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Cell of origin of glioma: biological and clinical implications

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The cellular origin of gliomas remains a topic of controversy in cancer research. Advances in neurobiology, molecular genetics, and functional genomics have ushered new insights through exploiting the development of more sophisticated tools to address this question. Diverse distinct cell populations in the adult brain have been reported to give rise to gliomas, although how these studies relate physiologically to mechanisms of spontaneous tumour formation via accumulation of tumour-initiating mutations within a single cell are less well developed. Recent studies in animal models indicate that the lineage of the tumour-initiating cell may contribute to the biological and genomic phenotype of glioblastoma. These results suggest that the cell of origin may not only serve as a source of diversity for these tumours, but may also provide new avenues for improved diagnostics and therapeutic targeting that may prolong the lives of patients.

Gliomas are the most common primary malignancies in the central nervous system (CNS). This heterogeneous group of tumours is characterised by their resemblance to glia that perform a variety of important functions including support to neurons. The World Health Organisation (WHO) classifies astrocytic gliomas based on histologic criteria from low-grade lesions (grades I–II) to high-grade (grades III–IV) malignancies. Glioblastoma multiforme (GBM) is a highly aggressive grade IV tumour that is the most prevalent and presents with the poorest prognosis, with a median survival of 15 months from the time of diagnosis. Typical histological features of GBM include regions of necrosis, microvascular proliferation, abundant mitoses, and pleomorphic cells (Wen and Kesari, 2008). These highly infiltrative tumours are resistant to conventional therapies, including chemotherapy and radiation, and surgical intervention fails to remove the entire tumour. The consequence is eventual tumour recurrence and death (Stupp *et al*, 2005).

GENETIC CHANGES AND MOLECULAR CLASSIFICATION

In recent years, extensive molecular profiling has afforded an increasing understanding of the genomic landscape of malignant gliomas. Large-scale sequencing analyses have provided new data on molecular changes in gene expression, copy number, somatic mutations, and epigenetic signatures in these tumours (TCGA,

2008; Brennan *et al*, 2013). These studies validate the most commonly mutated gene signatures in human GBM that most frequently include a subset of the following oncogenes and tumour suppressors: *TP53*, *PTEN*, *NF1*, *EGFR*, *ERBB2*, *RB1*, *PIK3R1*, and *PIK3CA* (TCGA, 2008). This diverse group of cancer-associated genes represents a core set of pathways that are commonly deregulated in GBM, including growth factor signalling (receptor tyrosine kinase (RTK)/phosphatidylinositol 3-kinase (PI3K)/Ras), p53, and Rb signalling pathways. These lead to aberrant signalling in proliferation, cell cycle regulation, senescence, and apoptosis, underscoring the importance of such pathways in the development of malignant gliomas (TCGA, 2008; Brennan *et al*, 2013). These GBM-associated genes are altered in a variety of ways, including gene amplification and deletion or mutation, and are present in more than three-quarters of patients. Isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) mutations have also emerged as important drivers of a more rare subset of gliomas, particularly low-grade gliomas and secondary GBM (Verhaak *et al*, 2010).

Based on signature genome-wide gene expression changes, coupled with somatic mutations and copy number changes, GBM is commonly classified into four subtypes: proneural, mesenchymal, classical, and neural subtypes (Verhaak *et al*, 2010; Brennan *et al*, 2013). In addition to exhibiting characteristic mutational and gene expression profiles, mutations in *NF1* are prevalent in the mesenchymal subtype, *EGFR* in the classical subtype, and *PGDRA*

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and *IDH1* in the proneural subtype. These subtypes are also found to bear resemblance with gene expression profiles of normal brain cells, with proneural tumours enriched for the oligodendrocyte development signature. Mesenchymal tumours are enriched for the cultured astroglia signature, although it remains unclear whether cultured astroglia represent any specific stem or progenitor cells *in vivo*. More recent analyses have been expanded to include other types of gliomas, such as grade II and III astrocytomas, oligodendrogliomas, or oligoastrocytomas. These glioma subgroups are based on newer criteria, such as the presence of *IDH1* and *TERT* promoter mutations and chromosome 1p/19q co-deletion, as well as telomere alterations and DNA methylation (Eckel-Passow *et al*, 2015; Ceccarelli *et al*, 2016). However, the reproducibility, clinical relevance, and functional basis of these subclasses remain to be established.

On the other hand, Glioblastoma multiforme is a highly heterogeneous tumour consisting of mutant cells with varying morphologies, levels of aneuploidy, and expression of different cell markers. The cellular and genetic heterogeneity is well illustrated using recently developed techniques involving single-cell sequencing analysis that has demonstrated that single GBM cells, even within tumour cells from the same patient, are not genetic phenocopies. Single cells are found to exhibit variable expression of transcriptional programs in cellular processes such as hypoxia, cell cycle, and immune signalling, underscoring the inherent heterogeneity and complexity of these tumours (Patel *et al*, 2014). Moreover, genomic analysis reveals divergence of presumed mutational and epigenetic drivers between therapy-naïve primary and recurrent GBM, as well as between low- and high-grade recurrent lesions, showing the versatility and evolutionary plasticity of these tumour cells (Johnson *et al*, 2014; Kim *et al*, 2015). These advances have shed light on the evolving genome of GBM, and speak to dynamic changes in the genomic make-up of tumour cells during malignant progression and in response to different therapies.

GENETICALLY ENGINEERED MOUSE MODELS

A variety of animal models have been developed that incorporate signature mutations found in human patients. Genetically engineered mouse models (GEMMs) are powerful tools in investigating the natural history of glioma development and also as preclinical models. The general approach involves loss of function of driver tumour suppressor genes, such as *Cdkn2a* (*Ink4A/Arf*), *Nf1*, *Trp53*, *Pten*, and *Rb1* and/or the expression of driver oncogenes, such as mutant epidermal growth factor receptor (e.g., *EGFR VIII*) and *Ras* (*K-Ras*, *H-Ras*), *Akt*, and platelet-derived growth factor (PDGF) ligands (Kegelman *et al*, 2014). Such mutations are introduced as conventional or conditional knockouts and transgenic models. Modifications include the RCAS (replication-competent ASLV long terminal repeat with splice acceptor) system that employs Rous sarcoma virus-based vectors that are then injected into mice expressing the TVA receptor under the control of cell type-specific promoters, and MADM (mosaic analysis with double markers), a cre-based genetic mosaic model (Liu *et al*, 2011; Kegelman *et al*, 2014). Adenoviral or lentiviral vectors expressing cre or any gene of interest, or cultured cells first transduced with virus, can also be directly injected into mice. The use of conditional knockouts target initiating mutations into discrete cell populations using specific promoters that limit the extent of cre-mediated recombination in restricted cell types. Inducible cre systems further provide temporal and spatial control of gene targeting that prevents the developmental phenotype and early lethality complications associated with conventional knockouts. Such methods have produced a plethora of mouse models that develop gliomas with a range of histologic types, penetrance,

and latencies, and which recapitulate in varying degrees the pathological hallmarks of human gliomas (Janbazian *et al*, 2014). Variations caused by differences in genetic mutations, cell of origin, and timing of initiation make it imperative that physiologic relevance and resemblance to human disease be established whenever animal models are used to study glioma pathophysiology.

CELLS OF ORIGIN OF GBM

The cell of origin refers to the normal cell that acquires the initial cancer-promoting genetic hit(s). The identity of tumour-initiating cells in different organ cancers is an area of intense study. To date, many examples exist demonstrating the potential of immature, proliferating cells in animal models of leukaemia, colon, breast, lung, and other cancers (Visvader, 2011).

In GBM, addressing this question requires an understanding of the hierarchy of cell lineages in the CNS. In the past several decades, advances in developmental neurobiology have identified various cellular compartments that allow targeting of discrete cell populations. In the adult mammalian brain, the two main neurogenic regions are in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampal formation. These adult neural stem cells (NSCs) are quiescent glial fibrillary acidic protein-positive (Gfap+) cells that exhibit unlimited self-renewal and multipotentiality. These give rise to proliferative progenitor cells that have more limited self-renewal and are fated to differentiate into different cell types. Whereas Gfap+ cells in the SVZ and SGZ identify quiescent neural stem cells, Nestin is widely expressed in both stem and progenitor cells; other markers that have been reported include Glast, Sox2, Tlx, Gli1, EGFR, PlexinB2, and Dlx1 (Doetsch *et al*, 1999; Canoll and Goldman, 2008; Mich *et al*, 2014). The bHLH transcription factor Ascl1 has recently been shown to mark a population of adult bipotential progenitor cells that can give rise to both the neuronal and oligodendrocyte lineage, as well as give rise to neurospheres *in vitro* (Kim *et al*, 2007; Mich *et al*, 2014). Neuronal progenitor cells in the SVZ give rise to new neurons that migrate through the rostral migratory stream and into the olfactory bulb, whereas those in the SGZ locally migrate into the granular cell layer. On the other hand, unipotent progenitors such as oligodendrocyte progenitor cells (OPCs) are broadly arrayed in the adult brain, where they comprise ~5% of CNS cells. These OPCs express lineage-specific proteins including NG2, PDGFR α , and Olig1, and typically give rise to mature oligodendrocytes in both the developing and adult brain (Nishiyama *et al*, 2009).

The identification of cell lineage markers has provided necessary tools for the interrogation of the cell of origin. Both stereotactic and genetic approaches have been used, and inducible cre lines have allowed cell type-specific targeting of initiating mutations at adult ages (Figure 1). Earlier models target mutations using cell type-specific promoters but in embryonic or early postnatal cells, which is not ideal as GBM is an adult disease, with a median age of 64 years at the time of diagnosis (Stupp *et al*, 2005; Wen and Kesari, 2008). Direct injection of cre recombinase-expressing adenovirus into the SVZ of adult mutant mice containing conditional tumour suppressor alleles of *Nf1*, *Trp53*, and *Pten* has been shown to induce GBM formation (Alcantara Llaguno *et al*, 2009; Jacques *et al*, 2010). Mutant mice with the *Nestin-creER^{T2}* transgene, which contains the *Nestin* promoter/enhancer with the intron 2 regulatory element that limits its expression in NSCs but not other cell types such as endothelial cells, when bred to contain the same tumour suppressor mutations and induced at adult ages (4–8 weeks of age), develop GBM (Alcantara Llaguno *et al*, 2009). On the other hand, the use of Gfap as a marker for

Figure 1. Lineage hierarchy of tumour-initiating cells in GBM. Neural stem cells (NSC) are activated to give rise to progenitor cells that exhibit varying levels of potentiality: multipotent progenitors (MPPs) that give rise to all CNS cell types; bipotent progenitors (BPP), such as *Ascl1* progenitors, that identify both adult oligodendrocyte progenitor cells (OPCs) and neural progenitor cells (NPCs); and unipotent progenitors (UPP) that differentiate into oligodendrocytes, neurons, or astrocytes. Dashed lines indicate that the hierarchical relationship between different progenitors is still unclear. Experiments using different inducible cre driver lines in conjunction with known driver mutations employed to target different adult brain cell types are shown. Models using *Nestin-creER^{T2}*, which is expressed in neural stem cells as well as multipotent progenitors, can give rise to GBM. The *Gfap-creERTM*, which is expressed in SVZ neural stem cells as well as a subset of mature astrocytes, develop tumours near the neurogenic niches. The *Ascl1-creERTM* model, which targets NPCs and OPCs, gives rise to two GBM subtypes. One of these subtypes appears similar to GBM that develop using *NG2-creERTM*. Such animal models in which cre recombination is temporally controlled in the adult stem and progenitor populations have been shown to induce GBM formation, although with different molecular subtypes.

quiescent NSCs is complicated by its expression in some mature astrocytes, particularly in the dentate gyrus and striatum. *Gfap-creERTM* transgene-mediated loss of *Pten*, *Trp53*, and/or *Rb* induces high-grade glioma formation, where a high proportion of tumours can be found in close association with proliferative niches in the SVZ-RMS and SGZ. This would be consistent with a scenario wherein the tumours originate from the NSC-targeted cells even though cre expression in stem cells was significantly lower than in non-neurogenic regions (Chow *et al*, 2011). The contribution of lineage-restricted progenitors is determined using the *Ascl1-creERTM* transgene that targets bipotent progenitors, whereby expression is observed in both adult neural and oligodendrocyte progenitors. *Ascl1-creERTM* mice carrying *Nf1*, *Trp53*, and/or *Pten* mutations develop GBM, as do *NG2-creERTM* mice, where adult oligodendrocyte progenitor cells alone are targeted (Galvao *et al*, 2014; Alcantara Llaguno *et al*, 2015).

On the other hand, the role of post-mitotic, fully differentiated CNS cells in GBM formation is still controversial. Whether astrocytes are tumour-initiating cells is debatable because markers for astrocytes, such as *Gfap* and *Glast*, are also expressed in neural stem cells (Chow *et al*, 2011), although reports using cultured proliferative, early postnatal astrocytes (or possibly astrocyte progenitors) may not replicate the characteristics of adult astrocytes *in vivo* (Bachoo *et al*, 2002). There is evidence, however, that direct

targeting of *Gfap* + parenchymal astrocytes in adult mice does not easily form glioma (Alcantara Llaguno *et al*, 2009; Jacques *et al*, 2010). Meanwhile, differentiated neurons have been reported to transform into tumours in the experimental setting (Friedmann-Morvinski *et al*, 2012); however, it is not clear whether the targeted cells are exclusively post-mitotic neurons. Confirmation of these findings using temporally controlled and more specific lineage-restricted drivers as well as interrogation of the molecular profiles of these probable differentiated cell type-initiated tumours would be important in addressing this question.

Whether specific cells of origin are susceptible to certain mutations is not settled but most data from animal models suggest that stem cells and restricted progenitors are widely susceptible to a variety of mutations (Visvader, 2011). It is possible that specific cell types may exhibit preferential vulnerability to certain mutations, and that certain cell of origin-mutation combinations lead to specific tumour types or subtypes (Jacques *et al*, 2010).

CELL OF ORIGIN AS A DRIVER OF INTERTUMOURAL DIVERSITY

The advent of advanced genomics has greatly increased our understanding of the molecular underpinnings of glioblastoma.

Although GBMs from human patients contain different types of driver mutations that may represent different subtypes, the contribution of the cell of origin is not known. It is interesting to note that the TCGA molecular subtypes are enriched for lineage markers characteristic of distinct CNS cell types, such as proneural tumours exhibiting enrichment for the oligodendrocytic progenitor signature, suggesting that the molecular and/or epigenetic signature of the tumour-initiating cell is maintained during tumourigenesis (Verhaak *et al*, 2010; Levine *et al*, 2015).

What are the consequences of driving the same initiating mutations in different cells of origin? In mouse models, tumour suppressor deletions in different stem and progenitor lineages all lead to histologic GBM. However, these same GBMs are found to be molecularly distinct and separable based on the cell of origin. Hence, GBM driven by *Nestin-creER*^{T2}, *Ascl1-creER*TM, and *NG2-creER*TM exhibit lineage-specific signatures in the context of *Nf1*, *Trp53*, and *Pten* loss (Alcantara Llaguno *et al*, 2015). Moreover, direct comparison of GBM from the *Ascl1-creER*TM model, where both adult neural and oligodendrocyte progenitors are targeted, demonstrate two subtypes of GBM, showing different predilection sites, marker expression, tumour boundaries, and gene expression profiles. Analysis of the mouse model subtype tumours also show similarities to the gene expression signatures of their normal progenitor cell counterparts. These observations suggest that the cell of origin can

be an important determinant of tumour phenotype and genotype in GBM, and thus plays an important role in its malignant behaviour.

These studies are reminiscent of findings reported for leukaemia and medulloblastoma. For example, leukaemic stem cells (LSCs) derived from haematopoietic stem cells transduced with mixed lineage leukaemia (MLL) rearrangements express high levels of *Evi-1* that has been correlated with adverse clinical outcome, are hypermethylated, and are more resistant to chemotherapeutic agents, whereas LSCs from granulocyte-macrophage progenitors exhibit low *Evi-1* expression and global methylation, and are less resistant to the same pharmacologic agents (Krivtsov *et al*, 2013). In medulloblastoma, molecular subtypes reflect distinct developmental origins. Sonic hedgehog (Shh) subtype medulloblastoma arising through aberrant Shh signalling (most often through inactivating mutations in *Patched*) in committed granule neuron precursor cells form within cerebellar hemispheres, whereas Wnt subtype medulloblastoma arises from progenitor cells of the dorsal brainstem in response to activating mutations in the Wnt effector pathway, and tend to infiltrate the brainstem (Gibson *et al*, 2010).

These studies from GBM and other model systems are consistent with the cell of origin as a source of intertumoural diversity, whereby mutations in different cell types form different molecular subtypes (Figure 2). However, these subtypes can also be influenced by genetic mutations, epigenetic changes, and the microenvironment. These

Figure 2. Lineage-based functional subtyping of GBM. (Upper left panel) Cell of origin model of intertumoural diversity. Mouse models developed from targeting of tumour-initiating mutations into different cells of origin give rise to different GBM subtypes (represented by different colours). The GBM subtypes from different mouse models are molecularly separable, as hypothetically shown via locally linear embedding (LLE) dimension reduction analysis (upper right panel), and gene expression profiling (lower right panel). Studies on the molecular subtypes of GBMs that express distinct biomarkers, activate or turn off specific signalling pathways, and exhibit differential response to different treatment modalities may pave the way for improved diagnostics and therapeutics.

influences are not mutually exclusive, however, and more likely than not, intertumoural heterogeneity is a function of the combined effects of these several factors. Moreover, the relationship between cell lineage and biological phenotype suggests that mechanisms operative in normal cells may contribute to tumourigenesis. Signalling pathways such as Wnt and Notch, which are developmentally regulated in specific cell types, and especially stem and progenitor cells, may play important roles in tumourigenesis (Visvader, 2011).

On the other hand, although it has not been experimentally proven for GBM, the lineage of the tumour-initiating cell may also directly influence the phenotype of the ensuing cancer stem cell that drives malignant behaviour and tumour progression that may also play a role in intratumoural heterogeneity.

CLINICAL CONSEQUENCES

Molecular subtyping of tumours, whether based on the lineage of the cell of origin, signature mutations, or a combination, provide important tools that can result in more refined diagnosis of brain tumours. Currently, the definitive clinical diagnosis for GBM is based on histopathologic examination by a neuropathologist. However, the subjective nature of this classic method makes it susceptible to bias and human error, in addition to relying on correlative and incomplete information. The incremental use of biomarkers and precision genomics will greatly supplement traditional histopathology in identifying gliomas. Molecular diagnostics will greatly advance the field, and facilitate the sharing of clinical and genomic information between academic and health centres. On the other hand, classification of these tumours into molecular subtypes using epigenetic, genetic, and gene expression profiling will further improve prognostication and lead to better stratification of patients for guided therapy (Figure 2). For example, the classical and mesenchymal subtypes have reportedly not only been associated with slightly better response to aggressive therapies, but also portend poorer prognosis compared with other subtypes (Verhaak *et al*, 2010; Ceccarelli *et al*, 2016). The CpG island methylator phenotype (G-CIMP), on the other hand, has been shown to represent a small subgroup of proneural gliomas that are associated with lower-grade lesions, *IDH1* mutations, and better prognosis (Noussimehr *et al*, 2010). Furthermore, although single-cell sequencing analyses have demonstrated the expected intra-tumoural genetic heterogeneity of GBM cells at pre- and post-treatment conditions, studies in leukaemias suggest that evolving tumour cells retain the genetic and epigenetic signatures of their cells of origin (Levine *et al*, 2015). The maintenance of these lineage identifiers in tumour cells can potentially be used to identify specific tumour subtypes and track the evolution of individual cells. With more sophisticated technology, this may allow early detection of incipient tumours, as well as pave the way for preventative therapies for patients at high risk of developing brain tumours, such as neurofibromatosis type 1 (NF1) patients.

On the other hand, the identity of the cell of origin informing GBM subtype suggests that there may be subtype-specific pathways that may be open to therapeutic exploitation in different subsets of patients. Improved stratification will prevent unnecessary delay in delivery of appropriate treatments to which patients will most likely respond. Molecular diagnosis and subtype-specific treatments in CNS tumours is well under way in medulloblastomas (Taylor *et al*, 2012) and the glioblastoma field should not be far behind.

Finally, the use of experimental systems that address the cell of origin has led to the development of needed preclinical models that can be used to test candidate therapies. Physiologically relevant animal models that mirror human GBM at the molecular and cellular level are being used in both high-throughput screens and

specific inhibitor studies in conjunction with patient-derived xenografts in drug development pipelines. Animal models provide large cohorts of *in vitro* and *in vivo* testing material that can be used to narrow down the most promising agents for subsequent testing in more limited patient samples. These molecularly defined subtype-specific mouse models can also provide unique insight into therapeutic response and resistance. Although mouse tumours do not always exhibit 100% equivalence with human GBM, these tools can be harnessed not only for drug validation but also inform target pathways that otherwise may not be uncovered by using only human tumour cells. The hope is that a deeper and more integrated understanding of the developmental origins, molecular underpinnings, and clinical behaviour of gliomas will pave the way for better therapeutic approaches that will benefit these patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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