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Glioblastoma Cancer Stem Cells: Biomarker and Therapeutic Advances

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Introduction

Glioblastoma multiforme (GBM) is a grade IV astrocytoma that is the most malignant primary brain tumor. In the United States approximately 12,000 new GBM patients are diagnosed annually (1), accounting for more than 50% of all detected malignant primary brain cancers and 20% of all primary intracranial tumors (2, 3). Astrocytomas are graded based on nuclear atypia, mitosis, vascular endothelial proliferation and/or necrosis, with three of the four characteristics required for GBM diagnosis (4). GBMs display a large degree of intra- and inter-tumor heterogeneity and are highly infiltrative into surrounding normal brain tissue (5). The current standard of care for GBMs established by Stupp et al. (2005) is maximal safe surgical resection followed by temozolomide chemotherapy and radiation (6). Unfortunately even after complete surgical removal and aggressive treatment, GBM inevitably recurs. The current standard therapeutic regimen has increased median survival for GBM from 12.1 months to 14.6 months with a median two-year survival of only 26% (6). These statistics suggest that novel therapeutic approaches are necessary to combat GBMs. While there are many therapeutic avenues that are being explored to advance the

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understanding and treatment of GBMs, in this review we will focus on methods to target GBM cancer stem cells (GSCs).

GBM Genome Analysis

Since cancer is a disease arising from mutations, much research has focused on identifying and studying neoplastic genetic events for possible therapeutic targeting. The Cancer Genome Atlas (TCGA) is an ongoing project to identify many mutations in a large cohort of GBMs (7). An interim report by the Cancer Genome Atlas Project showed successful high throughput genetic and epigenetic analyses of 206 GBMs and somatic mutational analysis of more than six hundred selected genes, and demonstrated several major classes of GBM-associated cellular tumorigenic mechanisms (7). Parson and colleagues have also reported sequencing and analysis of 20,661 genes and 689,071,123 nucleotides in 22 GBMs, resulting in detection of 2,325 somatic mutations (8). Both analyses provide a wealth of information on receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) and ERBB2, and their role in GBM proliferation and survival. Other major GBM-associated mutations were alterations in tumor suppressor genes, such as p53 (TP53) and retinoblastoma protein (RB1) and their related pathways. These systematic large-scale analyses revealed that 75% of GBMs have mutations in common, well-studied signaling pathways. However, these analysis techniques likely under-analyze sub-populations of tumor cells that are clinically relevant (such as therapeutically resistant cells or cancer stem cells), because only selected gross tumor specimens are examined. Mutational analyses highlight some of the key challenges that exist in understanding cancer therapy; however, they do not provide better comprehension of tumor initiating cells or cancer stem cell biology.

Cancer Stem Cell Hypothesis

Cancer stem cells (CSCs) or tumor initiating cells are a subset of tumor cells that possess the stem cell properties of self-renewal and multi-lineage differentiation, and functionally are highly efficient at initiating tumor xenografts *in vivo*. The CSC hypothesis postulates that the CSCs sit at the apex of a cancer cellular hierarchy, and the CSCs lose self-renewal and tumorigenic potential as they “differentiate” to molecularly diverse progeny of the tumor bulk. Therefore, eradication of CSCs is necessary and sufficient to halt tumor expansion or prevent re-growth after therapy (9). CSCs are thought to play a critical role in tumor initiation, recurrence, and metastasis. The cancer stem cell hypothesis was initially established for leukemia and lymphoma in the 1990's by J. Dick and colleagues (10). Ignatova et al. were the first to isolate GSCs via sphere culture in stem cell medium (11), and Singh et al. (2004) successfully identified brain tumor initiating cells directly from human surgical specimens by sorting for cells expressing the cell membrane marker AC/CD133 (12). GSC-derived tumor xenografts in immunodeficient mice were initiated with as few as 100-1000 AC/CD133⁺ GSCs, and resembled the parental human GBM from which they were derived; this xenograft initiation efficiency is about 1000-fold higher than implantation of traditional glioblastoma cell lines (13, 14). Lee et al. (2006) demonstrated that GSC lines are more similar to primary patient tumors than cultured cell lines in gene expression profiles (15). Glioblastoma cancer stem cells have been shown to be resistant to

conventional chemo- and radiotherapies (16, 17). Current standard treatments are thought to target partially differentiated tumor cells, but fail to kill glioblastoma cancer stem (or progenitor) cells leading to rapid cancer recurrence. In order to improve GBM treatment outcomes, all cells of the cancer must be targeted, including GSCs (18-21). Thus, a better understanding of GSC biology is critical in order to improve patient outcomes.

Controversy regarding CSC hypothesis

Many controversies exist regarding the cancer stem cell hypothesis. Identifying specific surface markers is necessary to isolate GSCs and subsequently characterize them for future GSC-targeted therapies. As mentioned above, AC/CD133 was one of the earliest markers used to isolate GSCs. More recently, stem cell/progenitor markers such as stage-specific embryonic antigen 1 (SSEA-1/CD15) (19, 22, 23), were also successfully used to enrich for GSCs. While these markers are able to enrich for GSCs, marker negative tumor cells often retain tumor initiation potential and these markers are only expressed in a subset of brain tumors.

Multiple GSC populations have been proposed to also exist within a given cancer, which distinctly drive tumor growth and metastasis (24). Glioblastoma cancer stem cells are most likely a cellular population with the ability to adapt through clonal evolution or epigenetic plasticity (25). Successful cancer treatments will likely have to eradicate all GSC sub-populations in addition to other tumor cells.

Furthermore, tumor heterogeneity makes it difficult to determine the origin of GSCs and still remains a topic of discussion. There is evidence that suggests GSCs arise from abnormal stem cells that have obtained oncogenic characteristics (26, 27). Expression of oncogenes driven by stem cell-specific promoters, such as nestin in brain tumors, results in recapitulated human tumors in mice (28, 29); however, progenitor cells that are more differentiated have also been shown to recapitulate other cancers in a similar manner (30, 31).

While controversy exists regarding the cancer stem cell hypothesis, the ability of GSCs to initiate a tumor in a highly efficient manner supports a role for GSCs in cancer initiation, progression and recurrence. Moreover, GSC resistance to radiation and chemotherapy (16, 17, 32) suggests that new therapeutic strategies need to focus on targeting GSC sub-populations.

GSC biology and GBM resistances

Although the median survival for GBM is 14.6 months, there are patient cohorts who survive much longer after current therapies documented by researchers (33, 34). This suggests there are differences between different patients' tumors in therapeutic responses. The current standard of care therapy includes temozolomide, an imidazotetrazine derivative acting as an alkylating agent. The principal mechanism responsible for temozolomide cytotoxicity against GBM is the creation of O6-methylguanine (O6MeG) lesions, resulting in DNA fragmentation, disrupting DNA replication, causing tumor suppression and apoptotic cell death (35). Hegi et al. (2005) reported a specific patient subgroup of long-term

GBM survivors, with a mean survival of 21.7 months and two-year survival of 46%. The extended survival of this subgroup was based on absent tumor expression of methylguanine-DNA methyltransferase (MGMT) (33). MGMT repairs DNA by removing temozolomide-added methyl groups and prevents tumor cell death (35). MGMT expression is repressed by methylation of its promoter. Thus, the GBM subgroup harboring MGMT promoter methylation (absent or reduced MGMT protein) showed improved survival after temozolomide chemotherapy, because GBMs with methylated MGMT promoters are not able to repair the temozolomide created DNA damage. This results in more tumor suppression, apoptotic tumor cell death and improve patient survival. The differences seen in patient survival due to MGMT status underscore the clinical significance of understanding tumor molecular biology. Sciuscio et al. (2011) later showed enriched MGMT promoter methylation in GSCs, suggesting differential MGMT expression within GBM cellular sub-populations, and that even a small percentage of MGMT methylation found in a GBM sample may predict better survival after temozolomide therapy (36).

Epidermal growth factor receptor family members are also linked to GBMs. The epidermal growth factor receptor (EGFR) is mutated in many cancers and is amplified or mutated in ~50% of GBMs (7). Twenty percent of tumors express constitutively active mutant EGFRvIII protein (37). Activation of EGFR leads to downstream activation of AKT and other signaling pathways, which promotes cell proliferation, survival and increased infiltration (38, 39). Clinical trials with single target agents against EGFR, such as erlotinib and gefitinib, have been conducted due to studies showing that many cancers overexpress EGFR (40, 41). While these EGFR inhibitors were successful in clinical trials to treat breast cancer and lung cancer, in GBM trials, they failed to improve patient survival (42). Failure of anti-EGFR therapies to increase survival in GBM despite high expression and dependence on EGFR suggest intrinsic or rapidly acquired resistance, possibly by GSCs. Our group has demonstrated that while normal neural stem cells do not survive without growth factors, GSCs still propagate and maintain tumorigenicity. We also found that EGFR receptor family members ERBB2 and ERBB3 were upregulated when EGFR signaling is decreased by exogenous EGF ligand removal or EGFR-targeted therapy (43). Compensatory activation of ERBB2 and ERBB3 preserves downstream signaling and AKT activation. This suggests that the multiple members of the epidermal growth factor family must be targeted for effective cancer stem cell therapy through drugs such as lapatinib, which targets EGFR and ERBB2. Lapatinib was further demonstrated to have the ability to reduce AKT expression and ablate cancer stem cell lines (43). Still, rare lapatinib-resistant cells were selected and isolated after extended time of therapy, further emphasizing a need for additional therapeutic approaches. Initial clinical trials testing lapatinib as a single agent or adjuvant with temozolomide/radiation were conducted with disappointing results showing no significant efficacy (44, 45). However, logistical issues may have played a role: including failure to achieve sufficient intratumoral concentrations due to the blood-brain barrier (44) or the need for proper patient selection since only ~50% of GBMs harbor EGFR amplification/mutation (7). Additionally, activation or selection of alterations in related receptor tyrosine kinases (RTKs) such as c-Met or platelet-derived growth factor receptor (PDGFR) may overcome EGFR inhibition to activate similar downstream signaling pathways (46). GBM often exhibits a high level of mosaicism in RTK expression,

amplification, and mutation (47), and it is likely that multiple RTKs will need to be targeted for clinical efficacy (48). Therefore, additional research is needed into GBM and GSC intrinsic and acquired resistance to chemotherapies, including molecular-targeted agents.

Heterogeneity of GBM cancer stem cells

Glioblastomas demonstrate a high level of intra- and inter-tumor heterogeneity, and it is beginning to be appreciated that the GSCs that drive GBM growth are also very heterogeneous. Glioblastoma stem cells in sphere culture show a mixture of cellular morphologies (49, 50) resembling normal neural lineages, and therefore potentially could be classified based on neural developmental markers (34). Our group has classified GSC lines isolated from different patients into three classes that exhibited differential expression of oligodendrocyte progenitor cell (OPC), astrocyte progenitor cell (APC), and neural progenitor cell (NPC) protein markers. Each of these classes is associated with differences in xenograft invasiveness and correlated with patient survival. GSCs that express OPC and NPC markers form focal lesions, NPC-positive GSC lines form minimally invasive lesions, and APC-positive lines form highly invasive lesions. Additionally, GSCs that form focal xenograft lesions were also 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) positive, while the GSCs that form invasive tumors were CNP negative. CNP expression was also correlated with better survival in both mice bearing GSC-derived xenografts and in humans based on analysis of a clinically annotated GBM tissue microarray (34). The aforementioned study identified a biomarker (CNP) from developmental biology that could potentially be used as a clinical prognosis marker for patient survival.

Phage display antibody libraries

While clinical trials using antibody-based targeting of GBM via adoptive T cells loaded with chimeric antigen receptors (CAR) (51) and bispecific T cell engaging antibodies (52) to target GBMs with EGFRvIII mutations, no global specific cancer cell targets exist. This is because current markers also distinguish normal neural stem cells and/or are only expressed in some brain tumors due to inter-patient variability. Since GSCs are the resistant subpopulation of cells to standard treatment, discovery of novel and specific membrane proteins for GSCs will create new avenues for antibody-based isolation, diagnosis, drug delivery, and treatment monitoring for GBM. Both gene and protein expression profiling can identify novel tumor specific antigens; however, without antibodies engineered to their cognate epitopes, therapeutic efficacy cannot be realized. Phage display recombinant antibody libraries along with a biopanning approach have rapidly become the tool of choice in many molecular biology laboratories. Phage display antibody technology was developed in 1985 (53). Even though it is a fairly new technology, nearly 30% of all human antibody therapies have come from phage antibody libraries (35).

Phage display technology presents antibodies with filamentous M13 or T7 bacteriophage using its coat protein III (54). Libraries are created by cloning single-chain variable fragments (scFv) from hundreds of human B cell donors into phage or phagemid vectors. These scFvs are displayed on the surface of the bacteriophage. scFvs are fusion proteins of the variable regions, heavy (V_H) and light chains (V_L), from immunoglobulins connected by

a glycine rich linker (55). *Escherichia coli* are used to create and amplify the phage antibodies, typically expressed in their periplasmic space (56, 57). Non-immune or naïve universal human antibodies are most desirable because they may be specific to virtually any antigen or epitope (58, 59). Naïve libraries are best against membrane proteins since they are a richer source of antibodies compared to immune libraries that have removed clones reactive to self-cell surface antigens during host maturation (60).

To select for unique antibody clones from phage display libraries against complex cell surfaces, a ‘biopanning’ and screening approach can be used. During biopanning, phage antibodies are incubated on the cell type or antigen of interest adhered to a plate. Unbound phage are washed away and discarded, while bound phage are recovered and infected into *E. coli* for amplification of desired clones. This is repeated for several rounds against the target antigen until an enriched population of antibodies is obtained. The result is a polyclonal pool, which is then screened and sequenced for individual clones. Unique clones can then be engineered into immunoglobulin formats to test for therapeutic efficacy (61, 62).

Two recent studies have explored phage display technology against GSCs. Zhu et al. (63), used phage display flow cytometric sorting of CD133⁺ GBM sphere cells. They identified an internalizing scFv clone that had therapeutic efficacy when cloned into a full-length human IgG1 format and evaluated in a sphere forming assay. The cognate antigen was not identified. In another study using a 7-mer phage peptide library, Beck et al. (64) identified a peptide that could be internalized and interact with nestin in GSCs. When conjugated with a fluorescent quantum dot, this peptide localized specifically to orthotopic GSC xenograft tumors. Both of these studies demonstrate the promise of therapeutic and diagnostic antibody-based modalities against GSCs.

There are several disadvantages of phage display libraries for proteomics. First, phage particles have a lot of non-specific binding to mammalian cells (65). Non-specific binding allows for amplification of false positive antibodies. Second, less than twenty unique and relevant antibodies are found after biopanning with phage (66, 67). This may be due, in part, to phage non-specificity. Finally, phage technology requires additional molecular cloning steps to obtain an antibody suitable for immunoprecipitation and antigen identification by mass spectrometry, which is not necessary with yeast display antibody platforms (67), as described below.

Yeast display antibody libraries

Beyond phage display antibody libraries, researchers have moved into other model organisms and platforms including bacterial display, yeast display, mammalian display, ribosomal display, puromycin display, and antibody arrays (68). The antibody format and their construction are also just as diverse, from naïve to immune (69, 70), synthetic to semi-synthetic (71, 72), and fragment antigen-binding (Fab) versions to single domain variable fragment (Fv) formats (60, 73, 74).

Similar to phage display, the yeast libraries can be engineered to express naïve (non-immune) scFvs. A diversity of 10^7 clones is hypothesized to be sufficient to recognize greater than 99% of epitopes (75), with affinities ranging from 1 to 1,000 nanomolar (76).

This type of platform has been well developed for flow cytometric and biopanning scFv screening (77), scFv maturation to femtomolar affinity (78), directed evolution mutagenesis of T cell receptors (79), and epitope specific selection (80).

Many of the limitations observed in phage display are resolved by yeast surface display technology for the identification of unique membrane proteins (81, 82). First, yeast are unicellular eukaryotic fungi containing a cell wall composed of hydrophilic polysaccharides (83, 84), and are flocculin deficient (85), which reduces non-specific binding on mammalian cell surfaces. Finally, as suggested before, yeast cells can be utilized as the immunoprecipitation agent for antigen pull-down and identification (67), which expedites the scFv discovery workflow.

Candidate GSC-specific binding scFv antibodies are isolated by screening roughly 500,000,000 unique human scFv antibodies expressed by yeast against glioblastoma cancer stem cells. Yeast binders are recovered and amplified overnight before subsequent rounds of selection. This is done over multiple steps to amplify and confirm binding specificity (Figure 1). Negative subtraction screening steps enrich for GSC-specific scFv antibodies by removing candidates that bind control neural stem cells, tumor cells and differentiated astrocytes. An initial quality control test is that only yeast induced for scFv expression bind the GSCs, whereas yeast that are not induced for scFv expression do not bind (86). Candidate scFv clones are isolated and sequenced. Cognate antigens may be identified via mass spectrometry in order to identify possible new or novel GSC antigens for further study and possible therapeutic targeting. Even without identifying the bound antigen, GSC-specific antibodies could be useful for rapid isolation and identification of GSC from surgical specimens, and may also be used for therapeutic delivery to cancer stem cells.

Altogether, the goal of biopanning with an scFv-expressing yeast antibody library against GSCs is to find an antibody toward a globally expressed GSC surface antigen, with no or minimal activity against normal neural cells. As a research tool, a GSC-specific antibody could be used for efficient and highly reproducible purification of GSCs toward uncovering key genes and proteins for their specific survival, growth, and treatment resistance. Currently used markers such as AC/CD133 (12, 16) and SSEA-1/CD15 (87) are expressed by normal stem cells, not found in every GBM, and do not encompass every GSC (i.e. AC/CD133⁺ GBM cells initiate tumors). Clinically, antibodies specific to GSC surface antigens could be used for rapid sorting of patient-specific GSCs and subsequent treatment sensitivity analysis (88). For therapy, an antibody-drug conjugate (ADC) to specifically kill GSCs could be constructed and used along with common treatments, and has demonstrated high efficacy in other cancers (89, 90). For successful therapeutic use of an ADC for GBM, the formidable obstacle of the blood-brain barrier (BBB) must be overcome; nevertheless, GBM generates a leaky and drug-penetrable environment. Antibody and biologic design is rapidly developing to incorporate BBB-penetrating regions or peptides (91), and some immunotherapies have demonstrated success in getting antibody-based immunotherapies to the central nervous system (92). However accomplished, destruction of the stem cell compartment has now been shown a viable and efficacious approach in pre-clinical models of many cancers (21, 93), including glioblastoma (94).

Discussion

Although recent advances have improved GBM patient survival, the median survival remains less than two years after diagnosis. The cancer stem cell hypothesis states that although a cancer may be cellularly diverse, there are a sub-population of cancer stem cells solely responsible for initiating a cancer and recapitulating it after treatment (9). Although controversial, mounting evidence supports the cancer stem cells hypothesis in glioblastoma and other cancer initiation, propagation, therapy resistance, and recurrence (10, 12, 95, 96), and therefore eradication of these cancer stem cells is critical for effective treatment (21, 93, 94). Novel therapeutic approaches that address glioblastoma cancer stem cells and GSC niches, in addition to the rest of the tumor, are necessary in order to target the entire cancer and prevent recurrence (Figure 2). Enriched, although not pure, glioblastoma stem cell cultures can be obtained via sphere culture in stem cell medium (11, 15, 34) or selected via cell surface markers such as AC/CD133 (12) or SSEA-1/CD15 (87). Although no cell culture is a perfect imitation of the in vivo system, GSCs propagated by the above methods better maintain patient genetic abnormalities and GBM phenotype when orthotopically reimplanted compared to GBM lines established via traditional cell culture methods (15). Such GSCs could potentially be used to uncover novel GBM and/or GSC cellular and molecular biology, for example potentially unique GSC intrinsic and acquired resistances to cytotoxic or molecular-targeted therapies (33, 97). Enriched GSC cultures could also be more amenable for large-scale drug discovery assays (98) or development of personalized patient treatment regimens (88), as compared to in vivo pre-clinical or clinical models. Novel methodologies such as yeast antibody display biopanning could identify new GBM cell surface antigens toward enabling pure GSC isolation and biological studies. Such GSC-targeted antibodies could also be used in developing drug-conjugates to kill the GSCs responsible for GBM recurrence while better sparing normal tissue compared to traditional therapies. However, there are still many major issues that need resolving prior to clinically efficacious GBM therapies based on GSCs. Genetic and epigenetic studies, such as The Cancer Genome Atlas project, are finding enormous heterogeneity in mutations and other genetic aberrations among GBM patients (7). This heterogeneity likely also exists among the patient GSCs (34, 50), making discovery of a single identifying surface marker incredibly difficult to near impossible. However, many of the genetic studies also successfully identify GBM subgroups (99-101), with GSCs that likely carry a common targetable antigen. Additionally, GBM like any cancer is dynamically evolving during progression (102, 103), with this change particularly evident after treatment with radiation and chemotherapy (7, 104). Although a large portion of GBM cell surface markers are likely changing, the GSCs that drive tumorigenesis or recapitulate the tumor after therapy maintain specific markers to maintain identity or support their self-renewal. For example, AC/CD133 has been shown to enrich for cancer stem cells in pediatric and adult GBM (12, 16), before and after chemotherapy and radiation (105), and even across different cancer types (12, 106, 107), suggesting preservation of stem cells and surface markers despite significant changes in the tumor bulk. Despite the challenges, we feel rapid isolation of primary glioblastoma cancer stem cells is necessary for proper, individualized therapeutic interventions. Phage and yeast display technologies provide a methodology to target GSCs while laying down a foundation to establish new therapeutic approaches to treating GBMs.

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Highlights

- The median survival for GBM patients is 14.6 months despite treatment advances.
- Therapies are needed to target GBM cancer stem cells, the cause of tumor recurrence.
- Yeast and phage display technologies are new ways to target GBM cancer stem cells.
- New therapies can be developed with yeast/phage display, improving GBM survival.

Figure 1.

GSC surface protein marker enrichment using a yeast display library. Enrichment for GSC-specific scFvs is accomplished by multiple positive selection rounds with yeast expressing scFv. GSC-bound scFvs are validated for GSC specificity by differential cell binding against GSCs, non-GSCs, and normal neural cells, followed by evaluation for scFv ability to discriminate tumor initiating and non-tumorigenic GBM cells. Adapted from Ebben et al. (108) and Wang et al.(77).

Figure 2.

Targeting glioblastoma cancer stem cells is necessary to prevent tumor recurrence. GBM cancer stem cells are resistant to the current standard of care: surgical resection, radiotherapy and temozolomide. Therapeutic approaches and strategies to target GSCs in addition to the differentiated tumor cells are necessary to effectively treat the entire cancer and prevent tumor recurrence.