in vitro of cells exhibiting stem cell characteristics. Under these culture conditions, the tumour cells generate gliomaspheres that can be serially passaged, as reported by Galli et al. (2004). Briefly, when the gliomaspheres have reached an adequate size (~150 μm diameter), they were dissociated using enzymatic digestion with a solution containing trypsin/ethylenediaminetetraacetic acid (0.05%) for 3–5 min. Finally, cells were washed, counted using trypan blue to exclude dead cells and replated in fresh media supplemented with epidermal growth factor and basic fibroblast growth factor. Using this technique, which has proven to be a more reliable model than traditional cancer cell lines to study cancer biology (Lee et al., 2006), we generated 20 patient-specific human glioblastoma gliomasphere cultures and one patient-specific grade III gliomasphere culture that we used in the current study. All lines used were passaged <20 times and none of the lines expressed any functional or phenotypic changes over this time span.

Growth rate assay

To measure cell proliferation and identify a slow-dividing population, cells derived from 20 human glioblastoma samples and one grade III glioma sample were loaded with CellTrace CFSE green fluorescent dye (Molecular Probes) according to the manufacturer’s instructions. After passage, cells were incubated with 5 μM CFSE. Slow-cycling cells (top 5%) and overall population (bottom 85%) were identified 5–10 days after CFSE staining based on their CFSE retention level during culture. Based on the fact that CFSE intensity decreased 2-fold every time a cell divides, we calculated the time to undergo cell division based on the decay rate of CFSE intensity, which we normalized to the fluorescence decay observed with non-proliferative mouse astrocytes. CFSE bright cells (top 5%) dilute the dye significantly slower than that of the overall population. Human glioblastoma cells cultured in the neurosphere assay were observed using a bright field/fluorescent microscope to monitor sphere formation and dye dilution on the day of loading and 24, 48, 96, 120 and 144 h after CFSE labelling. Growth rate between the different cell populations was also analysed by measuring the cell number obtained at each passage. The cellular fold expansion was measured by dividing the number of cells quantified at each passage by the number of plated cells.
Sphere forming frequency assay

Five to 10 days after passaging the cells, the spheres from seven different lines were isolated by flow sorting using Hoechst 33342 and stained using Alexafluor 488 or 568 (1:500, Invitrogen) to identify human glioblastoma cells. The frequency of tumour-initiating cells could be calculated in a statistically robust manner by combining a limiting dilution assay with rigorous statistical analysis (Hu and Smyth, 2009). Therefore, to quantify tumour formation ability, we have used the accepted limiting dilution transplantation assay (ranging from 10,000 to 200,000 cells injected) coupled with statistical analysis using the ‘StatMod’ package (Hu and Smyth, 2009) (http://bioinf.wehi.edu.au/software/lmdil/), part of the R statistical software project (http://www.r-project.org).

Sphere size measurement

Single cells from four different lines were seeded into 384-well dishes as above and allowed to proliferate to form spheres. Spheres were stained using Hoechst 33342 and imaged using a Zeiss Axio Observer. Sphere sizes were measured using ImageJ software.

Differentiation of stem cell progeny

To assess multipotency, cells were plated at a density of $2.5 \times 10^4$ cells/cm$^2$ onto poly-L-ornithine-coated glass coverslips in basal culture media lacking growth factors and containing 10% foetal calf serum (Singh et al., 2003). Multiple immunofluorescence assay for neural antigens was performed after 7–10 days (Deleyrolle and Reynolds, 2009).

Immunostaining and flow cytometry

Five to 10 days post-CFSE load, immunostaining was performed using antibody against CD133 (1:11, Miltenyi Biotec, 10 independent lines), CD15 (BD Pharmingen, 1:50, 18 independent lines), ABCG2 (BD Pharmingen, 1:50, nine independent lines) and mini chromosome maintenance 2 (MCM2) (1:500, Santa Cruz, 12 independent lines). Staining was quantified by flow cytometry (BD LSRII).

Multipotency assay

Five to 10 days post-CFSE loading, the CFSE$^{\text{high}}$ fraction was isolated by fluorescence-activated cell sorting (BD FACSAria Flow Cytometer) and plated in the neurosphere assay for in vitro expansion before being placed in differentiation conditions for 4–7 days. Multi-lineage differentiation potential was analysed by fluorescent microscopy using the antibodies anti-glial fibrillary acidic protein (1:500, Dako), TUJ1 (1:1000, Promega) and O4 (5 $\mu$g/ml, R&D Systems) to label astrocytes, neurons and oligodendrocytes, respectively.

To isolate and culture the in vivo (intracranial) slow- and fast-cycling cells, 6–9 weeks post-implantation, the transplanted tissue was mechanically and enzymatically dissociated (Galli et al., 2004; Deleyrolle and Reynolds, 2009). Single cells were stained with propidium iodide (1 $\mu$g/ml) to detect viable cells. We used a specific anti-human CD56 antibody (1:100, BD Biosciences) to identify human cells, which were isolated by fluorescence-activated cell sorting based on the CFSE level and subsequently cultured in the neurosphere assay. CD56 staining was also confirmed using fluorescent microscopy.

Immunohistochemistry

In situ tumour formation was confirmed using haematoxylin and eosin staining. Human glioblastoma cells were identified using an anti-human Nestin antibody (1:500, Millipore) alone or in combination with CD133 (1:300, Abcam). A human-specific MCM2 antibody (1:200, Santa Cruz) was used to identify human glioblastoma cells that were competent to divide. Immunocomplexes were visualized in 3,3’-diaminobenzidine using the ABC-Elite peroxidase method (Vector Laboratories) or using secondary antibodies conjugated to Alexafluor 488 or 568 (1:500, Invitrogen) together with DAPI (1:1000, Invitrogen).

Xenotransplantation assay

We used 6- to 10-week-old female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice for all surgeries, following institutional and national regulations. Two microlitres of cell suspension (5000–100,000 live cells/$\mu$l) were injected (using a 5 $\mu$l Hamilton syringe) into the striatum using a stereotactic apparatus. Injection coordinates were 2 mm lateral to Bregma and 3 mm deep. After tumour cell implantation, the animals were monitored for any neurological signs affecting their quality of life. When symptoms were observed (ataxia, lethargy, seizures or paralysis), the mice were sacrificed and tumour formation was confirmed by tissue analysis. Tumour-initiation ability of the slow-cycling fraction and the overall population has been analysed in three independent human glioblastoma cell lines and one grade III glioma cell line. Although historic publications have reported injecting as few as 100 cells and getting tumour formation (though not 100% of the time), this addresses the issue of the minimal number of cells sufficient to generate a tumour and does not provide the actual frequency of tumour-initiating cells. It was recently demonstrated that the frequency of tumour-initiating cells could be calculated in a statistically robust manner by combining a limiting dilution assay with rigorous statistical analysis (Hu and Smyth, 2009). Therefore, to quantify tumour formation ability, we have used the accepted limiting dilution transplantation assay (ranging from 10,000 to 200,000 cells injected) coupled with statistical analysis using the ‘StatMod’ package (Hu and Smyth, 2009) (http://bioinf.wehi.edu.au/software/lmdil/), part of the R statistical software project (http://www.r-project.org).

Generation of astrocytes

Murine astrocytes were generated as described in the Supplementary materials and methods of the Supplementary material.

5-Ethynyl-2’-deoxyuridine incorporation

5-Ethynyl-2’-deoxyuridine retention was measured by fluorescence-activated cell sorting in four different lines at different time points post-labelling (0, 48, 72 and 96 h) after a 45 min 5-ethynyl-2’-deoxyuridine pulse (5 $\mu$M). Labelling was performed according to the manufacturer’s instructions (Click-IT EdU, Invitrogen).

Quantitative polymerase chain reaction analyses

Total RNA was isolated from the sorted sub-populations of two cell lines on the basis of CFSE labelling, using the RNAqueous 2kit (Ambion, #AM1931). Complementary DNA production and quantitative polymerase chain reaction reactions were performed as described in the Supplementary material.
Results

Identification of a label-retaining sub-population in human glioblastoma

To identify and characterize label-retaining cells in human glioblastoma, cells derived from primary tumours cultured in the neurosphere assay (Galli et al., 2004; Singh et al., 2004; Bao et al., 2006; Lee et al., 2006; Piccirillo et al., 2006) were loaded with the non-selective cell-permeant fluorescent dye CFSE. This intracellular fluorescent dye is partitioned evenly between daughter cells upon cell division, resulting in a 2-fold dilution of the fluorescence intensity, thereby enabling proliferation kinetic quantifications (Lyons, 2000; Barnes and Melo, 2006). During 8–10 cell divisions, the original intensity decreases by $2^R$ to $2^D$ reaching a level equivalent to the autofluorescence of unlabelled cells (Lyons, 2000). We monitored gliosphere formation by CFSE-labelled human glioblastoma cells using dual bright field-fluorescent microscopy (Fig. 1A), and quantified the fluorescence intensity over time using flow cytometry (Fig. 1B). This process demonstrated the serial dilution of CFSE with each cell division and the subsequent growth of gliomaspheres. Figure 1C shows a typical flow cytometry histogram of CFSE levels within a gliosphere culture together with fluorescent micrographs of varying CFSE intensities. Using these methods, we were able to identify two populations in 20 individual human glioblastoma cell lines and in one grade III glioma line (Fig. 1C and Supplementary Fig. 1); a label-retaining population of cells (top 5% CFSE) and an overall population (bottom 85% CFSE), separated by a 10% gap to avoid overlap and contamination between the two fractions. Comparison of CFSE decay between both populations over time revealed that the label-retaining fraction diluted CFSE significantly less compared with the overall population (Fig. 1D). Importantly, CFSE decay in a non-proliferating control population was significantly reduced compared with the human glioblastoma cells (Fig. 1D and Supplementary Fig. 2), supporting the notion that loss of CFSE intensity observed in the tumour cells was driven by cell division (Fig. 1F). Altogether, these data validate the use of CFSE to identify cellular sub-populations within glioma cells cultured as gliomaspheres based on their rate of cell division.

Characterization of label-retaining human glioblastoma cells

Label retaining and the overall cell population were isolated using fluorescence-activated cell sorting and cultured in the neurosphere assay in which both populations generated spheres at a frequency of $7.70 \pm 0.97$ for the CFSE$^{high}$ versus $10.03 \pm 1.72$ for the CFSE$^{low}$ (Fig. 2A–C). The average size of gliomaspheres generated by the overall population was significantly higher than that of the gliomaspheres generated by the slow-cycling cell population, indicating lower cell division frequency occurring within the CFSE$^{high}$ spheres (Fig. 2D). Although long-term cell culture showed that both populations exhibited cardinal in vitro stem cell characteristics of extensive self-renewal, generation of a large number of progeny and multi-lineage differentiation potential (Fig. 2F and G), the slow-cycling cells expansion rate was significantly lower when compared with the overall population (Fig. 2E). The reduction in sphere size and expansion rate of CFSE$^{high}$ cells provides further evidence of the reduced proliferative rate of CFSE$^{high}$ progeny compared with the overall CFSE$^{low}$ population.

We then analysed CFSE-retaining cells by flow cytometry with a panel of markers. We demonstrated that human glioblastoma and grade III glioma CFSE$^{high}$ cells expressed cell surface markers used previously to identify tumour-initiating cells (Singh et al., 2004; Bleau et al., 2009; Son et al., 2009) (CD133$^+$ /CD15$^+$ /ABCG2$^+$) (Fig. 2H–K). Even though CD133 expression has been described in tumourigenic and non-tumourigenic cells, this marker is commonly used to enrich for tumour-initiating cells in human brain tumours (Singh et al., 2004; Bao et al., 2006; Shackleton et al., 2009). Importantly, the slow-dividing population was enriched in CD133$^+$ cells (Fig. 2H and I). When the entire population was evaluated for CD133 immunoreactivity, 14% of the CD133$^+$ fraction was slow cycling when compared with only 3% of the CD133$^-$ cells (Fig. 2M and N). Earlier reports showed that both CD15 (Son et al., 2009) and ABCG2 (Bleau et al., 2009) expression enrich for tumourigenic potential. Our data demonstrating a greater CD15$^+$ and ABCG2$^+$ fraction in the CFSE-retaining population indicate a potential enrichment for tumour-initiating cells within the slow-cycling compartment (Fig. 2H–K).

For analysis of proliferative capacity, the cells were tested for expression of proteins actively involved in cell proliferation. MCM proteins (including MCM2-7) are nuclear proteins involved in the proliferation licensing system by regulating DNA replication (Bly and Hodgson, 2002). While MCM2 is absent in differentiated cells, it is highly expressed in human cancer cells, and plays a vital role in genome duplication in proliferating cells (Lei, 2005). MCM family proteins (especially MCM2) can be used to identify cells competent to divide and have, therefore, been classified as cancer biomarkers (Bly and Hodgson, 2002; Semple and Duncker, 2004). Using flow cytometry, we quantified the cells competent to divide using labelling with MCM2. When MCM2$^+$ cells were quantified, we observed enrichment in the slow-cycling compartment for proliferative potential compared with the overall population (Fig. 2H and L).
Figure 1  Identification of slow-dividing cancer cells in human glioblastoma and grade III glioma. (A) Gliomasphere formation and fluorescence of CFSE-loaded human glioblastoma cultures were monitored at the indicated times. (B) CFSE intensity was recorded by fluorescence-activated cell sorting at the indicated times after load. (C) Flow cytometry histogram and fluorescent micrographs of CFSE intensity revealed in human glioblastoma and grade III glioma cell lines after 5–10 days of growth in the neurosphere assay. A slow-cycling population of cells and an overall population were identified in 20 independent human glioblastoma cell lines and one grade III glioma cell line based on their capacity to retain CFSE (CFSE\textsuperscript{high}-top 5% and CFSE\textsuperscript{low}-bottom 85%). (D) A time dependent decrease of mean fluorescence intensity (MFI) was observed over a period of 120 h after CFSE labelling. A non-proliferative mouse astrocyte culture was used to determine the baseline for CFSE dilution unrelated to cell division. **P < 0.005, n = 6–11, t-test, four independent cell lines. (E) Human glioblastoma populations’ division times were calculated from the CFSE intensity decay data and normalized to the decay obtained with the non-proliferative mouse cells. Based on the fact that at every cell division the fluorescence intensity is divided by two, we defined the following formula: $2^X = B$ with $X$ as the number of cell division and $B$ as the ratio (initial CFSE MFI)/(final CFSE MFI). Therefore, $X = \log_B / \log_2$. **P < 0.01, n = 9, t-test, four independent cell lines. (F) 5-Ethynyl-\textsuperscript{2\textprime}-deoxyuridine labelling (EdU) after 4 h of pulse and different chase period times revealed higher ability to retain 5-ethynyl-\textsuperscript{2\textprime}-deoxyuridine staining overtime in the CFSE\textsuperscript{high} fraction compared with the overall population supporting slower proliferation. *P < 0.001, **P < 1 \times 10^{-5}, ***P < 1 \times 10^{-10}, n = 6–12, t-test, four independent cell lines.
Enrichment in CD133+ and MCM2+ cells in the slow-cycling population was also confirmed by quantitative polymerase chain reaction (Fig. 2O). The overall population was used as calibrator for the quantitative polymerase chain reaction experiments, and the expression levels of CD133 and MCM2 were greater in the slow-cycling cells (7.83 ± 1.41 and 4.1 ± 1.1, respectively) (Fig. 2O).

Together, these results demonstrate that the label-retaining CFSEhigh cell population is enriched for replication-competent CD133, CD15 and ABCG2 immuno-reactive cells.
Table 1 Tumour formation ability of the slow-dividing cells

<table>
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<tr>
<th>Number of tumours/number of injections</th>
<th>Time frame for tumour formation (weeks)</th>
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<td><strong>200 000 cells per injection</strong></td>
<td><strong>200 000 cells per injection</strong></td>
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<td></td>
<td><strong>L0</strong></td>
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<tr>
<td>Overall population</td>
<td>36/36</td>
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<tr>
<td>Slow-cycling cells</td>
<td>13/13</td>
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Flow cytometrically isolated CFSE\textsuperscript{high} and CFSE\textsuperscript{low} cells derived from three independent human glioblastoma-derived cells (line 0, 1 and 2; respectively L0, L1 and L2) or one grade III glioma-derived cells (line 3, L3) were intracranially transplanted. Shown are the numbers of implanted animals and mice bearing tumours at the indicated times.

Label-retaining human glioblastoma cells have greater tumour-initiation ability

After demonstrating that, slow-cycling human glioblastoma cells are enriched in cells expressing tumour-initiating markers, we sought to assess their in vivo tumour-initiation capability. Transplantation of 200 000 CFSE\textsuperscript{high} or CFSE\textsuperscript{low} cells (derived from three glioblastoma or one grade III glioma cell line) into the striatum of immunocompromised mice (SCID) resulted in tumour formation and 100% mortality (Table 1). Our standard injection of 200 000 cells, based on previously published work (Galli et al., 2004; Piccirillo et al., 2006; Beier et al., 2007; Chen et al., 2010), was chosen as it generates a tumour ~100% of the time. The glioblastoma-derived tumours exhibited typical histopathological hallmarks that define high-grade glioma (Fig. 3A–G) (Galli et al., 2004; Lee et al., 2006). The slow-dividing cell-derived tumours displayed migratory and infiltration capability (Fig. 3A, F and G), nest-like formations (Fig. 3B), vascular proliferation and nuclear pleomorphism with mitotic figures (Fig. 3C) as well as areas of pseudo-palisading necrosis (Fig. 3D). Anti-human nestin staining confirmed that slow-cycling cell-derived tumours were composed of human glioblastoma cells (Fig. 3E). Nestin labelling demonstrated infiltration of the tumour cells into the parenchyma as a result of slow-cycling progeny that would cross the contralateral hemisphere, migrate along the sub-cortical white matter tracts towards the ventricular system (Fig. 3F), and infiltrate the overlying cerebral cortex (Fig. 3G). Each tumour contained cells competent to divide as evidenced by the expression of MCM2 (Fig. 3H). Tumours derived from the slow-cycling fraction were also immunoreactive for the brain tumour-initiating marker CD133 (Fig. 3I).

Next, we compared the tumour-initiating efficiency between CFSE-retaining cells and the overall population by performing limiting dilution transplantation assay into the striatum of immunocompromised mice (Fig. 4). This experiment showed that 0.01% of the CFSE\textsuperscript{high} cells could initiate a tumour whereas only 0.003% of the CFSE\textsuperscript{low} exhibited this ability (Fig. 4 and Supplementary Fig. 3).

Together, these results reveal that the label-retaining human glioblastoma sub-population is enriched in tumour-initiating cells, and as the slow-cycling sub-population contains significantly more cells able to initiate the generation of high-grade brain tumours than the overall tumour population.

In vivo CFSE-retaining cells display tumourigenic potential

To address the question of whether label-retaining human glioblastoma cells exist in vivo, freshly stained CFSE cells derived from human glioblastoma gliomasphere cultures were injected into the striatum of immunodeficient mice (200 000 cells/mouse). All animals developed invasive tumours and a sub-population of CFSE-retaining cells was clearly evident 6 weeks after implantation, confirming that label-retaining human glioblastoma cells exist in vivo after intracranial transplantation (Fig. 5A). One of the technical challenges in using flow cytometry for the analysis of human cells in xenograft models in vivo is that the implanted tumour cells migrate and infiltrate the surrounding host tissue, thereby creating a chimeric population of human and host cells. This problem can be overcome by using a human-specific CD monoclonal antibody (CD56) that recognizes virtually 100% of the human glioblastoma cells but not mouse cells (Supplementary Fig. 4). Using this approach, implanted human glioblastoma cells (expressing CD56 antigen) were separated from the host tissue (debris and mouse cells). The in vivo slow-cycling (CFSE\textsuperscript{high}) and faster cycling (CFSE\textsuperscript{low}) cells were then isolated and individually cultured (Fig. 5B and C). Both slow- and fast-cycling in vivo cells exhibited expansion in culture. Like in vitro CFSE-retaining cells, in vivo slow-cycling cells generate gliomaspheres (Fig. 5D) and can drive long-term expansion; however, they have a significantly reduced mean fold expansion when compared with the in vivo CFSE\textsuperscript{low} population (Fig. 5E). Finally, to confirm in vivo tumourigenicity, we re-implanted the cells (200 000 cells/animal) derived from in vivo CFSE\textsuperscript{low} population (Fig. 5E). Following re-transplantation, all animals (Fig. 5F and G) developed large tumours displaying human glioblastoma-like features (i.e. vascular proliferation, pseudopalisading necrosis and nuclear pleomorphism) resulting in the death of the animals (Fig. 5H). Together, these results show that tumour-initiating label-retaining cells are present in vivo.

To our knowledge, the results presented here provide the first evidence for the existence of a label-retaining tumour-initiating
cell population within gliomasphere-derived human glioblastoma cells.

Discussion

The notion that a self-renewing, infrequently cycling, cancer stem-like cell population is responsible for tumour initiation is well-established in leukaemias (Holyoake et al., 2001; Graham et al., 2002). While an infrequently cycling compartment has also been described in solid tumours such as breast cancer (Krishnamurthy et al., 2008; Pece et al., 2010), pancreas adenocarcinoma (Dembinski and Krauss, 2009) and melanoma (Roesch et al., 2010), a similar population has yet to be identified in brain tumours.

Here, we identified a sub-population of label-retaining cells within human glioblastoma that exhibited a lower frequency of cell division, compared with the bulk of the tumour cells, along
with the expression of CD133, CD15 and ABCG2, as well as an enhanced ability to form tumours in vivo; features that are consistent with a tumour-initiating cell. To track cell divisions and identify slower cycling cells, we used functional labelling with the lipophilic, non-fluorescent precursor, carboxyfluorescein diacetate succinimidyl ester (CFDASE). The probe is activated by intracellular esterase activity converting it to fluorescent CFSE while covalently coupling it to amino groups where it becomes cell permanent and is diluted in half at each cell division (Lyons, 2000). Cells exhibiting higher CFSE epifluorescence over time corresponded to slow-dividing cells, which was confirmed using nucleoside analogue incorporation [bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU)] followed by a chase period, correlating label-retaining cells with CFSE intensity (Golmohammadi et al., 2008). This shows that CFSE retention directly correlates with 5-ethynyl-2'-deoxyuridine retention (Fig. 1F), providing direct confirmation for the hypothesis that a subset of human glioblastoma cells cultured as gliomaspheres retain CFSE due to reduced cell division. In sum, these results, together with the extensive literature using CFSE to track cell division (Lyons, 2000; Graham

Figure 4  Tumour initiating frequency of human glioblastoma slow-cycling cells. (A) Limiting dilution assay. From 10 000 to 200 000 cells, the slow-cycling cells or the overall population derived from one cell line were injected into the striatum of NOD/SCID mice. The percentage of animals bearing brain tumours was recorded. (B) The frequency of the cells able to generate a tumour was calculated based on the numbers presented in the table. Tumour-initiating cell frequency (evaluated using likelihood ratio tests) was greater in the slow-cycling fraction compared with the overall population ($\chi^2 = 4.45, *P = 0.0349$). Time frame for tumours to develop is presented as average ± SEM.
**Figure 5** *In vivo* CFSE-retaining cells show stem cell features and tumour formation ability. (A) CFSE*+* cells were observed at the injection site 6 weeks post-transplant of CFSE-loaded cells into the brain of NOD/SCID mice, demonstrating the existence of slow-cycling cells *in vivo*. (B) Human-specific anti-CD56 antibody was used to separate the donor cells from the host tissue. (C) *In vivo*, slow-cycling cells and overall population were identified from the CD56*+* fraction and isolated based on their CFSE level. (D) Biopsy samples from brains of mice implanted with slow cycling and overall population were re-cultured in the neurosphere assay. The micrograph shows gliomaspheres derived from the *in vivo* slow-cycling cells. (E) Re-cultured human glioblastoma cells exhibited long-term self-renewal and ability to generate large number of progeny. Like *in vitro*, the *in vivo* CFSE-retaining cells showed a lower fold expansion rate measured over a number of seven passages. ***P < 0.01, n = 13–24, t-test. (F–G) The progeny of the *in vivo* CFSE*high* or CFSE*low* cells cultured in the neurosphere assay were re-implanted in the striatum of immunocompromised animals. All the animals that had transplants developed tumours. Survival was also analysed, *P < 0.436, Log-rank test, two independent cell lines. (H) Like the *in vitro* slow-cycling cells, the *in vivo* CFSE-retaining cells give rise to progenies able to generate tumours exhibiting human glioblastoma features when transplanted into the striatum of NOD/SCID mice. *In vivo* slow-dividing cells were isolated by flow cytometry and cultured *in vitro* in the neurosphere assay for several passages and their progenies were subsequently transplanted intracranially. Vascular proliferation (black arrow), necrosis (arrowhead) surrounded by pseudo-palisade (asterisk), nuclear pleomorphism and mitosis (yellow arrow) were evident 13 weeks post-transplantation using haematoxylin and Eosin staining. (H') Higher magnification of the box presented in (H).
et al., 2002), demonstrate CFSE labelling as a valid approach to identify and isolate sub-fractions of cells based on the frequency of cell division.

In support of the cancer stem cell hypothesis, we demonstrate that label-retaining cells, isolated from cultured human glioblastoma-derived spheres using this methodology (CFSE<sup>high</sup>), possess the characteristics of long-term proliferation, extensive self-renewal, generation of large number of progeny and multipotency (Fig. 2). As previously demonstrated in leukaemia (Holyoake et al., 1999), breast (Krishnamurthy et al., 2008) and pancreatic cancers (Dembinski and Krauss, 2009), where low-frequency cell division was associated with tumorigenicity, we show here that the human glioblastoma and human grade III glioma infrequently dividing sub-population is enriched in CD133, CD15 and ABCG2 (Fig. 2), markers used to identify brain tumour-initiating cells with enhanced competencies for self-renewal, tumour formation and treatment resistance (Singh et al., 2004; Bao et al., 2006; Bleau et al., 2009; Son et al., 2009). As relative quiescence would be a functional characteristic providing protection against conventional treatments (such as radiation) targeting actively dividing cells (Anderson et al., 2003; Zhou et al., 2003), our findings suggest an additional mechanism underpinning resistance to such treatment of the CD133<sup>+</sup>, CD15<sup>+</sup> or ABCG2<sup>+</sup> fractions compared with their respective negative populations reported in the literature (Bao et al., 2006; Bleau et al., 2009).

While there is an overlap with formerly identified tumour-initiating cell markers, the infrequently cycling CFSE-retaining cells define a definite and unique population of tumour-initiating cells. Therefore, this study constitutes a relevant step towards characterizing the biological activities of sub-populations within the extensive heterogeneous tumour environment, and provides further evidence for heterogeneity in solid tumours based on the functional criteria (i.e. the frequency of cell division).

Importantly, not only in vitro, but also in vivo, the CFSE<sup>high</sup> fraction demonstrated the ability to establish tumours. Similar experiments have been described in an in vivo breast cancer model using vibrant CM Dil (Chloromethyl 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) to isolate slow-cycling cells (Krishnamurthy et al., 2008). Our study reports on an in vivo model using CFSE as a tracker in a CNS-derived tumour. These results further validate the generality of selecting sub-populations of cancer cells based on their rate of division as determined by the ability to retain CFSE, which does not reflect a culture-specific phenomenon. This notion is additionally supported by the data shown in Supplementary Fig. 5 that demonstrate a random distribution of the CFSE-retaining cells within gliomaspheres invalidating the hypothesis of either nutrient or oxygen deprivation leading to a reduced proliferation rate of this population due to its concentration in the core of the spheres.

Importantly, the ability of label-retaining cells to recapitulate tumours that harbour extensive similarities to the original disease (i.e. high mitotic activity, pseudopalisading necrosis, vascular proliferation and invasion) render the slow-cycling population an essential candidate to brain tumour initiation and progression, identifying them as a potential novel therapeutic target. Additionally, these data support the relevance of studying cancer cell biology using serum-free culture conditions (i.e. neurosphere assay) as sub-population of cells from gliomaspheres derived from primary human glioblastoma biopsies demonstrate the capability to recapitulate the overall in vivo phenotype of the parental tumour (Lee et al., 2006). Moreover gliomasphere formation ability has been associated with clinical outcome in malignant glioma demonstrating the neurosphere assay as a tumour relevant methodology (Laks et al., 2009). However, a limitation of our study is that our observations included only cells propagated in the neurosphere assay; therefore, one cannot exclude the possibility that a slow-dividing cell population lacking gliomasphere-generating ability may exist in vivo.

While the progeny of the slow-cycling cells divided less frequently in culture (Figs 2D and E and 5E), this compartment demonstrated proliferative and tumour-initiating potential, as evidenced by an enrichment in cells that were competent to divide (i.e. increased MCM2 immunoreactivity) and in expression of CD133, CD15 and ABCG2, respectively (Fig. 2H–O). On the surface, the decreased effective proliferation of the slow-cycling progeny in culture (Fig. 2D and E) appears to contradict the in vivo tumour initiation (Fig. 4) and progression (Fig. 5G) properties; however, these results may be reconciled by appreciating that tumour initiation and progression are not solely influenced by cell proliferation. Tumour cell invasion into the healthy brain, angiogenesis and the tumour cell niche are all likely to contribute to driving tumour initiation and progression (Brennan et al., 2009; Witz, 2009; Wong et al., 2009; Qian and Pollard, 2010), and our study suggests that a high-proliferative rate is not a primary driver of these mechanisms.

The use of single cell-surface marker expression to identify and characterize putative tumour-initiating cells in tumours remains controversial (Bidlemaier et al., 2008; Cheng et al., 2009). This is particularly true for CD133, the marker most widely used to identify brain tumour-initiating cells. CD133 is a cholesterol-binding membrane protein of unknown biological function and has been shown by several groups to be preferentially expressed in tumour-initiating cells (Singh et al., 2003, 2004; Collins et al., 2005; Suetusgu et al., 2006; Ricci-Vitiani et al., 2007; Eramo et al., 2008; Suva et al., 2009). However, recent studies have questioned the utility of using CD133 as a marker for tumour-initiating cells, as CD133 negative cells have been demonstrated to be efficient at initiating tumours in a variety of tumour types, including human glioblastoma tumours (Beier et al., 2007; Bidlemaier et al., 2008; Joo et al., 2008; Ogden et al., 2008; Wang et al., 2008; Cheng et al., 2009; Kelly et al., 2009; Nishide et al., 2009; Shackleton et al., 2009; Son et al., 2009). In addition, CD133 expression has been reported to increase in response to cellular stress, further confusing its utility as a robust marker for tumour-initiating cells (Grieger et al., 2008). It, therefore, remains an open question as to whether CD133 expression can be used to unambiguously identify tumour-initiating sub-fraction in human glioblastoma. A very recent study may help explain these conflicting results (Chen et al., 2010). These data suggest that the ability of cells to move between CD133<sup>+</sup> and CD133<sup>−</sup> sub-populations is a better indicator of tumour-initiation ability that CD133 expression per se. This interpretation is consistent with our finding that although the number of CD133<sup>+</sup> cells is increased within the...
label-retaining population; it is not increased to the extent one would expect if only CD133 expressing cells can initiate tumours. In contrast, label retention has consistently enriched for tumour initiation across multiple tumour types (Krishnamurthy et al., 2008; Pece et al., 2010), supporting the utility of this approach for the identification of tumour-initiating cells. However it is important to note that our analyses have revealed that the label-retaining population is made up of several tumour cell sub-populations (L. P. Deleyrolle et al., unpublished data). Future work analysing lineage relationships and isolating a more pure tumour-initiating sub-population based on multiple functional and phenotypic parameters is now underway to formally identify and quantify the prevalence of tumour-initiating cells within primary tumours. Nevertheless our study, in combination with recent publications, reveals label retention as an effective marker for enriching tumour-initiating cells from the total human glioblastoma cell population for further study.

In conclusion, our results show that label-retaining cells, defining a slow-cycling fraction, exist within human glioma (under the experimental paradigms used) and that this population in human glioblastoma cells is enriched in tumour-initiating cells expressing tumour-initiating cell markers CD133, CD15 and ABCG2 and exhibiting functional characteristics expected of a tumour-initiating cell population in culture. These findings, together with data from a growing number of studies (Graham et al., 2002; Krishnamurthy et al., 2008; Dembinski and Krauss, 2009; Pece et al., 2010; Roesch et al., 2010), provide a strong rationale for the contribution of label-retaining cancer cells towards tumour initiation in cancer. Therefore, identifying agents that effectively target the label-retaining fraction may lead to improving outcomes in patients when combined with conventional treatments that target the rapidly dividing population (Reya et al., 2001). Glioblastoma tumours are characterized by the presence of a multitude of cell types, and this tumour complexity is thought to contribute to the rapid rate of therapeutic failure. Defining and understanding tumour heterogeneity by the identification of clinically relevant cellular sub-networks is important to design combinatorial therapeutic interventions enhancing disease outcome. The results presented here demonstrating the isolation and characterization of a sub-compartment of infrequently dividing cells, consistently identified as tumourigenic, constitute an important step towards the comprehension of the pattern of diversity encountered in glioblastoma tumours.

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Supplementary material

Supplementary material is available at Brain online.

References

Lyons AB. Analysing cell division in vivo and in vitro using flow cytometry.


