

Identification of Internalizing Human Single-Chain Antibodies Targeting Brain Tumor Sphere Cells

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Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive form of primary brain tumor for which there is no curative treatment to date. Resistance to conventional therapies and tumor recurrence pose major challenges to treatment and management of this disease, and therefore new therapeutic strategies need to be developed. Previous studies by other investigators have shown that a subpopulation of GBM cells can grow as neurosphere-like cells when cultured in restrictive medium and exhibits enhanced tumor-initiating ability and resistance to therapy. We report here the identification of internalizing human single-chain antibodies (scFv) targeting GBM tumor sphere cells. We selected a large naive phage antibody display library on the glycosylation-dependent CD133 epitope-positive subpopulation of GBM cells grown as tumor spheres and identified internalizing scFvs that target tumor sphere cells broadly, as well as scFvs that target the CD133-positive subpopulation. These scFvs were found to be efficiently internalized by GBM tumor sphere cells. One scFv GC4 inhibited self-renewal of GBM tumor sphere cells *in vitro*. We have further developed a full-length human IgG1 based on this scFv, and found that it potently inhibits proliferation of GBM tumor sphere cells and GBM cells grown in regular nonselective medium. Taken together, these results show that internalizing human scFvs targeting brain tumor sphere cells can be readily identified from a phage antibody display library, which could be useful for further development of novel therapies that target subpopulations of GBM cells to combat recurrence and resistance to treatment. *Mol Cancer Ther*; 9(7); 2131–41. ©2010 AACR.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive form of primary brain tumor, accounting for 52% of all primary brain tumor cases and 20% of all intracranial tumors. The median survival time for a newly diagnosed patient is ~1 year (1). Treatment can involve chemotherapy, radiotherapy, and surgery, all of which are acknowledged as palliative measures but are not curative (2–5). Even with complete gross surgical resection of the tumor, combined with the best available treatment, recurrence has been nearly impossible to prevent, and consequently the long-term survival rate for GBM patients is extremely low.

It has been hypothesized that tumors are heterogeneous and only some of the tumor cells with stem-like properties are able to initiate and sustain tumor development (6–8). It has been further postulated that current

therapies targeting the bulk tumor are unable to completely eliminate these tumor-initiating subpopulations, leading to tumor recurrence and treatment failure (9, 10). Therapies optimized for eliminating these subpopulations would therefore be more effective in preventing recurrence than those targeting the bulk tumor (9, 11). Tumor-initiating cells have been identified initially in hematologic malignancies and later in many types of solid tumors, including brain tumors. A subpopulation of cells that are positive for the glycosylation-dependent conformational CD133 epitopes (AC133 and/or AC141) have been identified in malignant gliomas, and this subpopulation has been shown to have significantly enhanced potency for initiating tumors in immunocompromised mice (12–16). In one study, as few as 100 CD133 epitope-positive human glioma cells were shown to be capable of tumor initiation in immunocompromised mice, whereas 100,000 CD133 epitope-negative cells isolated from the same tumor mass were nontumorigenic (14).

Glioma tumor-initiating cells can be enriched by culturing primary tumor cells in serum-free medium supplemented with epidermal growth factor and basic fibroblast growth factor (15). In this serum-free selective culture system, a subpopulation of GBM cells proliferate and form tumor spheres. Moreover, these GBM tumor sphere cells more closely resemble the phenotype of primary tumors than do serum-cultured cell lines (17), and show enhanced ability to self-renew and to give rise to differentiated progenies. As a result, these GBM tumor

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sphere cells provide an attractive target for the development of therapies targeting brain tumor-initiating cells.

Due to accessibility to externally administered agents, cell surface antigens are attractive targets for the development of therapeutics that require intracellular delivery of payloads (18, 19). We and others have previously selected internalizing phage antibodies against a panel of tumor cell lines (20–25) and tumor cells *in situ* (26). Here, we have derived GBM tumor spheres from human brain tumor specimens and targeted the glycosylation-dependent CD133 epitope-positive subpopulation (from here on, we simply refer to this population as “CD133 positive”) for phage antibody selection by fluorescence-activated cell sorting (FACS) to recover internalizing scFvs. We have identified a panel of internalizing human scFvs that bind to CD133-positive GBM tumor sphere cells, and one of these scFvs is capable of inhibiting the growth of GBM tumor sphere cells *in vitro*. We have further developed a full-length human IgG1 molecule based on this inhibitory scFv and found that human IgG1 inhibits the proliferation of both GBM tumor sphere cells and primary GBM cells. Thus, we have shown that internalizing scFvs targeting GBM tumor sphere cells can be readily identified from a phage antibody display library. These fully human antibody fragments can be used in the future to develop targeted therapeutics attacking the subpopulation of tumor-initiating cells.

Materials and Methods

Culture of primary glioblastoma cells and tumor sphere cells

Human GBM tissues obtained from the operating room were either processed immediately or transplanted and maintained in nude mice as xenografts as described (27). The protocol for human tissue acquisitions was approved by the institutional review board and in accordance with an assurance filed with and approved by the Department of Health and Human Services. Animal studies were approved by the institutional review board and adhered to the USPHS Policy on Humane Care and Use of Laboratory Animals. The procedure for obtaining tumor sphere cell cultures is the same for fresh GBM tissues or GBM xenografts. Briefly, GBM tissues were minced and enzymatically dissociated by sequential digestion with collagenase (1 mg/mL, Invitrogen) and trypsin (0.05%, Invitrogen), and passed through a cell strainer (BD Biosciences) to obtain monodispersed cells. These cells were divided into two groups and were either (a) resuspended in DMEM/F12 containing 2% fetal calf serum (FCS) and then plated at a density of 1×10^6 cells per 75 cm² flask to obtain primary GBM cell monolayer cultures, or (b) seeded into tumor sphere-forming medium (TSFM) containing serum-free Complete NeuroCult medium (StemCell Technologies) supplemented with 10 ng/mL basic fibroblast growth factor (Millipore/Chemicon), 10 ng/mL epidermal growth factor (Sigma), and 0.2% heparin/PBS

(StemCell Technologies). For serial propagation, primary tumor spheres were redissociated into monodispersed cells by trypsin digestion and further cultured in TSFM.

Cloning efficiency assay

Tumor spheres were treated with 0.05% trypsin and passed through a cell strainer to obtain monodispersed cells and plated into a 96-well plate (1,000 cells per well) and cultured in 200 μ L TSFM for 14 days (28). Cells were fed with 25 μ L of TSFM by changing the medium every 2 days. Experiments were done in triplicates. The number of tumor spheres were counted and averaged to determine the cloning efficiency (15, 29).

Flow cytometry analysis of CD133 epitope expression

Primary GBM and tumor sphere cells were dissociated by 0.05% trypsin and washed with PBS, and 100 μ L aliquots of 10^5 cells in PBS were placed in 96-well V-bottomed plates and incubated on ice for 30 minutes with phycoerythrin (PE)-conjugated anti-CD133 antibody (Miltenyi Biotec) or an isotype control PE-labeled mouse IgG (BD/Pharmingen). Cells were washed twice with PBS and analyzed by FACS (LSRII, Becton Dickinson).

Selection for internalizing scFvs targeting GBM tumor sphere cells

A multivalent fd phage antibody display library containing the Sheets repertoire (30, 31) was used for selection on GBM tumor sphere cells that express high levels of the CD133 epitope. For the first round of selection, the library was counterselected on a panel of control cells to remove nonspecific binders as described (22, 24, 26), and incubated for 2 hours at 4°C with 10^5 monodispersed GBM sphere cells, washed three times with ice-cold PBS, then incubated with prewarmed (37°C) DMEM/F12 containing 10% FCS at 37°C for 1 hour to allow receptor-mediated phage antibody internalization (22, 24). Noninternalized phage were removed by washing cells with 50 mmol/L glycine/150 mmol/L NaCl (pH 2.8). Internalized phage were recovered and propagated as described previously (22, 24, 26). For the second round of selection, tumor sphere cells were incubated with polyclonal first-round phage under internalizing conditions as described above and further incubated with the PE-conjugated anti-CD133 antibody to identify subpopulations expressing high levels of CD133, which were sorted by FACS Aria (Becton Dickinson) along with internalized phage. The process was repeated for the third round of selection. Monoclonal phage binders were identified by FACS-based 96-well plate screening using biotin-labeled anti-fd antibody (Sigma) and streptavidin-R-PE (Invitrogen) as previously described (22, 24, 26).

Double labeling of the CD133 epitope and antigens bound by phage antibodies

Phage antibodies were fluorescently labeled using 6-[fluorescein-5-(and-6)-carboxamido]hexanoic acid, succinimidyl ester [5(6)-SFX; Invitrogen] and purified as

previously described (22, 32, 33). Monodispersed GBM sphere cells were incubated with biotin-labeled anti-CD133 monoclonal antibody (mAb; 5 $\mu\text{g}/\text{mL}$, Miltenyi Biotec) and FITC-labeled phage antibodies. Streptavidin-647 (Invitrogen) was used to detect bound biotin-labeled anti-CD133 antibody.

Phage antibody binding to antigens other than CD133

Caco-2 cells that express high levels of CD133 were incubated with phage antibodies along with the positive control PE-conjugated anti-CD133 antibody at 4°C for 1 hour, washed twice with PBS, and bound phage were detected by biotin-labeled anti-fd antibody followed by streptavidin-R-PE, and analyzed by FACS. For immunoprecipitation studies, 10^5 Caco-2 cells were lysed in 1% NP40, 0.15 mol/L NaCl, and 0.01 mol/L sodium phosphate (pH 7.2) on ice for 2 hours. Lysates were precleared and incubated with 50 $\mu\text{g}/\text{mL}$ scFvs bearing a hexahistidine tag (see below) at 4°C for 1 hour, and captured onto Ni-NTA+ beads (Qiagen). As a positive control, pre-cleared Caco-2 lysates were incubated with rabbit anti-CD133 antibody (Cell Signaling Technology) followed by capturing with protein A beads (Thermo Fisher Scientific). All immunoprecipitation products were analyzed by 4% to 20% gradient SDS-PAGE, followed by Western blot with mouse anti-CD133, followed by horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) using ECL Plus (GE Healthcare).

Expression and purification of recombinant scFvs

scFvs were subcloned from the phage vector into a bacterial expression vector, resulting in the addition of a c-myc epitope and a hexahistidine tag at the COOH terminus as described (22). scFv was harvested from the bacterial periplasm (22) and purified using AKTAprime (GE HealthCare) by immobilized metal affinity chromatography (HiTrap His, GE HealthCare) and gel filtration (PD10, GE HealthCare), and analyzed by reducing 10% SDS-PAGE (22, 23).

scFv internalization by tumor sphere cells

scFvs were labeled with Q-dot (655) using a two-step conjugation procedure as described (25), incubated with tumor sphere cells at 37°C for 1 hour, washed with PBS and fixed in 2% paraformaldehyde (Sigma), and viewed under a fluorescence microscope (Olympus) and a confocal microscope (Zeiss). 4',6-Diamidino-2-phenylindole (nucleus)- and Q-dot-labeled phage antibodies were imaged in separate channels and merged to show scFv sub-cellular location. To broaden applicability, we labeled scFvs with FITC using 5(6)-SFX and repeated the binding and internalization experiments.

Quantification of internalized phage antibodies was done as described (22). Briefly, 1×10^5 GBM sphere cells were incubated with phage antibodies at 37°C for 1 and 4 hours, respectively, washed with PBS, and divided evenly into two groups, one for quantification of the total

phage bound and the other for the internalized fraction following surface stripping with 50 mmol/L glycine/150 mmol/L NaCl (pH 2.8).

Inhibition of self-renewal of GBM tumor sphere cells by recombinant scFvs

One thousand monodispersed tumor sphere cells were incubated with GC4 scFv (50 $\mu\text{g}/\text{mL}$) in 200 μL TSMF. The medium was changed every 2 days by replacing 25 μL of old medium with fresh medium containing 50 $\mu\text{g}/\text{mL}$ scFvs. Colonies (tumor spheroids) were counted after 14 days.

Generation of a recombinant human GC4 IgG1 molecule

The sequence information of GC4 scFv was used to develop human IgG1 as described previously (34). Briefly, GC4 VH was amplified using primer pairs with overhangs containing *MluI* and *NheI* sites, digested with *MluI* and *NheI*, and ligated into a mammalian expression vector to create in frame fusion with the human heavy chain constant region. The VL gene was amplified with the primer pairs with overhangs containing *DraIII* and *AvrII*, and ligated into *DraIII*- and *KpnI*-digested plasmid containing the GC4 heavy chain gene to create a plasmid that expresses both heavy and light chains, which was used to transfect CHO-DG44 cells by electroporation and to establish stable lines by selecting with G418 as described previously (34, 35). GC4 IgG1 was purified on a protein A column using the AKTA protein purification system (GE Healthcare), and analyzed by SDS-PAGE under reducing conditions. IgG concentration was measured using Nanodrop (Thermal Fisher Scientific).

Affinity measurement

Varying concentrations of GC4 IgG1s were incubated with 10^5 GBM cells at room temperature for 1 hour in PBS containing 0.25% bovine serum albumin. Bound IgG1s were detected by PE-conjugated goat anti-human antibody (Jackson ImmunoResearch) following incubation at room temperature for 30 minutes. Apparent dissociation constant was determined by FACS with mean fluorescence intensity data fitted using GraphPad Prism (GraphPad Software). The apparent dissociation constant of GC4 scFv was measured similarly except that bound scFvs were detected by mouse anti-hexahistidine antibody (AbD Serotec/MorphoSys) followed by PE-conjugated goat anti-mouse antibody (Jackson ImmunoResearch).

Inhibition of GMB cell proliferation by human GC4 IgG1

To test the effect of GC4 IgG1 on tumor sphere cells, 1,000 monodispersed tumor sphere cells were incubated with 50 $\mu\text{g}/\text{mL}$ GC4 IgG1 in 200 μL TSMF and placed in a 96-well plate. There were no significant aggregations of tumor sphere cells under this condition. The medium was changed every 2 days by replacing 25 μL of old medium with fresh medium containing 50 $\mu\text{g}/\text{mL}$ GC4

IgG1. Tumor spheres were counted after 14 days. The assay was done in parallel with a control nonbinding human IgG1 (34). To test the effect of GC4 IgG1 on GBM cells grown in nonselective medium, soft agar assays were performed. Briefly, 300 μ L melted 0.5% agarose was added to a 24-well plate and allowed to solidify. To prepare the top layer, 2.5% SeaPlaque low-melting temperature agarose (Cambrex Bioscience) was melted and diluted (v/v 1:7, final 0.35%) in RPMI 1640/10% FCS medium, and kept at 42°C in a water bath. Ten thousand monodispersed GBM cells were mixed with human IgG1s (at a final concentration of 50 μ g/mL) in 300 μ L 0.35% low-melting temperature agarose. The mixture was applied to the top of the solidified agarose in the 24-well plate and kept at room temperature for 30 minutes to allow solidification of the top layer. The plate was then incubated at 37°C and colonies were counted after 14 days (36).

Growth inhibition of established tumor spheres

Monodispersed tumor sphere cells were placed in six-well plates in TSFM at a density of 200 cells per well and allowed to grow for 2 to 3 days to form tumor spheroids. GC4 scFv was added to a final concentration of 50 μ g/mL, and the incubation continued for an additional 4 to 5 days. The number of tumor spheres with a diameter >50 μ m were counted under an inverted microscope (Nikon).

Statistics

Student's *t* test was used to analyze a pair of variables, and a *P* value of <0.05 was considered statistically significant.

Results

Isolation and characterization of GBM tumor sphere cells

We first isolated GBM tumor spheres from five fresh human surgical specimens, as well as from nine human

GBM tissues maintained as xenografts in immunocompromised mice, by growing dissociated GBM cells in restrictive medium containing basic fibroblast growth factor and epidermal growth factor. Tumor spheres consisting of a few hundred to a few thousand cells became visible after 7 to 14 days of culture (Fig. 1). We next compared the cloning efficiency of tumor spheres grown in restrictive medium to bulk tumor cells from fresh surgical GBM specimens and GBM xenografts grown in medium containing serum. GBM tumor sphere cells have higher cloning (self-renewal) capacity than either of the bulk tumor cells (~10–20% versus 1–2%), consistent with reports by others (15). Next, we analyzed the expression of the CD133 epitope (AC141) on GBM tumor sphere cells and GBM cells grown as monolayer cultures in nonselective medium containing 2% FCS. We found detectable levels of CD133 epitope expression in 11 of 14 GBM cultures (grown as spheres or monolayer cells; Fig. 1; Table 1A). In most cases (10 of 11) where epitope expression was evident, tumor sphere cells expressed higher levels of the CD133 epitope than the equivalent cells cultured as a monolayer in nonselective medium. In addition, the expression of the CD133 epitope usually remained stable for a longer period in tumor sphere cells compared with monolayer primary tumor (4–6 months compared with ~1–2 months).

Selection of internalizing human scFvs targeting the CD133-positive subpopulation of tumor sphere cells

For phage antibody library selection (Fig. 2A), we initially targeted CD133-positive tumor sphere cells derived from fresh human GBM tissues (GBMh2; Table 1B) to increase the likelihood of identifying scFvs that target clinically represented markers. For subsequent rounds of selection, we targeted the CD133-positive fraction of tumor sphere cells derived from GBM tissues maintained as xenografts (GBMx-8; Table 1B). To broaden applicability, positive binders were retested on additional GBM tissues (Table 1B).

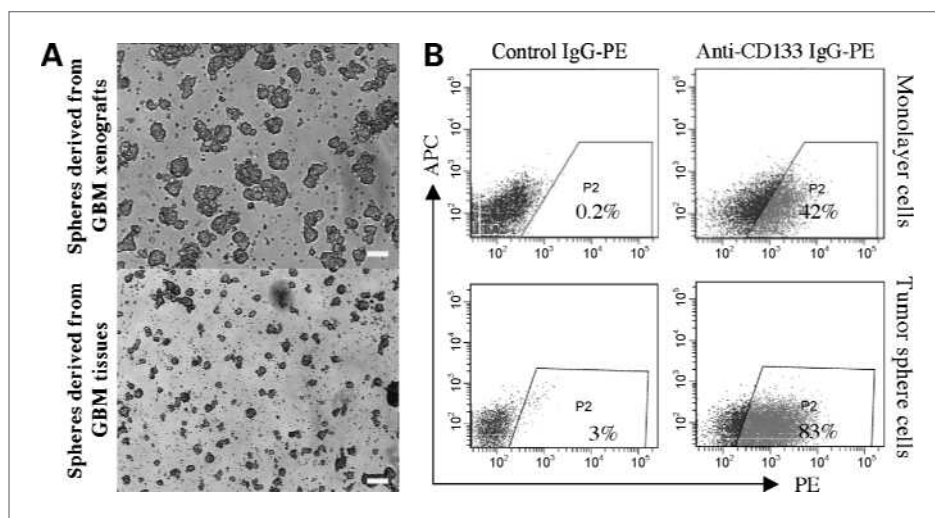


Figure 1. Characterization of GBM tumor spheres. A, morphology of GBM tumor spheres derived from xenografts and fresh human tissues after 14 days of incubation in serum-free restrictive medium. Scale bar, 500 μ m. B, CD133 epitope expression by GBM cells grown as a monolayer in serum-containing medium or as tumor spheres in serum-free medium.

Table 1. Selection of human scFvs targeting human GBM tumor spheres

(A)					
GBM tissues	Tumor sphere formation	CD133 epitope expression	Target tissues for initial selection	Tissues used for retest	
GBMx-1	++	80%		+	
GBMx-2	++	50%		+	
GBMx-3	++	2%		+	
GBMx-4	++	0*		+	
GBMx-5	++	3%		+	
GBMx-6	++	4%		+	
GBMx-7	++	15%		+	
GBMx-8	++	70%	+	+	
GBMx-9	++	0*			
GBMh-1	+	0*			
GBMh-2	++	12%	+	+	
GBMh-3	+	8%			
GBMh-4	++	3%		+	
GBMh-5	+	6%			

(B)					
	H3	GC4	GB1	GB8	GH9
Binding to GBM sphere cells	100%	50–99%	Variable*	Variable*	Variable*
Binding to nonsphere GBM cells	Unchanged (100%)	Reduced	Reduced concurrently with CD133	Reduced concurrently with CD133	Reduced concurrently with CD133
Relationship to CD133 ⁺ cells	Cover CD133 ⁺	Cover CD133 ⁺	A subset of CD133 ⁺	A subset of CD133 ⁺	Costain with CD133 ⁺
Target antigen	CD166	ND	ND	ND	ND

NOTE: (A) Tumor sphere formation and CD133 epitope expression. ++, large-sized spheres containing >100 cells per sphere after 14 days of incubation. +, small-sized spheres containing <100 cells per sphere after 14 days of incubation. 0*, expression levels indistinguishable from that of the control (control isotype-matched mouse IgG stain). GBMx-8 and GBMh-2 were used for initial phage antibody library selection. Positive binders were retested on additional GBM tissues as indicated. (B) Summary of binding results for the five unique human scFvs studied. Costaining with CD133 is shown in Fig. 3. Nonsphere GBM cells are derived from sphere cells cultured in differentiation-promoting medium containing serum.

Abbreviations: GBMh, human GBM tissues; GBMx, GBM maintained as xenografts in nude mice; ND, not determined.

*Variable binding patterns were shown in Supplementary Table S1.

To begin, we first counterselected the 500-million-member naïve phage antibody display library on a panel of nontumorigenic cell lines to remove nonspecific binders, and then selected the library on GBM tumor sphere cells under internalizing conditions to enrich polyclonal phage that target internalizing epitopes. The polyclonal phage antibodies were then used as the input for further selection by FACS to target the CD133-positive cells. After two rounds of FACS-based selection, we analyzed the selection output for binding to GBM sphere cells. About 45% (83 of 186) of phage antibodies bound to GBM sphere cells but not control cells such as BPH-1. There were 20 unique scFvs among 60 that were sequenced. The phage antibodies exhibit a

range of binding patterns, binding from ~3% to 99% of the GBM sphere cell population (Fig. 2B). Five scFvs with the highest frequency of representations in the output were chosen for further studies (Table 1B). They have been retested and validated on additional GBM tissues not used in the initial selection (Table 1B). By scanning a sequence database of scFvs previously identified in our laboratory, one scFv that binds to all GBM sphere cells was found to be identical to H3, an scFv that we have previously identified and shown to bind to an internalizing epitope on ALCAM/CD166 (26, 34). To simplify the nomenclature, we used the original name H3 to designate this scFv. The other four scFvs have not been previously identified.

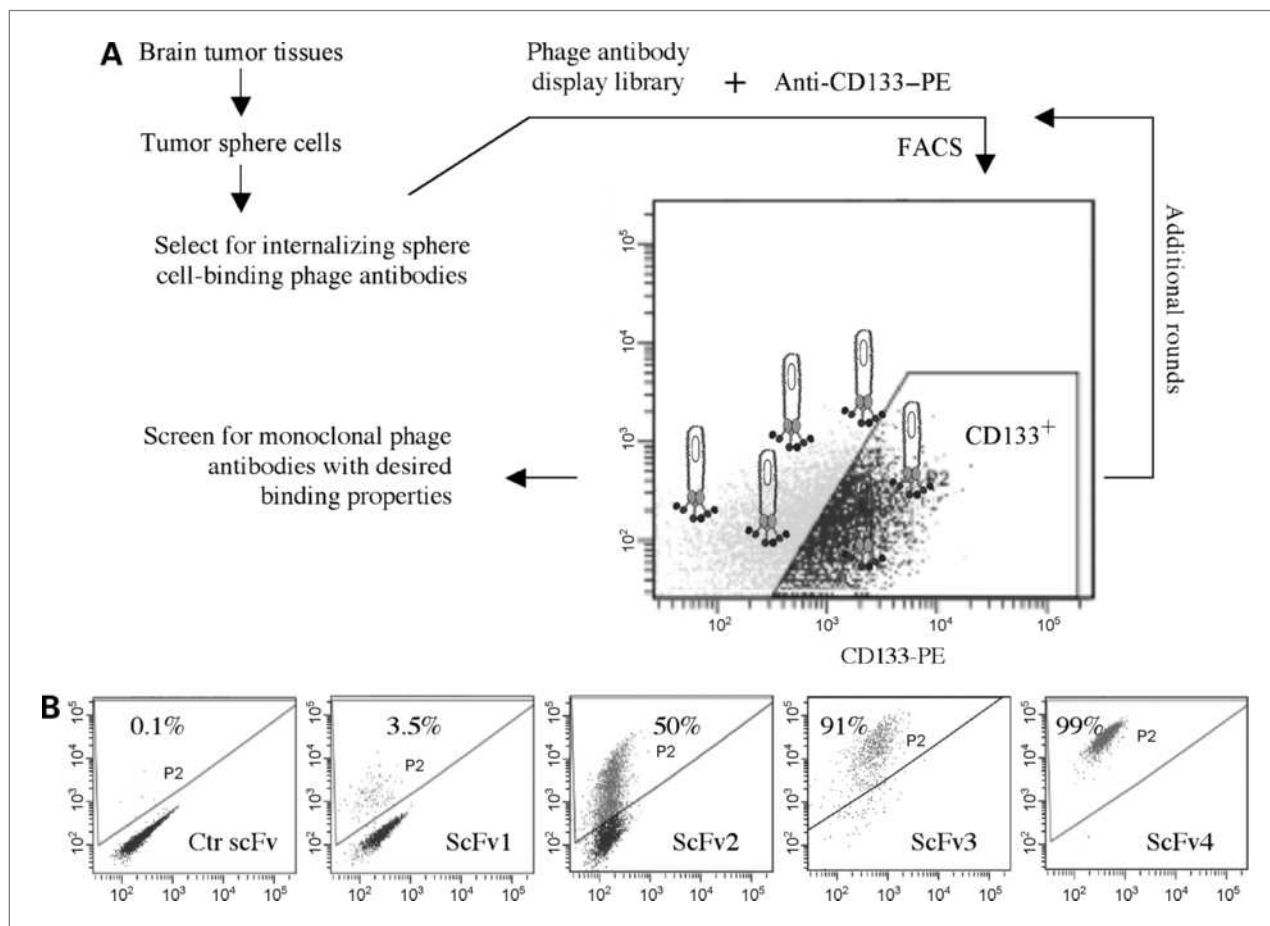


Figure 2. Selection of phage antibodies binding to the CD133⁺ cell population. **A**, outline of the selection scheme. Selection was done by FACS on cells derived from multiple GBM cases. CD133⁺ cells were gated by comparing with results from staining with an isotype-matched PE-conjugated control mouse mAb. Outputs from third-round selections were screened to identify monoclonal phage antibodies. **B**, patterns of phage antibody binding to GBM cells. The percentage of positive cells is indicated. GBMx8 tumor sphere cells were used in the analysis.

Analysis of coexpression of scFv-targeted antigens and the CD133 epitope

Because the selection scheme was designed to capture binders to the CD133-positive fraction of GBM sphere cells, we used double-labeling analysis to determine if the phage antibodies that bind a subset of the cell population bind preferentially to the CD133-positive population. We tested GB8 and GH9 phage antibodies and found that they costained with the anti-CD133 epitope mAb (Fig. 3A), whereas very little phage binding to CD133 epitope-negative cells was observed (<1% for GB8 and <6% for GH9). Thus, GB8 and GH9 bind to antigens expressed mainly on the CD133⁺ subpopulation of GBM tumor spheres, indicating that our original selection scheme was successful. Neither GB8 nor GH9 phage antibodies compete with the anti-CD133 epitope mAb for binding (Fig. 3A; no reductions in percentage of CD133-APC positive cells were observed), indicating that these phage antibodies bind to novel epitopes that do not overlap with the widely used CD133 epitopes.

To further determine if our scFvs bind to CD133 through a different epitope, we first studied binding of scFvs to Caco-2 cell line that expresses a high level of CD133 (Supplementary Fig. S1A). As shown in Supplementary Fig. S1B, none of our scFvs bind to Caco-2 cells, whereas the positive control anti-CD133 antibody binds strongly to Caco-2 cells. We further performed immunoprecipitation studies and found that our scFvs do not pull down the CD133 molecule, whereas the positive control antibody does (Supplementary Fig. S1C), consistent with results of the FACS analysis. We therefore conclude that our scFvs do not bind to CD133.

Phage antibodies target antigens preferentially associated with tumor sphere cells grown in selective medium

To test if antigens bound by phage antibodies are preferentially expressed by GBM cells growing in restrictive medium as tumor sphere cells, we compared expression of the CD133 epitope and scFv-targeted antigens by GBM cells grown in selective versus nonselective medium. We

observed concurrent downregulation of the CD133 epitope and phage antibody-bound epitopes for all the scFvs tested (Fig. 3B and C), suggesting that these phage antibody-targeted antigens are preferentially associated with GBM tumor sphere cells growing in selective medium.

Antibody internalization by GBM tumor sphere cells

Our phage antibodies were selected for internalizing functions with the goal of developing potential tumor stem cell-targeted therapies based on intracellular delivery strategies. To confirm the internalizing function of selected scFvs, we labeled scFvs with quantum dots (Q-dot 655) and visualized cellular uptake by fluorescence microscopy. An example of the experiment with GC4 scFv is shown in Fig. 4A. When incubation was at 4°C, GC4 scFv was found to bind to the surface of GBM sphere cells. When incubation was at 37°C, which permits receptor-mediated endocytosis, GC4 scFv was efficiently internalized (Fig. 4A) into intracellular compartments. To rule out potential Q-dot labeling artifacts, the experiment was repeated with FITC-labeled scFvs, and the same results were obtained (data not shown).

To quantify the internalization efficiency, we compared the cell-associated phage antibodies before and after treatment with a low-pH buffer that strips off surface-bound noninternalized phage antibodies. As shown in Fig. 4B, all four scFvs studied were efficiently internalized. Significant internalization was seen as early as 1 hour. By 4 hours, most phage antibodies were internalized (Fig. 4B), demonstrating that our selection strategies were effective at enriching internalizing human scFvs that target the GBM tumor sphere cells.

GC4 scFv inhibits GBM tumor sphere cell self-renewal

We next tested the ability of the scFvs to inhibit tumor sphere self-renewal. We incubated affinity-purified recombinant scFvs with dissociated GBM tumor sphere cells for 7 days, and determined the efficiency of clonal expansion. GC4 scFv caused a significant inhibition of GBM sphere growth *in vitro* (Fig. 5A), suggesting that it modulates the activity of a cell surface antigen that plays a significant role in GBM tumor sphere cell self-renewal. None of the other scFvs tested showed inhibitory effects under identical conditions (Fig. 5A). To test for

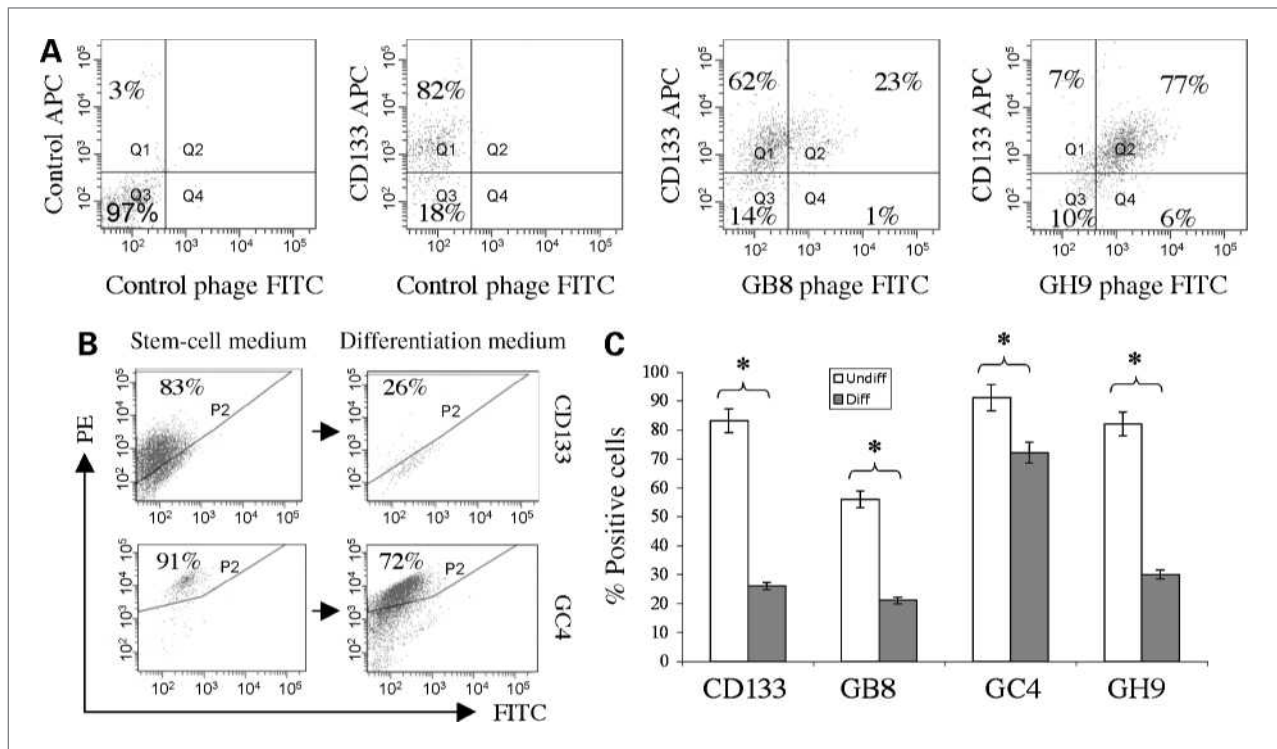


Figure 3. Coexpression of the CD133 epitope and phage-bound antigens. A, FITC-labeled phage antibodies and biotin-labeled anti-CD133 mAb were studied on GBM sphere cells. A typical result from studies on GBMx-1 sphere cells is shown. The percentage of total cells is indicated for each quadrant. B, concurrent downregulation of the CD133 and the phage-bound epitopes. FACS analysis of the expression of the CD133 epitope (top panels) and the antigen bound by the GC4 phage antibody (bottom panels). Concurrent downregulations were observed when GBM sphere cells were removed from TSM and cultured in medium that promotes differentiation. C, quantification of binding status of the anti-CD133 epitope mAb and phage antibodies to GBM tumor sphere cells cultured in TSM that promote maintenance of the undifferentiated state (Undiff) and nonselective medium that promote differentiation (Diff). Experiments were done in triplicates, and SD values are shown. *, $P < 0.05$.

nonspecific cell toxicity, we studied the effect of GC4 scFv on cell lines that were not bound by this scFv (SKOV3 and PC3, see Supplementary Table S2). No inhibition was observed in these control experiments.

To further determine if GC4 scFv is capable of inhibiting the growth of established GBM tumor spheres, we grew monodispersed GBM cells in TSMF to obtain tumor spheroids, and treated them with recombinant GC4 scFv (100 μ g/mL). As shown in Supplementary Fig. S2, treatment with GC4 but not the control scFv significantly reduced the growth of established tumor spheres.

GC4 IgG1 inhibits proliferation of GBM tumor sphere cells

Because GC4 scFv inhibits proliferation of GBM tumor sphere cells, we sought to develop full-length human GC4 IgG1 for further *in vivo* applications. Using the VH and VL sequences from GC4 scFv, we constructed GC4 IgG1 in a mammalian expression vector, transformed CHO-DG44 cells, and produced full-length recombinant human GC4 IgG1. Analysis of purified IgG1 by reducing SDS-PAGE showed heavy and light chains with appropriate sizes (Fig. 5B). For quality control, we tested and confirmed binding of the recombinant GC4 IgG1 to GBM tumor sphere cells (Fig. 5C). In addition, GC4 IgG1 competed specifically with the parent GC4 scFv for binding to GBM sphere cells (Supplementary Fig. S3), demonstrating that IgG and scFv have the same specificity,

consistent with our previous experience with recombinant IgG development (34). We further measured cell-binding affinity by FACS and found that GC4 IgG1 binds with greater affinity than the parental scFv [the binding constant measured at room temperature is 79 nmol/L for IgG1 (Supplementary Fig. S4) versus 222 nmol/L for scFv (Supplementary Fig. S5)]. We next tested the inhibitory effects of GC4 IgG1 on GBM tumor sphere cells grown in selective medium. Like the parental GC4 scFv, recombinant GC4 IgG1 inhibited self-renewal of GBM tumor sphere cells (~60% inhibition, similar to that of GC4 scFv).

To determine if this inhibitory effect is also applicable to GBM cells grown under nonselective culture conditions, we treated GBM cells cultured in nonselective medium and measured cloning efficiencies using the soft agar assay. As shown in Fig. 5D, GC4 IgG1 inhibited the clonogenic activity of GBM cells. Compared with controls, GC4 IgG1-treated GBM cells formed significantly fewer colonies. Moreover, the colonies formed by GC4 IgG1-treated cells are significantly smaller than those formed by cells treated with the control IgG (Fig. 5D). This experiment was repeated on GBM cells derived from five independent cases. Inhibitory effects were observed in four of these cases. Again, no inhibitory effects were observed on cell lines to which the GC4 antibody does not bind (SKOV3 and PC3; nonbinding cells are listed in Supplementary Table S2). These results showed that GC4 human IgG1 is a potent inhibitor of *in vitro* GBM cell proliferation in nonselective medium.

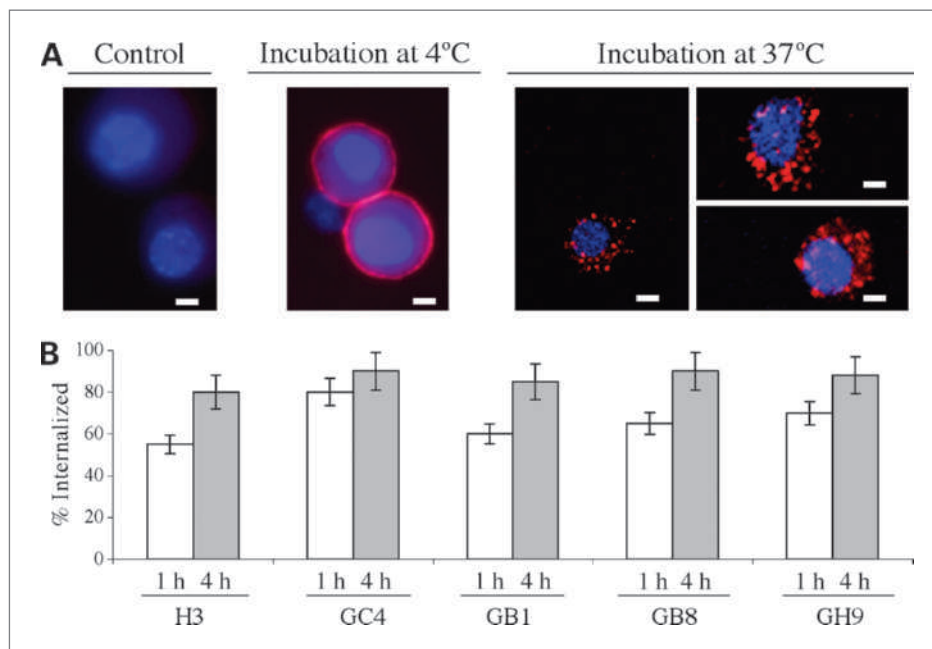


Figure 4. Internalization by GBM tumor sphere cells. A, internalization of Q-dot 655-labeled GC4 scFv by GBM tumor sphere cells. Representative images are shown for each indicated incubation condition. Control, GBM sphere cells incubated with Q-dot 655-labeled control nonbinding scFv (N3M2). This nonbinding scFv was originally picked from the unselected naïve library and has been used as a negative control in our previous studies (25, 50). Scale bar, 2 μ m. B, quantification of percent internalized. Following incubation with phage antibodies, data from two time points (1 h and 4 h) were collected and analyzed. Surface-bound phage antibodies were removed by washing with a low-pH glycine buffer. The percentage of internalized phage is calculated based on the ratio of internalized over total cell-associated phage antibodies. The experiments were done in triplicates. Error bars, SD.

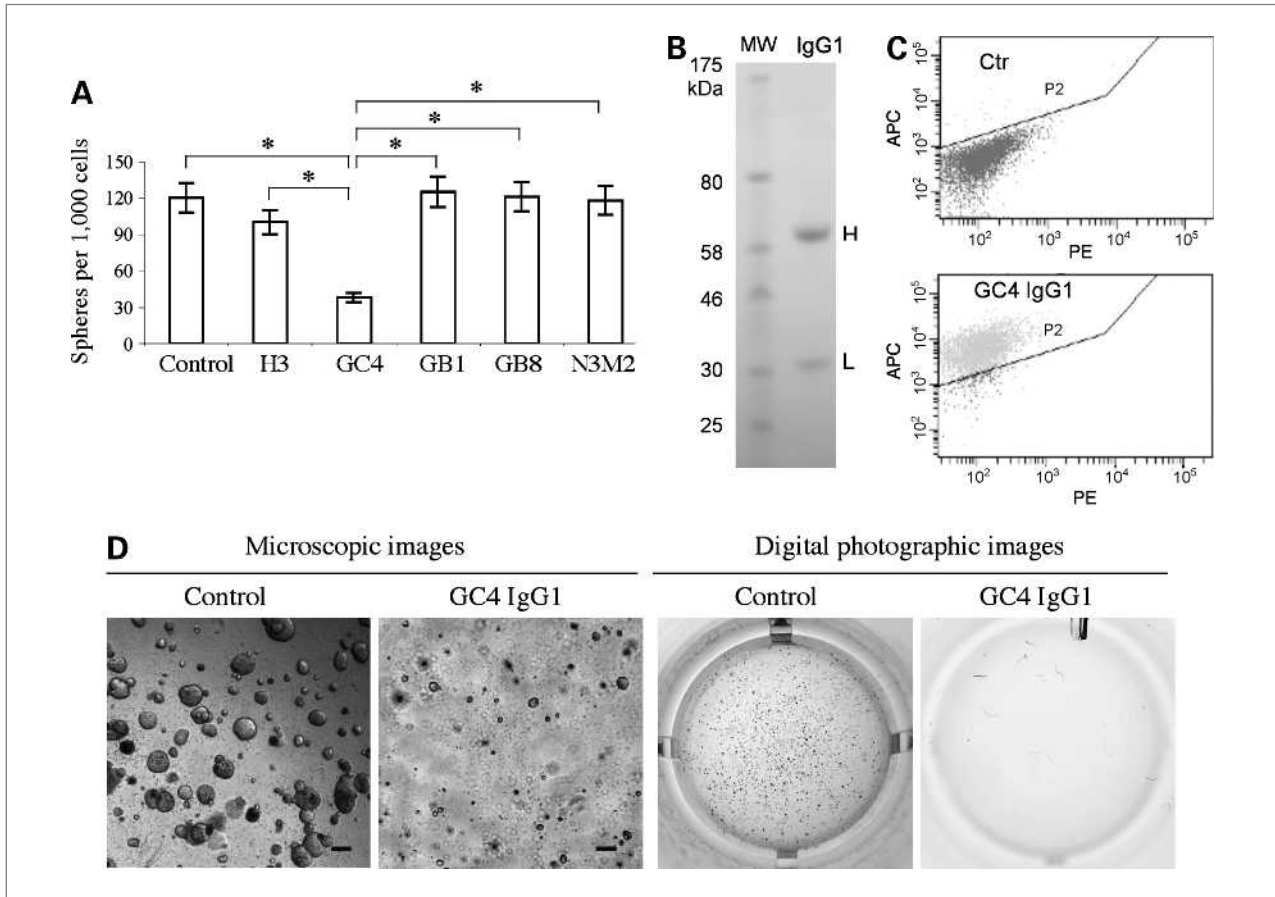


Figure 5. Inhibition of self-renewal of GBM tumor sphere cells. **A**, GC4 scFvs but not other GBM tumor sphere cell-binding scFvs significantly inhibited GBM sphere cell proliferation. Cloning efficiencies were calculated from tumor spheres formed per 1,000 GBM cells seeded. Control, untreated sphere cells. N3M2, a nonbinding scFv control. Experiments were done in triplicates; error bars, SD. *, $P < 0.05$ for pairs indicated. **B**, production of recombinant GC4 IgG. H, heavy chain; L, light chain; MW, molecular weight. **C**, quality control study confirming that GC4 scFv-derived IgG1 retains the ability to bind GBM sphere cells. Ctr, a nonbinding human IgG1. **D**, GC4 IgG1 inhibits colony formation of GBM cells in the soft agar assay. Both microscopic and digital photographic images of GC4 and control IgG1-treated wells are shown. Scale bar, 250 μ m.

Discussion

It has been hypothesized that bulk tumor is sustained by a subpopulation of tumor-initiating cells, and their elimination would promote collapse of the tumor hierarchy, overcoming drug resistance and tumor recurrence associated with current anticancer therapies. Although conceptually appealing, therapies that target these tumor-initiating cells have yet to be developed for use in clinical practice. To develop human mAb-based therapies that target prospective brain tumor-initiating cells, we selected a large naïve phage antibody display library on GBM tumor sphere cells to identify novel internalizing human scFvs that target potential brain tumor-initiating cells. We have shown that our antibody library-based approach is effective in identifying novel human antibodies targeting the whole as well as subpopulations of GBM tumor sphere cells. To our knowledge, this is the first time that a phage antibody library selection has been used for this purpose.

GBM tumor-initiating cells have been well studied and are generally defined in two ways: by the expression of stem cell markers, such as CD133 epitopes, and/or by the ability to grow in restrictive medium (tumor sphere formation assay; refs. 14, 15). Both methods have been widely used to enrich brain tumor-initiating cells, but each by itself may have limitations (16). Thus, to more precisely target GBM tumor-initiating cells, we used GBM tumor sphere cells that express high levels of the CD133 epitope as our selection target. The use of two criteria to define prospective GBM tumor-initiating cells used for antibody selection helps increase the chances of recovering antibodies targeting these cells.

We performed antibody library selection on tumor sphere cells derived from fresh human GBM tissues and GBM tissues maintained in immunocompromised mice. Primary GBM tumors maintained as xenografts are useful for these studies for several reasons. First, these tumors have been well characterized and shown to have the same gene alterations identified in their corresponding patient

tumors (37). Second, a previous study has shown that brain tumors maintained as xenografts retain many features of the original tumors (27). Finally, these tumors can be propagated indefinitely and thereby exist as a sustainable source of tissue for extended studies. Xenografts therefore provide a continuous supply of tissues with relatively consistent characteristics, and facilitate experimental reproducibility (27, 38). Similar approaches that aim to identify and characterize tumor-initiating cells have been used by others for investigating pancreatic cancer (38).

The GBM tumor sphere cell-targeting scFvs we have identified provide an opportunity to develop novel mAb-based therapeutics. For example, we have identified a scFv that inhibits self-renewal of GBM tumor sphere cells. We have further developed a full-length human IgG based on this scFv and shown that like the parental scFv, this recombinant human IgG inhibits proliferation of GBM tumor sphere cells. The availability of a full-length human IgG with a demonstrated inhibitory activity should allow further testing of antitumor efficacy *in vivo* using appropriate animal models. Even scFvs with no apparent antiproliferation effects *in vitro* could nonetheless be useful for therapeutic development. Because our panel of scFvs has been selected for internalizing functions, they can be used to develop therapies based on intracellular delivery strategies. For example, these internalizing scFvs can be conjugated to drugs or drug-loaded nanoparticles, engineered toxin molecules, or appropriate radionuclides to impart a targeted cell killing function (39, 40). These targeted agents could in theory offer an opportunity to determine if *in vivo* ablation of tumor-initiating cells results in improved therapeutic effects such as suppression of recurrence.

In addition to therapeutic development, our panel of GBM tumor sphere cell-targeting scFvs could be useful in identifying additional tumor-initiating cell markers other than the CD133 epitopes, which could provide new insights into tumor heterogeneity, facilitate improvement of selection and enrichment protocols for GBM tumor subpopulations, and allow development and optimization of new treatment strategies targeting these antigens.

We would like to point out that although numerous independent studies by others have shown that GBM tumor sphere cells and CD133-positive GBM cells can initiate tumor formation *in vivo* and thus are operationally defined as GBM cancer stem cells (16, 41), we have not repeated the *in vivo* tumor initiation experiment using GBM tumor sphere cells that we have isolated according to published protocols. We have thus refrained from referring to these GBM tumor sphere cells as cancer stem cells, although they showed higher self-renewal ability *in vitro*, a property commonly associated with cancer stem cells.

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The strategy of using antibodies to target tumor subpopulations, although conceptually appealing, is likely to encounter similar challenges associated with antibody targeting of the bulk tumor. For example, issues such as tissue penetration and binding site barrier need to be addressed (42–45). Optimizing antibody-target interactions and pharmacokinetics have been shown to improve efficiencies of antibody-based tumor targeting (44, 46, 47). It should be pointed out that in most cases, antibody therapy is most effective in treating solid tumors following volume reduction by surgery, chemotherapy, or radiotherapy. In addition, small size lesions such as micrometastasis can be effectively targeted by mAbs. Because tumor-initiating cells are hypothesized to be the primary force driving recurrence, targeting these cells by antibodies in the above two situations is feasible and should result in improved treatment outcome. Finally, recent studies have shown that at least for some cancers, including GBM, tumor-initiating cells exist in a special vascular “niche” (48, 49), which would render them accessible to intravenously injected therapeutics such as mAbs.

In summary, our studies show that it is feasible to use the combinatorial antibody library approach to select internalizing scFvs targeting GBM tumor sphere cells and furthermore a subpopulation of these tumor sphere cells that express high levels of the CD133 epitope. In addition, inhibitory scFvs can be identified, and their full-length inhibitory human IgG derivatives can be readily developed. Our methods should be generally applicable to the development of internalizing human mAbs targeting subpopulations of heterogeneous tumor cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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