Glioblastoma Cell Malignancy and Drug Sensitivity Are Affected by the Cell of Origin

Graphical Abstract

Highlights
- The cell of origin affects mouse glioblastoma development and phenotype
- A neural stem-cell-like origin produces higher malignancy and drug sensitivity
- The mouse cell origin signature separates distinct phenotypes of human glioblastoma
- The mouse cell origin signature predicts glioblastoma cell response to temozolomide

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In Brief
The contribution of the glioblastoma cell of origin for disease progression and treatment response remains unclear. Jiang et al. find that the originating cell type affects mouse glioblastoma tumorigenicity and drug sensitivity and validate this using transcriptome and functional analyses on a large panel of human glioblastoma cell lines.

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Glioblastoma Cell Malignancy and Drug Sensitivity Are Affected by the Cell of Origin

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SUMMARY

The identity of the glioblastoma (GBM) cell of origin and its contributions to disease progression and treatment response remain largely unknown. We have analyzed how the phenotypic state of the initially transformed cell affects mouse GBM development and essential GBM cell (GC) properties. We find that GBM induced in neural stem-cell-like glial fibrillary acidic protein (GFAP)-expressing cells in the subventricular zone of adult mice shows accelerated tumor development and produces more malignant GCs (mGC1GFAP) that are less resistant to cancer drugs, compared with those originating from more differentiated nestin- (mGC2NES) or 2',3'-cyclic nucleotide 3'-phosphodiesterase (mGC3CNP)-expressing cells. Transcriptome analysis of mouse GCs identified a 196 mouse cell origin (MCO) gene signature that was used to partition 61 patient-derived GC lines. Human GC lines that clustered with the mGC1GFAP cells were also significantly more self-renewing, tumorigenic, and sensitive to cancer drugs compared with those that clustered with mouse GCs of more differentiated origin.

INTRODUCTION

Glioblastoma (GBM) is the most frequent and aggressive primary malignant brain tumor (Ostrom et al., 2015). It is highly treatment resistant, has very poor prognosis, and is essentially uniformly lethal (Ostrom et al., 2015). A great challenge for therapy development is the large degree of GBM heterogeneity, both within and between tumors. An extensive body of work has shown that the GBM intertumor heterogeneity to a large extent is caused, to a large extent, by the genetic and epigenetic alterations that are found in these tumors (Brennan et al., 2013; TCGA, 2008; Verhaak et al., 2010). Despite the high diversity, adult GBMs can be divided into four to five molecular subtypes based on gene expression profiling (Brennan et al., 2013; Verhaak et al., 2010). These subtypes help delineate the major molecular pathways of GBM biology but are, as yet, of little relevance for treatment or prognosis. Studies of intratumor heterogeneity have shown that different molecular subtypes are present in different parts of the same tumor (Sottoriva et al., 2013) or even in individual cells within a single tumor (Patel et al., 2014). Another cause of GBM heterogeneity is the presence of GBM stem cells (GSCs) (Singh et al., 2004). GSCs are a subset of glioma cells that have the capacity to regenerate orthotopic glioma in immune-deficient mice showing the same characteristics as the primary tumor (Vescovi et al., 2006). They have been shown to escape irradiation (Bao et al., 2006) and chemotherapy (Bleau et al., 2009), and are believed to give rise to tumor recurrence (Chen et al., 2012). GSCs, which are most effectively maintained in culture using NSC (neural stem cell) medium (Lee et al., 2006), also share other features with NSCs (Vescovi et al., 2006) such as expression of various NSC markers, which have been used to prospectively sort GSCs from the tumor bulk (Singh et al., 2004). However, to date, no universal GSC marker has been identified, which further underlines the large heterogeneity of GBM.

The cell of origin for GBM has not been formally proven but is proposed to be an NSC, glial precursor cell (GPC), or more differentiated glial cell type. This generally accepted view is based on numerous observations such as: (1) the morphology and CNS marker expression in GBM tissues and cells are similar to that of normal neural and glial cell types (Louis et al., 2007); (2) the relationship between the transcriptomes of different GBM subtypes and those of various normal glial cell types (Verhaak et al., 2010); and (3) extensive in vivo modeling of glioma in mice showing that many CNS cell types such as NSCs (Aicantara Liaguno et al., 2009; Chen et al., 2012; Zhu et al., 2006), gliarial
precursor cells (Alcantara Llaguno et al., 2015; Friedmann-Morvinski et al., 2012; Holland et al., 1998), and oligodendrocyte precursor cells (OPCs) (Lindberg et al., 2009; Liu et al., 2011; Persson et al., 2010) have the capacity to develop glioma. In addition, the fact that isocitrate dehydrogenase (IDH)-mutated GBMs are molecularly, biologically, and clinically very different from IDH wild-type tumors (Ceccarelli et al., 2016) and the recent finding proposing that the cell of origin may determine the molecular subtype (Alcantara Llaguno et al., 2015), further support that GBM could stem from several cell types and suggest that the originating cell could have important implications for GBM biology and heterogeneity (Alcantara Llaguno et al., 2015; Sutherland and Visvader, 2015). We have also recently shown that the cell of origin affects the tumor phenotype in mouse models of pediatric supratentorial high-grade glioma (Sreedharan et al., 2016). Together, this argues for the importance of a deeper knowledge about the nature and consequences of the cell of origin in GBM.

By using a cross-species strategy, we addressed the question of how different glial cell types contribute to the heterogeneity of GBM cell (GC) phenotypes. We have performed in-depth analyses of the consequences of different cells of origin for mouse GBM development and GC properties. Through differential gene expression analyses of mouse GCs, we identified a gene signature that was used to stratify human GCs into subgroups.

**RESULTS**

**GBM Development in Adult Mice Is Accelerated When Induced in More Immature Glial Cells**

We used the replication-competent leukemia virus splice acceptor (RCAS)/tv-a mouse glioma model, where transgenetic expression of the avian tv-a receptor renders cells susceptible to RCAS viral vectors. Three different tv-a transgenic mouse lines were used in which expression of tv-a is under the control of the glial fibrillary acidic protein (GFAP) promoter in the G/tv-a line (Holland and Varmus, 1998), the nestin (NES) promoter in the N/tv-a line (Holland et al., 1998), or the 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) promoter in the C/tv-a line (Lindberg et al., 2009), directing RCAS infection to GFAP-, NES-, or CNP-expressing cells, respectively. All mice were in a homozygous p19Arf-deficient (Arf−/−) background. To obtain a localized RCAS infection, chicken fibroblast cell line (DF-1) chicken fibroblasts producing RCAS virus were transplanted through intracebral stereotactic injections to adult mice. RCAS is replication incompetent in mammalian cells (Federspiel et al., 1994), and infection of target cells will occur during a few days after injection.

First, we characterized the mouse CNS cells infected by RCAS in the different mouse strains. To target NSCs (Doetsch et al., 1999) and OPCs (Komitova et al., 2009), DF-1 cells producing RCAS-eGFP were injected in the subventricular zone (SVZ) of adult G/tv-a;Arf−/− (G/tv-a;SVZ) and C/tv-a;Arf−/− mice (C/tv-a;SVZ), respectively. To target other types of glial cells, RCAS-eGFP was also injected in the retrosplenial cortex (CTX) of adult N/tv-a;Arf−/− mice (N/tv-a;CTX), a location at a distance from and superior-anterior to the SVZ. The brains were analyzed 1 week post-infection by immunostainings for GFP and various neural and glial markers (Figures 1A, 1C, 1E, 1G, and 1I). Infected mouse cells could easily be distinguished from the residual, small, round, and intensely GFP-positive injected DF-1 cells by their larger, more heterogenous morphology with ample extensions, further supported by double-immunofluorescence stainings for chicken vimentin (c-vim, specific to DF-1 cells) and GFP (Figure S1A). The results from the double immunostainings for neural and glial markers and GFP were quantified by relating the number of double-positive cells to the number of GFP single-positive cells with neural or glial morphology (Figures 1B, 1D, 1F, 1H, and 1J). In G/tv-a;SVZ and N/tv-a;CTX brains, but not in C/tv-a;SVZ brains, we detected double-positive cells for both GFP and GFAP, and GFP and NES (Figures 1A–1D). This was expected because it is well established that GFAP and NES have an overlapping expression in adult NSCs and GPCs (Doetsch et al., 1999). Expression of the late OPC marker CNP was specific for the C/tv-a;SVZ brains (Figures 1E and 1F), whereas the more immature OPC marker OLIG2 (Zhou et al., 2001) was co-detected with GFP in N/tv-a;CTX and C/tv-a;SVZ brains (Figure 1G), but only rarely in G/tv-a;SVZ brains (Figure 1H). SRY-box2 (SOX2), a marker for neural stem and progenitor cells (Graham et al., 2003) could be found in all three mouse lines (Figure 1I). However, the number of GFP and SOX2 double-positive cells was significantly higher in G/tv-a;SVZ compared with N/tv-a;CTX or C/tv-a;SVZ brains (Figure 1J). Additionally, we stained for NG2, CD15, and TUJ1, but could not detect any GFP-positive cells expressing these markers (Figures S1B–S1D). Taken together, RCAS-infected CNS cells in G/tv-a;SVZ brains displayed an immature glial cell phenotype (GFAP+/NES+/CNP-/OLIG2-/SOX2+), reminiscent of an NSC or an early OPC. In contrast, RCAS-infected cells in N/tv-a;CTX brains displayed a OPC-like phenotype (GFAP+/NES+/OLIG2+/SOX2−), and in C/tv-a;SVZ brains the infected cells displayed clear features of an OPC (GFAP+/NES−/OLIG2−/SOX2+). We concluded that RCAS delivery in G/tv-a;SVZ, N/tv-a;CTX and C/tv-a;SVZ resulted in robust infection of distinct cell types along the glial cell differentiation axis.

Next, we induced tumors in adult G/tv-a;Arf−/−, N/tv-a;Arf−/−, and C/tv-a;Arf−/− mice by injecting DF-1 cells producing RCAS-PDGFB (platelet-derived growth factor B) in the same locations as above (G/tv-a;SVZ, N/tv-a;CTX, and C/tv-a;SVZ) (Figure 2A). PDGFB has a retention motif at the C-terminal domain that causes binding of secreted platelet-derived growth factor B homodimers (PDGF-BB) to the outer cell membrane of producing cells (LaRochelle et al., 1991); thus, tumors will be induced by autocrine PDGF-BB stimulation of RCAS-infected cells. Injected mice developed brain tumors with significantly different survival, with G/tv-a;SVZ mice showing the shortest latency (Figure 2B). To analyze whether the different microenvironments in CTX and SVZ locations would affect tumor development, we also injected RCAS-PDGFB in the SVZ of N/tv-a;Arf−/− mice and found no difference in survival (Figure S2). Tumors induced in G/tv-a;SVZ and N/tv-a;CTX mice tended to be more malignant (although not significant) than those developing in C/tv-a;SVZ mice.
Figure 1. Characterization of RCAS-eGFP-Infected Cells in G/tv-a;Arf^{-/-} Mice Injected in the SVZ (G/tv-a_SVZ), N/tv-a;Arf^{-/-} Mice Injected in the CTX (N/tv-a_CTX), and C/tv-a;Arf^{-/-} Mice Injected in the SVZ (C/tv-a_SVZ).

(A and B) Immunofluorescence staining for GFAP and GFP (A) and quantification of double-positive cells (B).

(C and D) Immunofluorescence staining for NES and GFP (C) and quantification of double-positive cells (D).

(E and F) Immunofluorescence staining for CNP and GFP (E) and quantification of double-positive cells (F).

(G and H) Immunofluorescence staining for OLIG2 and GFP (G) and quantification of double-positive cells (H).

(I and J) Immunofluorescence staining for SOX2 and GFP (I) and quantification of double-positive cells (J).

At least one section from at least two different mouse brains of each experimental group was analyzed in the quantification. The ratio of cells that were double positive for GFP and each of the glial cell lineage markers related to the total number of GFP-positive infected mouse cells (i.e., GFP-positive cells with glial morphology) was calculated. Values represent the mean ± SEM. Scale bars, 20 μm. Representative pictures are shown for each staining. White arrows indicate cells that are double positive for GFP and glial markers. The unpaired t test was performed, and statistical significance is indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. See also Figure S1.
This study is focused on GBM (grade IV glioma) and when analyzing grade IV tumors only, the significant difference in survival remained (Figure 2E). GBMs of different origin were histologically inseparable (Figure 2D), and from many of them GC cultures were established in serum-free defined NSC medium, but without addition of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) (Jiang et al., 2011). The GC cultures from G/tv-a\_SVZ, N/tv-a\_CTX, and C/tv-a\_SVZ were named mGC1\_GFAP, mGC2\_NES, and mGC3\_CNP, respectively, to indicate the different GBM origins (Figure 2A). At least three cultures from independent tumors in each group (mGC1\_GFAP, mGC2\_NES, and mGC3\_CNP) were established and used in the subsequent analyses. All cultures displayed tumor-initiating capacity following intracranial injection of $1 \times 10^4$ cells to syngeneic newborn mice (Figure 2F). In accordance with the survival of the primary RCAS-induced GBMs (Figure 2E), mGC1\_GFAP cells generated secondary tumors at a significantly faster rate than mGC2\_NES and mGC3\_CNP cells (Figure 2F). This argued that the accelerated primary tumor development in G/tv-a\_SVZ mice compared with N/tv-a\_CTX and C/tv-a\_SVZ mice (Figure 2A) was likely due to inherent properties of mGC1\_GFAP cells, making them more malignant and not the result of, e.g., a higher number of RCAS-infected cells in the G/tv-a\_Arf\^-/- mice. It also suggested that important intrinsic tumor cell properties could be maintained in stem cell culture.

**The Cell of Origin for GBM Affects Self-Renewal, Proliferation, and Differentiation of GCs**

To analyze the cause for the varying tumorigenic capacities of mouse GCs of different origin, we assessed the sphere-forming ability and proliferation rate of primary GC cultures at clonal density (1,000 cells/mL). Freshly explanted mGC1\_GFAP cells showed a significantly higher primary sphere-forming ability (Figure 3A) and generated larger spheres (Figures 3B and 3C) than mGC2\_NES and mGC3\_CNP cells. This difference was even more pronounced when analyzing secondary sphere-forming ability (Figure 3D), and only mGC1\_GFAP cells could be extensively passaged as spheres (data not shown). Thus, all subsequent experiments had to be performed on adherently cultured cells. We analyzed the sphere-forming ability and proliferation rate of primary GC cultures at clonal density (1,000 cells/mL). Freshly explanted mGC1\_GFAP cells showed a significantly higher primary sphere-forming ability (Figure 3A) and generated larger spheres (Figures 3B and 3C) than mGC2\_NES and mGC3\_CNP cells. This difference was even more pronounced when analyzing secondary sphere-forming ability (Figure 3D), and only mGC1\_GFAP cells could be extensively passaged as spheres (data not shown). Thus, all subsequent experiments had to be performed on adherently cultured cells. We
Figure 3. Self-Renewal, Proliferation, and Differentiation Analyses of mGCs Originating from GFAP-Expressing Cells, Nestin-Expressing Cells, and CNP-Expressing Cells

(A) Primary sphere-forming ability. The number of primary spheres formed per $1 \times 10^3$ freshly dissociated GCs.
(B) Phase-contrast photographs of representative primary spheres. Scale bars, 30 μm.
(C) The diameter of primary spheres. Around 40 spheres were counted for each culture.
(D) Secondary sphere-forming ability. The number of secondary spheres formed per $1 \times 10^3$ dissociated primary sphere cells.
(E) Limiting dilution analysis demonstrating the number of cells required to form at least one sphere per well (14, 39, and 33 cells for mGC1GFAP, mGC2NES, and mGC3CNP, respectively).
(F) Proliferation analysis showing the total number of cells after 7 days of adherent culture.
(G–I) Differentiation analysis of cells cultured with or without 5% FBS for 7 days. (G) Representative micrographs of immunofluorescence stainings for human influenza hemagglutinin (HA) (surrogate marker for RCAS-PDGFB expression) and BrdU. Scale bars, 30 μm. (H and I) Quantification of BrdU-positive cells in cultures (H) without FBS and (I) with 5% FBS. Approximately 300 cells were counted from each condition.

All experiments were performed at least three times except for the differentiation analysis that was repeated twice. Values represent the mean ± SEM. The unpaired t-test was performed, and statistical significance is indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. n, number of independently established cell cultures that were examined. See also Figures S3–S5.
found that also in adherent cultures, the difference in sphere-forming ability remained between the groups. Results from limiting dilution assays showed that mGC1GFAP cells had a significantly higher self-renewal capacity than mGC2NES and mGC3CNP cells (Figure 2E). A proliferation assay sustained the notion that mGC1GFAP cells had a higher proliferation rate than the mGC2NES and mGC3CNP cells (Figure 3F).

The difference in self-renewal indicated that the mGC1GFAP cells would be more stem-like. Therefore, we analyzed the protein expression of the NSC markers NES, SOX2, BMI1, and MSI1, and found that all mGC cultures, regardless of origin, had an equally high expression in essentially all cells of these markers (Figures S3A–S3D). Furthermore, there was no difference in primary and secondary sphere-forming ability or the size of the spheres in NSC cultures established from the SVZ of N/tv-a;Arf−/−, G/tv-a;Arf−/−, and C/tv-a;Arf−/− mice (Figures S4A–S4D), excluding that the different genetic backgrounds of the mouse strains had produced the phenotypes observed in the mGC cultures.

The ability of aberrant differentiation when cultured in fetal bovine serum (FBS) is a key characteristic of GSCs. Consistent with previous findings (Jiang et al., 2011), we found that all of our mGC cultures displayed dramatic changes in morphology and expression of glial and neuronal markers such as GFAP, TUJ1, CNP, SOX2, and NES when cultured in 5% FBS (Figure S5). Interestingly, a 16 h pulse of bromodeoxyuridine (BrdU) showed that, although proliferation was almost completely suppressed in the serum-cultured mGC2NES and mGC3CNP cells, mGC1GFAP cells maintained a considerable proliferation activity (Figures 3G and 3I). Thus, GCs of a less differentiated origin were remarkably more resistant to proliferative arrest in response to serum-induced differentiation.

Taken together, our in vitro cell analyses showed that a GBM originating from an immature NSC-like cell had a higher frequency of GSCs in their tumors, and cultured cells from these tumors displayed increased self-renewal and proliferative capacities compared with those of a more differentiated, glial precursor cell origin.

Transcriptome Analysis Identifies a 196 Mouse Cell Origin Gene Signature

Collectively, our data suggested that the cell of origin could determine important GBM cell properties. To uncover the molecular events underlying the different phenotypes, we performed transcriptome analyses on three independent GC cultures of each cell of origin group (mGC1GFAP, mGC2NES, and mGC3CNP) and three NSC cultures (mNSCgtv-a, mNSCnvt-a, and mNSCtv-a), one from each of the three mouse lines. Principal component analysis (PCA) performed on the global gene expression profiles showed a clear separation between the mGC groups of different origin (mGC1GFAP, mGC2NES, and mGC3CNP), as well as a separation between all mGCs and the mNSC group of cells, collectively called mNSC4 (Figure 4A). A list of 196 genes that were differentially expressed among the mGC1GFAP, mGC2NES, and mGC3CNP groups was identified and referred to as the 196 mouse cell origin (MCO) gene signature (Table S1). A heatmap of Z scores for the 196 MCO signature genes across the 12 mouse samples showed different patterns of gene expression between the different mGC and mNSC groups (Figure 4B).

The 196 MCO genes were distributed throughout the mouse genome with the largest numbers on chromosomes 7 and 10 (14 each). To identify candidate markers that could best differentiate between mGC groups, we sorted the log2 fold-change (logFC) values of the 196 MCO genes and selected the top 25% and bottom 25% genes in each mGC group, resulting in a list of 98 genes (top-98). A Venn diagram shows the overlap of the top-98 genes, where 48 genes were over- or under-expressed in only one mGC group (Figure S6A). Functional annotation of the top-98 genes from each mGC group (Table S2) by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway using DAVID (Huang da et al., 2009) detected a number of enriched GO terms associated with signaling, morphogenesis, and differentiation, and KEGG pathways such as hedgehog signaling and pathways in cancer (e.g., DCC, GLI3, and GPC3) are putative markers of the mGC1GFAP cluster. Moreover, over-representation analysis of the entire MCO gene list using the ConsensusPathDB (Kamburov et al., 2013) and the Reactome pathway-based sets produced nine enriched pathways (Figure S6B) including several signaling pathways. Functional annotation with Disease Ontologies using FunDO (Du et al., 2009) (Figure S6C) showed that several MCO genes were associated with terms such as “brain tumor,” “breast cancer,” and “cancer.” In all, these analyses supported that mouse GBMs of different origins are transcriptionally distinct and suggested that the 196 MCO gene signature could represent a basis for stratifying other GBM cases.

Molecular Subtype Prediction Reveals a Relation of Mouse GCs to Human GBM Subtypes That Is Dictated by the Cell of Origin

To investigate the relationship of our mouse GBM models to The Cancer Genome Atlas (TCGA) GBM subtypes, we compared the expression profiles of the human genes used for molecular subtype classification (Verhaak et al., 2010) in mRNA data from 515 human TCGA GBM samples (of which the vast majority had wild-type IDH1/IDH2; see Supplemental Experimental Procedures) and our 9 mGC samples (3 mGC1GFAP, 3 mGC2NES, and 3 mGC3CNP) and 3 mNSC samples (mNSCgtv-a, mNSCnvt-a, and mNSCtv-a) (Figure 4C). An isomap analysis (Tenenbaum et al., 2000) (a dimensionality reduction method well suited to visualize similarities and differences between groups of cancer samples; Nilsson et al., 2004) demonstrated that while the mGC1GFAP and mGC3CNP groups clustered together with proneural GBM samples, the mGC2NES group was closer to mesenchymal GBMs. The mNSC cultures clustered close to the classical GBMs that have a higher frequency of epithelial growth factor receptor (EGFR) alterations compared with the other subtypes (Brennan et al., 2009; Verhaak et al., 2010). This seemed reasonable given that these immortal (because of Arf loss) NSCs, unlike the mGCs, were dependent on EGF and had to be cultured in regular NSC medium with EGF and FGF2. The cross-species analysis showed that our mouse models could capture a considerable part of the complexity of human GBM and suggested that the cell of origin has a direct influence on the subtype of the tumor, in line with a recent
Figure 4. Transcriptome Profiling, Identification of the 196 MCO Gene Signature, and TCGA Subtype Clustering of mGC1 GFAP, mGC2 NES, and mGC3 CNP Cell Cultures

(A) Principal component analysis of the global gene expression profiles of mGC1 GFAP, mGC2 NES, mGC3 CNP, and mNSC4 cells. The axes depict the first three principal components, accounting for around 80% of the variance in the data.

(B) Heatmap of the Z scores for the 196 MCO genes across the mouse samples.

(C) Isomap analysis showing the relationship between the mouse GCs of different origin and 515 IDH1/IDH2 wild-type TCGA GBM samples of known subtype. The analysis is based on the Z scores of mRNA expression for 728 TCGA subtype classifier genes that were represented on the Affymetrix mouse array. For each mGC group, three independent cultures were included in the analysis. The differential gene expression analysis was performed using the three mNSC cultures (mNSCtv-a, mNSCtv-ar, mNSCtv-ar, i.e., one from each of the Gtv-a;Arf−/−, Ntv-a;Arf−/−, and Ctv-a;Arf−/− mouse lines) as reference. See also Figure S6 and Table S1.
finding using a different mouse GBM model (Alcantara Llaguno et al., 2015).

**The 196 MCO Gene Signature Can Be Used to Separate Molecularly Distinct Groups of Patient-Derived GC Lines**

Next, we applied the 196 MCO gene signature on transcriptome data from 61 patient-derived stem cell cultured GCs of defined TCGA subtypes (Xie et al., 2015) (Table S3). Thirty MCO genes had to be excluded because they were not represented on the microarray used for human cell line transcriptome profiling.

The expression values of the remaining 166 MCO genes were extracted from the transcriptome data and converted into Z scores within each dataset, across the 61 human glioblastoma cell cultures (hGC) lines and across the 12 mouse samples (3 mGC1GFAP, 3 mGC2NES, 3 mGC3CNP, and 3 mNSC) respectively, and combined (Figure 5A). The resulting matrix was subjected to consensus clustering (Monti et al., 2003) with an optimal \( k = 4 \) (Figure 5B). The resulting four clusters were called cluster 1GFAP, cluster 2NES/CNP, cluster 3, and cluster 4 (Figure 5C). Clusters 1–3 contained hGCs of different TCGA subtypes (Figure 5D), and cluster 4 consisted of mNSC cells only. Cluster 1GFAP was the largest (\( n = 27 \)), followed by cluster 2NES/CNP (\( n = 18 \)) and cluster 3 (\( n = 15 \)). The MCO clusters showed a clear separation also in an isomap analysis using the same combined matrix of Z scores for the 166 MCO genes (Figure 5E), providing further support for the grouping.

**Human GC Lines of Cluster 1GFAP Have Higher Self-Renewal and Are More Malignant Than Those of Cluster 2NES/CNP**

We reasoned that the molecular differences between hGC lines of cluster 1GFAP and cluster 2NES/CNP would also be reflected in their phenotypes. At least six hGC lines from each cluster were analyzed, and we aimed to cover the proportion of TCGA subtypes present in each cluster. Immune-deficient mice were injected intracerebrally with either hGC1 or hGC2 lines, and approximately one litter of mice was injected with each cell line (Figure S7). Analysis of survival comparing mice injected with hGC1 or hGC2 lines showed that cell lines of the hGC1 group produced a significantly shorter survival (Figure 6A), which is in line with comparing the mGC1GFAP cells with the mGC2NES or mGC3CNP cells (Figure 2F). Furthermore, we could also show that the hGC1 lines contained a significantly higher frequency of self-renewing cells than the hGC2 lines by limiting both dilution assay (Figure 6B) and primary (Figure 6C) and secondary sphere-forming assays (Figure 6D), also mimicking the corresponding mouse data (Figures 3E, 3A, and 3D, respectively). However, unlike the mGC cells (Figure 3F), human GC lines of the hGC1 group did not show a proliferation advantage over those in the hGC2 group (Figure 6E). The fact that hGC1 lines had higher self-renewal and were more malignant than hGC2 lines prompted us to analyze the survival of all patients from which the hGC1 and hGC2 cell lines were derived. This showed, somewhat surprisingly, that the more malignant phenotype of the cell lines in the hGC1 group were not reflected in different (shorter) survival of hGC1 patients (Figure 6F).

**Human GC Lines of Cluster 2NES/CNP Display a Higher General Resistance to Drugs Compared with Those of Cluster 1GFAP**

The absence of survival advantage for hGC2 patients despite their less malignant GCs proposed that there could be a difference in treatment response between patients in the hGC1 and hGC2 groups. To test the general drug resistance of mouse and human GCs of different origin, we used a panel of 26 compounds chosen to target a broad range of mechanisms relevant to cancer cells (Tables S4–S6). We tested three cell cultures each from the mGC1GFAP, mGC2NES, and mGC3CNP groups comparing the response of mGC1GFAP with the combined response of mGC2NES+mGC3CNP cells (Table S4), and seven hGC1 lines that were compared with six hGC2 lines (Tables S5 and S6). From the dose-response curves for each cell line, the area under the curve (AUC) was calculated. AUC measures both potency and efficacy of a drug, and is often used to compare the effect across multiple cell lines (Fallahi-Sichani et al., 2013). AUC values for each drug were compared using a two-sample unpaired Student’s t test and a significance level of 0.05 (Figures 7A and 7B). For mGCs, 13 out of 26 drugs showed a significant difference in drug response comparing mGC1GFAP with the combined result of mGC2NES and mGC3CNP cells (Figure 7A). For human GCs, there was a significant difference in drug response between the hGC1 and hGC2 groups for melphalan, temozolomide, 6-thioguanine, and b-AP15 (Figure 7B). In all these cases, the mGC1GFAP/hGC1 cells were more sensitive than the mGC2NES+mGC3CNP/hGC2 cells. To investigate the general trend in drug response, we plotted, for each drug, the median AUC values for mGC1GFAP or hGC1 cells (x axis) versus the median AUC values for mGC2NES+mGC3CNP or hGC2 cells (y axis) (Figures 7C and 7D). The Spearman’s rank correlation coefficient rho was equal to 0.91 (\( p = 9.5 \times 10^{-11} \)) for mGCs and 0.81 (\( p = 6.6 \times 10^{-7} \)) for hGCs, and the fitted linear regression line had a slope of 0.74 for mGCs and 0.65 for hGCs, which indicated (slope value < 1) that the mGC1GFAP and hGC1 cells were generally more sensitive to the compounds than mGC2NES+mGC3CNP and hGC2 cells, respectively.

Figure 5. 196 MCO Gene Signature-Based Cluster Analysis of 61 Human GC Lines

(A) An overview of the strategy.
(B and C) Consensus clustering of 61 human GC lines alongside the 9 mGC cultures (3 each of mGC1GFAP, mGC2NES, mGC3CNP, and the 3 mNSC reference cultures. (B) The Delta area plot showing the relative change in the area under the empirical cumulative distribution function (CDF) for each k versus k – 1, indicating that the optimal k = 4. (C) Consensus matrix showing four well-defined clusters.
(D) Distribution of the TCGA subtypes of the human GC lines in each of the resulting MCO clusters.
(E) Isomap analysis of Z scores of expression profiles for 166 MCO genes across 61 human GC lines, 9 mouse GC lines (3 each of mGC1GFAP, mGC2NES, and mGC3CNP), and 3 control mNSC cultures.

See also Tables S1 and S3.
Taken together, our results show that the originating cell type may contribute to the general drug response phenotype of GCs, where cells originating from immature glial cells tend to be more sensitive to drugs than those originating from more differentiated cell types.

DISCUSSION

We have assessed how three different cells of origin targeted by the same oncogenic alterations contribute to GBM development and the phenotypic properties of GBM cells. The cell of origin in this study was defined as the cell that receives the initial oncogenic mutation (the RCAS-infected cell). Our data demonstrate a functional relationship between the cell of origin for mouse GBM and the phenotype of GBM cells that could be molecularly captured in the 196 MCO gene signature. By cross-species analysis, we used the 196 MCO signature to separate patient-derived GBM cells into functionally distinct groups. Our findings may provide the basis for an improved patient stratification and for identifying new targets for therapy.

Relating the mouse GBM models used to the TCGA subtypes showed that mGC1GFAP and mGC3CNP cultures clustered with the proneural subtype, in line with previous data (Ozawa et al., 2014). Curiously, we found that the mGC2NES cultures were grouped with the mesenchymal subtype. Mesenchymal GBMs have been shown to be molecularly related to cultured astroglial cells (Verhaak et al., 2010), which are suggested to represent astrocyte precursor cells (Cahoy et al., 2008). This fits well with the origin of the mGC2NES cells being glial precursor cell-like toward the astrocyte lineage. The fact that we could model different GBM subtypes by initiating the tumor in different cell types suggests that the combination of cell of origin and oncogenic mutations are key factors in defining tumor phenotypes, in line with previous studies (Alcantara Llaguno et al., 2015; Ghazi et al., 2012; Wang et al., 2013). This also clearly demonstrated, as we had expected, that the three mouse models used could not cover the full spectrum of human GBM diversity. In that sense, our study should be viewed as a proof of concept, and further GBM modeling using different mutations will be needed in order to generate a comprehensive stratification based on the cell of origin.

Self-renewal and tumorigenicity are required characteristics of GSCs (Singh et al., 2004; Vescovi et al., 2006), and our results showed that these properties were strongly associated with the differentiation stage of the cell of origin. An immature, NSC-like origin generated primary GBMs faster, and the resulting mouse GCs had higher self-renewal abilities and accelerated tumor-initiating properties than those of glial precursor cell origin. Interestingly, GCs of all origins displayed uniform and...
Figure 7. Comparison of Cell Viability of Mouse and Human GCs Belonging to the mGC1GFAP/hGC1 or mGC2NES+mGC3CNP/hGC2 Groups in Response to 26 Anti-cancer Compounds

All experiments were performed twice using duplicate samples. The compounds were applied for 24 hr, and cell survival was measured by FMCA after 72 hr. Dose-response curves were plotted and AUC values were calculated. The higher the AUC value, the more viable are the cells.

(A and B) Boxplots of AUC values (median ± SD) from (A) three lines each of the mGC1GFAP, mGC2NES, and mGC3CNP groups, and (B) seven hGC1 lines and six hGC2 lines. A two-sample Student’s t test or a Kruskal-Wallis non-parametric test in the case of two drugs (found significant by the t test, but for which the normality assumption did not hold true as evaluated by a Shapiro-Wilk normality test) was used to determine significant differences in response: *p < 0.05.

(legend continued on next page)
high levels of stem cell markers, although the initially infected cell
did not. This suggested that the stem-cell-like phenotype of GCs could be a
general consequence of oncogenic transformation. NSC markers are frequently
being used to identify and analyze the presence of GSCs in tumors and cultures. Our
results showed that the influence of the originating cell type is subtle
and that stemness of GCs cannot be determined by NSC marker
expression only, but have to be uncovered by functional anal-
yses. Also, the 196 MCO gene signature could separate human
GBM cells based on their stemness phenotype.

One putative cause of the inability of the MCO stratification to
predict patient survival between cluster 1GFAP/hGC1 cells and
cluster 2NES/CNP/hGC2 cells, in spite of the hGC1 cells being signif-
cantly more malignant in experimental analyses, was the differ-
ence in drug sensitivity. The dose-response analysis of 13 hGC
lines to 26 compounds, most being in clinical use or in clinical trials
for treating cancer, clearly showed that the hGC1 lines had a higher
general sensitivity to drugs than the hGC2 lines. Furthermore,
hGC1 cell lines were significantly more sensitive than hGC2 lines
to temozolomide, which indicates that hGC1 patients could have
responded better to therapy than hGC2 patients, providing an
explanation to the lack of survival difference between the MCO
groups. This suggests that understanding the mechanisms regu-
ulating the interplay between stemness and drug sensitivity could
be important for developing more effective drugs against sub-
groups of GBM. Further exploration of the MCO gene signature
has the potential to uncover genes and pathways that could be
of value as functional and/or predictive biomarkers in GBM.

The highly inter- and intratumoral heterogeneous nature of this
disease has posed a great challenge to the development of more
efficient and specific therapies. Our results suggest that if the
functional consequences of the originating cell type can be deci-
phered, it could add to the understanding of the mechanisms
behind intertumoral heterogeneity and contribute to future evolu-
tion of targeted therapies for stratified groups of GBM patients.

**EXPERIMENTAL PROCEDURES**

For detailed descriptions, see Supplemental Experimental Procedures.

**Infection of tv-a Transgenic Mice**

Animal experiments were performed in accordance with the rules and regula-
tions of Uppsala University and were approved by the local animal ethics
committee. Six- to eight-week-old Ntv-a;Arf+/− (Jhrbom et al., 2003), G/tv-a;
Arf+/− (Jhrbom et al., 2005), and C/tv-a;Arf+/− (Lindberg et al., 2014) mice were
used. RCAS-producing DF-1 cells (RCAS-eGFP or RCAS-PDGF-B-HA;
gifts from Eric Holland) were transplanted by stereotactic injections of 106 cells
in 2 μL PBS. In G/tv-a;Arf+/− and C/tv-a;Arf+/− mice, the coordinates were:
0.5 mm anterior of bregma, 1.1 mm lateral, and 2.5 mm ventral ( = SVZ). In
Ntv-a;Arf+/− mice, the coordinates were: 3 mm posterior of bregma,
0.5 mm lateral, and 1 mm ventral (= CTX). Mice injected with RCAS-eGFP
were killed 1 week after injection, and mice injected with RCAS-PDGF-B-HA
were monitored every second day and killed upon symptoms of illness.

**Mouse GBM Cell and NSC Cultures**

Tumor and control cells were explanted and cultured from mouse primary tu-
more tissues or the SVZ of uninjected mice as described previously (Jiang et al.,
2011). Tumor cells were cultured without adding exogenous EGF and FGF2,
whereas non-tumor cells were cultured with addition of EGF and FGF2.

**Human GBM Cell Cultures**

All handling of human tissues and data were in accordance with the protocol
approved by the Uppsala ethical review board (2007/353). Samples from pa-
tients diagnosed with primary GBM were anonymized, coded, explanted,
cultured in serum-free defined NSC medium as previously described
(Kie et al., 2015). All experiments were performed on cells at passages 8–15.

**Intracranial GBM Cell Transplantation**

Intracranial cell transplantations of mouse or human GBM cell cultures were
performed in neonatal syngeneic (for mGCs) or non-obese diabetic-severe
combined immunodeficiency (NOD-SCID) (for hGCs) mice as previously
described (Jiang et al., 2011).

**Sphere-Forming, Proliferation, and Differentiation Analyses**

Single-cell suspensions from primary mouse GBMs or human GBM lines were
seeded at 1,000 cells/well or 500 cells/well, respectively, in 24-well plates.
For mGCs, the number and size of spheres were determined on day 7, after
which they were dissociated and seeded for secondary sphere-forming assay.
For hGCs, the number of spheres was determined on day 14, after which sec-
ondary sphere-forming analysis was performed. For proliferation analysis, 105
mGCs or hGCs were seeded in six-well plates and the total cell number was
determined on day 7. For differentiation analysis, mGCs were seeded onto
extracellular matrix (ECM)-coated glass coverslips and cultured in NSC medium
with or without 5% fetal bovine serum for 7 days. BrdU was added
16 hr before fixation. All experiments on mGCs were performed in at least du-
uplicates on at least three cell cultured NSC independent tumors in each group
(mGC1GFAP, mGC2NES, and mGC3CNP) and were repeated at least twice. All ex-
periments on hGCs were performed in at least duplicates and repeated twice.

**Microarray Analyses**

Total mRNA was extracted using the QIAGEN RNeasy kit (QIAGEN) from
9 mouse GC cultures, 3 mouse NSC cultures, and 61 human GBM cell lines.
The RNA was labeled and hybridized on Affymetrix Mouse Gene ST 1.0 arrays
(mouse samples) or Affymetrix Human Transcriptome 2.0 arrays (human sam-
ple) at the SciLifeLab Array and Analysis Facility. The raw data were normal-
ized using the Affymetrix Expression Console software (http://www.affymetrix.
com/estore) using the robust multi-array average method (Li and Wong, 2001).
All subsequent data processing and analyses were carried out in R using pack-
ages available from the Bioconductor project (https://www.bioconductor.org)
and MATLAB (version R2014b; The MathWorks).

**Identification of the 196 MCO Gene Signature**

To identify the genes that distinguish among the mGC1GFAP, mGC2NES,
and mGC3CNP cells, while excluding the influence of the genetic background, we
performed a differential gene expression analysis using the R limma package
(Wittenhall and Smyth, 2004) between the samples in each group and the
mNSC samples used as reference. We selected the genes that showed, in
any of the three comparisons, a log2 fold-change (logFC) greater than 1 or
smaller than −1 (at an adjusted p < 0.01 corresponding to a false discovery
rate [FDR] of 1%). For each of these genes we computed the difference in
logFC values between pairs of groups (mGC1GFAP versus mGC2NES,
mGC2NES versus mGC3CNP, and mGC1GFAP versus mGC3CNP) and selected
those for which the difference was at least greater or smaller than one unit. We

(C and D) The median AUC values were plotted for (C) mGC1GFAP (on the x axis) against mGC2NES+mGC3CNP (on the y axis) for each of the 26 drugs, and for (D)
hGC1 (x axis) against hGC2 (y axis). This highlights the general trend showing that most drugs produce a lower median AUC value (higher drug sensitivity) in
mGC1GFAP/hGC1 cells. A diagonal line (corresponding to a hypothetical case of equal median values between groups) is plotted in red, and the linear regression
line fitted to all the median pairs is plotted in blue.

See also Tables S3–S6.

988 Cell Reports 18, 977–990, January 24, 2017
further filtered this list to eliminate non-coding sequences and genes with an SD greater than 1.5 among the Z scores of the replicates within a group.

**Drug Response Analysis**
We used a panel of 26 selected anti-cancer agents and experimental compounds (Tables S4–S6). The experiment was done in duplicates and repeated twice for all GBM cell lines. Cell survival was measured using the non-clonogenic fluorometric microculture cytotoxicity assay (FMCA).

**Statistical Analyses**
Statistical analysis were performed using GraphPad Prism (GraphPad Software) and R (for the Kruskal-Wallis test, Shapiro- Wilk test, Spearman’s rank correlation, and linear modeling). Figures containing data from multiple experiments are presented as mean ± SD, and the statistical significance of differences between group means was determined with the two-tailed unpaired Student’s t test. For the drug analysis, Student’s t test was used for 20 drugs that fulfilled the normality assumption and the non-parametric Kruskal-Wallis for the other 6. The survival curves of mice and patients were analyzed by log rank test. The following p values indicate statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

**ACCESSION NUMBERS**
The accession number for the gene expression profile data generated by microarray analysis reported in this paper is NCBI GEO: GSE91393.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.01.003.

**AUTHOR CONTRIBUTIONS**

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