

Targeting a Glioblastoma Cancer Stem-Cell Population Defined by EGF Receptor Variant III

David R. Emler¹, Puja Gupta¹, Marina Holgado-Madruga⁶, Catherine A. Del Vecchio⁷, Siddhartha S. Mitra³, Shuang-Yin Han⁸, Gordon Li⁴, Kristin C. Jensen^{2,5}, Hannes Vogel², Linda Wei Xu⁴, Stephen S. Skirboll^{4,5}, and Albert J. Wong¹

Abstract

The relationship between mutated proteins and the cancer stem-cell population is unclear. Glioblastoma tumors frequently express EGFRvIII, an EGF receptor (EGFR) variant that arises via gene rearrangement and amplification. However, expression of EGFRvIII is restricted despite the prevalence of the alteration. Here, we show that EGFRvIII is highly coexpressed with CD133 and that EGFRvIII⁺/CD133⁺ defines the population of cancer stem cells (CSC) with the highest degree of self-renewal and tumor-initiating ability. EGFRvIII⁺ cells are associated with other stem/progenitor markers, whereas markers of differentiation are found in EGFRvIII⁻ cells. EGFRvIII expression is lost in standard cell culture, but its expression is maintained in tumor sphere culture, and cultured cells also retain the EGFRvIII⁺/CD133⁺ coexpression, self-renewal, and tumor initiating abilities. Elimination of the EGFRvIII⁺/CD133⁺ population using a bispecific antibody reduced tumorigenicity of implanted tumor cells better than any reagent directed against a single epitope. This work demonstrates that a mutated oncogene can have CSC-specific expression and be used to specifically target this population. *Cancer Res*; 74(4); 1238–49. ©2013 AACR.

Introduction

The cancer stem cell (CSC) hypothesis has been a useful concept for explaining several biologic properties of tumors, including the observation that stem-like cells are tumorigenic while most of a tumor consists of differentiated cells with low tumor-forming ability (1). Whether this principle can be exploited to enhance therapy is not clear. The CSC population may not necessarily represent the original tumor-initiating cell (2) and there is plasticity in the general tumor mass such that cells can reacquire a CSC-like phenotype. The repertoire of

markers available for identification of CSCs also complicates therapy. Stem-cell markers and other cellular proteins have been shown to enrich for CSCs but these otherwise normal proteins lack tumor specificity and no marker has precisely been selected for these cells (3–5).

Mutations or rearrangements in genes that potentially drive neoplasia can be identified in many cancers. *In vitro* work has shown that the resulting oncogenic proteins can contribute to CSC-related pathways (6). It stands to reason that the products of such altered genes could be used to identify and potentially target CSCs. In practice, this has been difficult to establish because driver mutations are present in cells throughout the mass and typically are not specific to any subpopulation. Thus, mutant proteins may not have any direct role in CSCs and perhaps only generally potentiate tumor growth (7). In addition, most altered proteins are intracellular.

Although not all tumors follow a CSC model, glioblastoma (GBM) has been strongly associated with the presence of CSCs (3, 8). Amplification of the *EGFR* gene is common in this tumor, and 20% to 40% of glioblastomas express EGFRvIII, an altered form of the *EGFR* gene, that arises via gene rearrangement and amplification (9). Some studies have seen EGFRvIII expression as high as 70% in glioblastoma (10). In addition to glioblastoma, EGFRvIII has been found in a high percentage of breast (11, 12), lung (13), head and neck, ovarian, and prostate cancers. Importantly, it is rarely found in normal tissue (11) and this almost exclusive expression in tumors makes it an intriguing target for therapy (14). The presence of EGFRvIII correlates with a worse prognosis for patients with both glioblastoma and anaplastic astrocytoma (15, 16). EGFRvIII expression is strongly associated with the

Authors' Affiliations: ¹Brain Tumor Research Laboratories, Program in Cancer Biology, Stanford University Medical Center, Stanford; ²Department of Pathology, Stanford University Medical Center, Stanford; ³Institute of Stem Cell Biology and Regenerative Medicine, Stanford University Medical Center, Stanford; ⁴Department of Neurosurgery, Stanford University School of Medicine, Stanford; ⁵Veterans Affairs Palo Alto Health Care System, Palo Alto, California; ⁶Department of Physiology and Pharmacology, Institute for Biomedical Research, School of Medicine, University of Salamanca, Salamanca, Spain; ⁷Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, Massachusetts; and ⁸Department of Gastroenterology, Henan Provincial People's Hospital, Zhengzhou, Henan, People's Republic of China

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D.R. Emler, P. Gupta, M. Holgado-Madruga, C.A. Del Vecchio, and S.S. Mitra contributed equally to this article.

Corresponding Author: Albert J. Wong, Program in Cancer Biology, Stanford University Medical Center, 1201 Welch Road, MSLS P105, Stanford, CA 94305-5327. Phone: 650-736-4220; Fax: 650-498-7761; E-mail: ajwong@stanford.edu

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"classical" molecular subtype of glioblastoma in which it is found in conjunction with *PTEN* mutations but is mutually exclusive with *P53* or *IDH1* mutations (17). Other laboratories and ours have shown that a peptide vaccine targeting the EGFRvIII antigen can effectively reduce tumor progression in preclinical models (18). Human clinical trials have demonstrated improved overall survival and an EGFRvIII-specific immune response in patients treated with the vaccine in several phase II trials (14, 19).

Despite this improvement in patient survival, a paradoxical observation is that the typical expression pattern for EGFRvIII in positive tumors is either sporadic cells or focal areas of positive cells, unlike wild-type (wt) EGFR, which is usually broadly seen across the same tumor (20, 21) despite prevalence of the gene rearrangement/amplification (22). Interestingly, gene amplification in glioblastoma is a clonal event (23) in which only one gene rearrangement is seen in EGFRvIII⁺ tumors (9, 24). These observations point to EGFRvIII being an early development in tumorigenesis. Thus, the restricted expression of EGFRvIII may reflect its association with the CSC population. CSCs show enhanced resistance to radiotherapy and increased DNA-repair mechanisms (25) and interestingly, EGFRvIII⁺ cells are also highly resistant to ionizing radiation due to increased DNA-repair mechanisms (26). On the other hand, EGFRvIII expression may only promote growth or have a less-specific paracrine function via expression of cytokines (7). Because EGFRvIII is the result of an early genetic alteration and is a transmembrane receptor, it provides a unique opportunity to test whether mutated oncogenes can indeed play a role in CSCs.

Materials and Methods

Dissociation of primary human brain tumors and culture

Freshly resected human glioblastoma tumor samples were obtained from the Stanford University tissue and brain bank under Institutional Review Board–approved protocols. Dissociated tissue samples were cultured on nonadherent plates using defined media containing EGF, basic fibroblast growth factor (bFGF), and heparin. For neurospheres from nonneoplastic tissue, recombinant human leukemia inhibitory factor was also added. For experiments in which tumor spheres were induced to differentiate, cells were cultured in the same media without EGF and fibroblast growth factor (FGF) plus the addition of either 5% FBS and 5% horse serum, or by a cocktail of ciliary neurotrophic factor, brain-derived neurotrophic factor (BDNF), and retinoic acid.

Flow cytometry

Freshly dissociated cells were costained with a monoclonal anti-EGFRvIII antibody (G100; ref. 13) or rabbit anti-EGFRvIII and CD133/1-APC and CD133/2-APC. Cells from the primary tumor itself were used for compensation using an anti-MHC I biotin antibody. Appropriate isotype controls were used to control for nonspecific isotype background. Sorted cells were collected in tumor stem media and used for orthotopic intracranial transplantation or *in vitro* assays.

Limiting dilution and tumor sphere formation analysis

Limiting dilution analysis (LDA) was done as described previously. An extreme LDA algorithm was used to determine the frequency of renewing cells (27). To estimate the ability to form tumor spheres after antibody-dependent cell-mediated cytotoxicity (ADCC), natural killer (NK) cells were separated from glioblastoma cells using the MACS Lineage Depletion Kit (Miltenyi Biotec). For the secondary sphere assay, spheres generated during the LDA were redissociated and plated (10 cells per well) on a 96-well plate and the number of spheres were counted after 8 weeks.

Intracranial transplantation and tumor formation

Six- to 8-week nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were intracranially injected and monitored for 22 to 26 weeks or until neurologic defects were observed. For experiments to test for an antitumor effect of the bispecific antibody (BsAb), human glioblastoma tumor cells were mixed with either BsAb, human IgG1-Fc, di-EGFRvIII, or di-CD133 at a final concentration of 0.01 µg/100,000 cells before injection without preincubation.

Immunohistochemistry and immunofluorescence

Immunohistochemistry (IHC) was performed according to the standard protocols. For the anti-CD31 and anti-EGFRvIII double labeling, the MACH 2 Double Stain Kit was used using the Vulcan Fast Red chromagen to detect the anti-CD31 antibody. For staining of cultured tumor spheres, spheres were fixed with paraformaldehyde, permeabilized with Triton X-100, and subjected to a standard PBS-based immunofluorescence protocol. For immunofluorescence, samples were visualized using a Leica SP2 confocal microscope with a ×63 oil-immersion objective.

Creation, expression, and purification of antibody constructs

Anti-CD133 single chain variable fragment (scFv) was obtained by PCR amplification of the variable regions of the light and heavy chains from AC133.1 hybridoma (ATCC No. HB-12346). Assembly of the scFv was done by splicing using overlap extension PCR and the purified PCR product was cloned into phagemid pAK100 (kind gift from Prof. Andreas Pluckthun, Biochemical Institute, University of Zurich, Zurich, Switzerland). Anti-EGFRvIII scFv was artificially synthesized on the basis of the published sequence available from Genbank (accession no. U76382). A pBudCE4:Her2:CD16 bispecific minibody vector was used as the base vector and was a kind gift from Dr. Louis Weiner, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC. This construct has scFv against Her2/neu and CD16 in a bicistronic vector and the CH3 region has a "knob-into-hole" configuration to enhance heterologous dimer formation. We replaced the scFvs for Her2/neu with the scFv of anti-EGFRvIII antibody and anti-CD16 scFv with the scFv of anti-CD133. V5 and His6 tag sequences are present at the 3'-end of the anti-EGFRvIII binding arm, and Myc and His6 tag sequences are present at the 3'-end of the anti-CD133 binding arm. As controls, antibodies containing either two scFv chains against AC133 or EGFRvIII were also created. The constructs were stably transfected into HEK 293 cells.

Antibodies were purified from prepared supernatant using a HisTrap column protocol (Amersham Biosciences).

Plasma membrane preparation and ELISA for BsAb analysis

Plasma membrane protein was quantitated and adsorbed onto 96-well ELISA plates. Wells were blocked with human γ -globulin and incubated with different concentrations of the antibody. The secondary antibody used to detect the bound antibody was THE Anti-c-Myc-tag [HRP] monoclonal antibody (mAb; GenScript). Plates were developed using the SureBlue TMB Microwell Substrate (KPL).

Live cell immunoprecipitation

Cell lines expressing EGFRvIII, CD133, or both were dissociated, blocked with human γ -globin, and counted. Viable cells were incubated with various concentrations of antibodies. For a qualitative analysis of affinity, the cells were lysed and processed for immunoblot analysis with anti-CD133, anti-EGFRvIII, or anti-V5 antibodies. For quantitative analysis of the surface-bound antibody, live cells were eluted with the glycine stripping buffer. Eluted antibody was neutralized and adsorbed on 96-well plates and detected using horseradish peroxidase (HRP)-labeled anti-myc antibody.

Competitive sandwich ELISA assay

U87 cell lines expressing either or both epitopes (10^4 to 10^9 cells) were incubated with 400 nmol/L of BsAb and then added to 96-well plates coated with the membrane fraction from U87 cells transfected with both EGFRvIII and CD133 ($\sim 10^5$ cells). The cells were then washed off and membrane fraction-bound BsAb was quantitated.

NK cell purification and culture

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density-gradient centrifugation. NK cells were purified using anti-CD56-coated microbeads, followed by two rounds of positive selection using autoMACS. CD3⁺ cells were subsequently depleted using anti-CD3-coated magnetic beads.

Cytotoxicity assay

The antibodies were tested for their ability to induce ADCC using NK cells as the effectors against target cells. The aCella-Tox kit was used to quantitate ADCC (Cell Technology, Inc.). The protocol was carried out according to the manufacturers' recommendation and luciferase activity was measured using a luminometer.

Cell lines and expression plasmids used

U87 cells expressing EGFRvIII (U87vIII) were a kind gift from Dr. Donald O'Rourke, Department of Neurosurgery, Penn Neurological Institute, University of Pennsylvania, Philadelphia, PA. U87-EGFRvIII/CD133 double expressing cells lines were generated by stable transfection of EGFRvIII and CD133. The CD133 clone was obtained from Plasmid Information Database (PlasmID) maintained by Dana-Farber/Harvard Cancer Center DNA Resource Core. The CD133 gene was PCR-amplified and subcloned into the pCR3.1 vector. EGFRvIII was also subcloned into the pCR3.1 vector (28).

Statistical analysis

To validate the significance of the observed differences, we analyzed variables using two-sided *t* tests or two-way ANOVA. Differences in survival estimates were calculated using the log-rank test.

Results

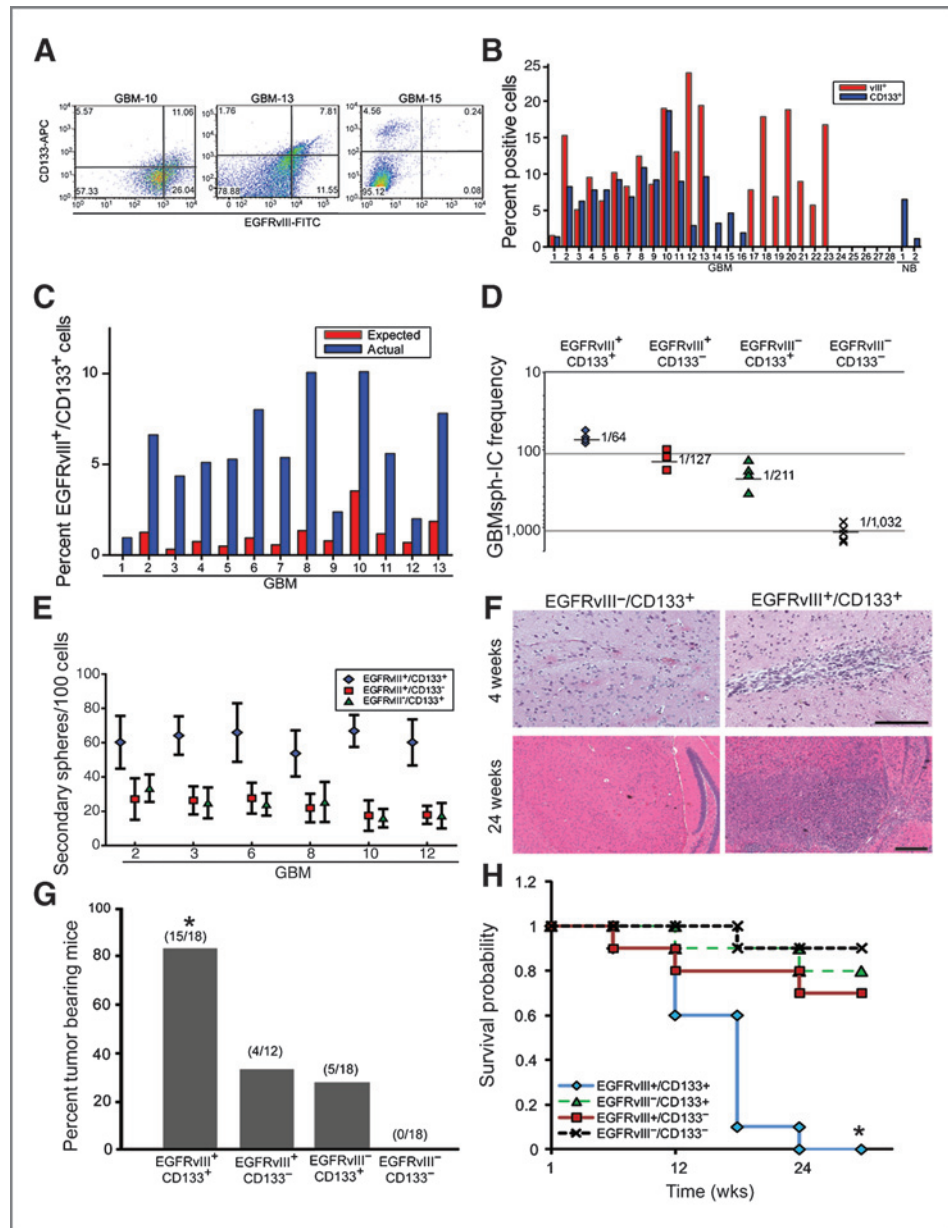
EGFRvIII is found in primary human glioblastoma CSCs

CD133 has been widely used for the identification of glioblastoma CSCs (3). Although some studies have found it to be problematic (29–31), it still remains one of the most generally used markers and has now been used to identify CSCs from colon, lung, ovary, and prostate tumors among many others. We reasoned that if EGFRvIII is found in CSCs, then it may show an association with CD133. We first analyzed 28 freshly resected glioblastoma samples by flow cytometry to assess EGFRvIII and CD133 expression. Figure 1A shows representative flow cytometry plots from three tumors, two showing coexpression of EGFRvIII and CD133 (GBM-10 and GBM-13) and one EGFRvIII⁻/CD133⁺ tumor (GBM-15). The gating strategy for these results is demonstrated in Supplementary Fig. S1. We found that 57% (16 of 28) of tumors were CD133⁺ (as well as two normal brain samples from adult resections of epileptic seizure foci), 71% (20 of 28) were EGFRvIII⁺, and 81% (13 of 16) of the CD133⁺ tumors also expressed EGFRvIII (Fig. 1B). Patient time-to-recurrence, survival, EGFRvIII/CD133 status, tumor location, and treatment data for available samples are shown in Supplementary Table S1, and EGFRvIII/CD133 expression in all samples is shown in Supplementary Table S2. We consistently observed a significant fold increase in the actual percentage of cells that express both proteins as compared with the coexpression expected through random coincidence (Fig. 1C, *P* < 0.001 for average of entire set), demonstrating that EGFRvIII is preferentially expressed with CD133 in a subset of tumor cells.

Tumor-derived neurospheres, or tumor spheres are widely used as an *in vitro* model for studying CSCs, and EGFRvIII has been shown to be expressed in these cells (32, 33). We hypothesized that the EGFRvIII⁺ cells should show enhanced tumor sphere-forming ability. Using LDA of the four sorted populations from six primary glioblastoma samples, we observed that EGFRvIII⁺/CD133⁺ and EGFRvIII⁺/CD133⁻ cells had the highest frequency of glioblastoma sphere-initiating cells (1 of 64 and 1 of 127 respectively) as compared with EGFRvIII⁻/CD133⁺ (1 of 211), and EGFRvIII⁻/CD133⁻ cells (1 of 1,032; Fig. 1D). Redissociated tumor spheres from EGFRvIII⁺/CD133⁺ and EGFRvIII⁺/CD133⁻ cells consistently showed the highest potential for sphere self-renewal compared with the EGFRvIII⁻/CD133⁺ populations (Fig. 1E). In addition, we observed in EGFRvIII⁺/CD133⁻ tumors, that selection for EGFRvIII⁺ resulted in a population capable of self-renewal, whereas the EGFRvIII⁻ population showed no self-renewal (4 of 4 tumors analyzed).

An essential property of CSCs is their tumor-forming ability in mice. CD133⁺ cells from primary glioblastoma tumors show enrichment for tumor formation when implanted intracranially into NOD/SCID mice (3). Figure 1F shows representative tumors formed 4 and 24 weeks after injection with EGFRvIII⁺/CD133⁺ versus EGFRvIII⁻/CD133⁺ primary glioblastoma

Figure 1. EGFRvIII expression and functional characteristics. Primary glioblastoma and normal brain samples were dissociated and analyzed. **A**, flow cytometry plots of EGFRvIII (x-axis) and CD133 (y-axis) surface expression in live dissociated glioblastoma cells. Representative plots are shown from 28 samples analyzed. **B**, percentage of EGFRvIII- and CD133-expressing cells in all samples tested, including two normal brain samples (NB) from adult seizure focus resection of epileptic brain. **C**, comparison of actual versus expected coexpression. **D**, an extreme LDA algorithm was used to determine the frequency of GBMsp-forming EGFRvIII⁺/CD133⁺ cells (◆), EGFRvIII⁺/CD133⁻ cells (■), EGFRvIII⁻/CD133⁺ cells (▲), and EGFRvIII⁻/CD133⁻ cells (X). **E**, neurospheres from **D** were redissociated without sorting to assay for self-renewal of EGFRvIII⁺/CD133⁺ cells (◆), EGFRvIII⁺/CD133⁻ cells (■), and EGFRvIII⁻/CD133⁺ cells (▲). Plotted is the mean ± SEM. **F**, sorted primary glioblastoma samples were intracranially injected into NOD/SCID mice. Shown is representative hematoxylin and eosin staining of brains injected with 1,000 EGFRvIII⁺/CD133⁺ or EGFRvIII⁻/CD133⁺ cells at 4 weeks and 24 weeks post injection. Scale bars, 500 μm. **G**, tumor incidence from injection of sorted cells into the brains of NOD/SCID mice. *, *P* < 0.001. **H**, Kaplan–Meier survival probability estimates of mice injected with sorted cells. *, *P* < 0.001. See also Supplementary Figs. S1 and S2 and Supplementary Tables S1 and S2.



cells. Review of the histopathology of these tumors shows several classic features of glioblastoma, including increased vascularity, pseudopalisades, increased mitoses, and varied cellular morphology, but there did not seem to be any consistent difference in tumor histology among the immunophenotypic groups. Also, it has been reported that glioblastoma CSCs exist in a perivascular niche (34) and can transdifferentiate (35, 36) into endothelial cells. Interestingly, when both primary human glioblastoma samples and mouse xenografts from cultured human glioblastoma tumor spheres were stained for EGFRvIII alone or EGFRvIII in combination with the endothelial marker CD31, they occasionally revealed the presence of EGFRvIII around and in vessels (Supplementary Fig. S2). In primary human glioblastoma samples, this staining pattern was observed in 23% of the human glioblastoma

tumors analyzed (5 of 21), demonstrating another property that EGFRvIII⁺ cells have in common with CSCs. In agreement with previous reports (20, 21), overall EGFRvIII staining appeared to be either sporadic or focal with no strong association with the edges of necrotic areas.

Using six glioblastoma samples, we found that 83% (15 of 18) of mice receiving EGFRvIII⁺/CD133⁺ cells, and 33% (4 of 12) of mice receiving EGFRvIII⁺/CD133⁻ cells developed tumors, whereas only 28% (5 of 18) of mice receiving EGFRvIII⁻/CD133⁺ cells showed tumors (Fig. 1G). None of the mice (0 of 18) injected with EGFRvIII⁻/CD133⁻ cells developed tumors even with doses as high as 100,000 cells. The survival probability of EGFRvIII⁺/CD133⁺ cell-injected mice was also significantly decreased as compared with mice injected with the EGFRvIII⁺/CD133⁻ or EGFRvIII⁻/CD133⁺ population (Fig. 1H).

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EGFRvIII⁺ tumor spheres recapitulate properties observed with primary tumor cells

EGFRvIII amplification and expression is rapidly lost when glioblastoma tumors are grown under standard serum-based culture conditions (37). However, because tumor spheres represent the CSC population in cell culture if EGFRvIII expression marks a CSC population, then its expression should be retained in spheres grown under stem-cell culture conditions. EGFRvIII expression was found in all EGFRvIII⁺/CD133⁺ cultured tumor spheres (GBMsp) derived from EGFRvIII⁺/CD133⁺ primary glioblastomas when examined by immunoblot, reverse transcriptase (RT)-PCR, flow cytometry, and immunofluorescence (Figs. 2A–C, 3 and 4). Expression was not detected in the cultured spheres derived from normal tissue or primary gli-

blastoma tumors that were EGFRvIII⁻ by flow cytometry. These data demonstrate that EGFRvIII does not arise spontaneously during culture, and also suggest that it is the selection for CSC properties that preserves EGFRvIII expression in tumor spheres. EGFRvIII⁺ tumor sphere cells also coexpress CD133 (Fig. 2C), maintain greater than expected coexpression of both receptors (Fig. 2D), and are the highest self-renewing population of cells (Fig. 2E), just as in the original tumors. Finally, EGFRvIII⁺ cultured tumor spheres are also tumorigenic, as orthotopic transplantation of 10⁴ cells into NOD/SCID mice cortex formed high-grade glioma tumors (Fig. 2F). These data extend the results of others, showing EGFRvIII expression in spheres (38, 39) by suggesting that it is the EGFRvIII⁺ cells that are the key cells within glioblastoma tumor spheres for self-renewal and

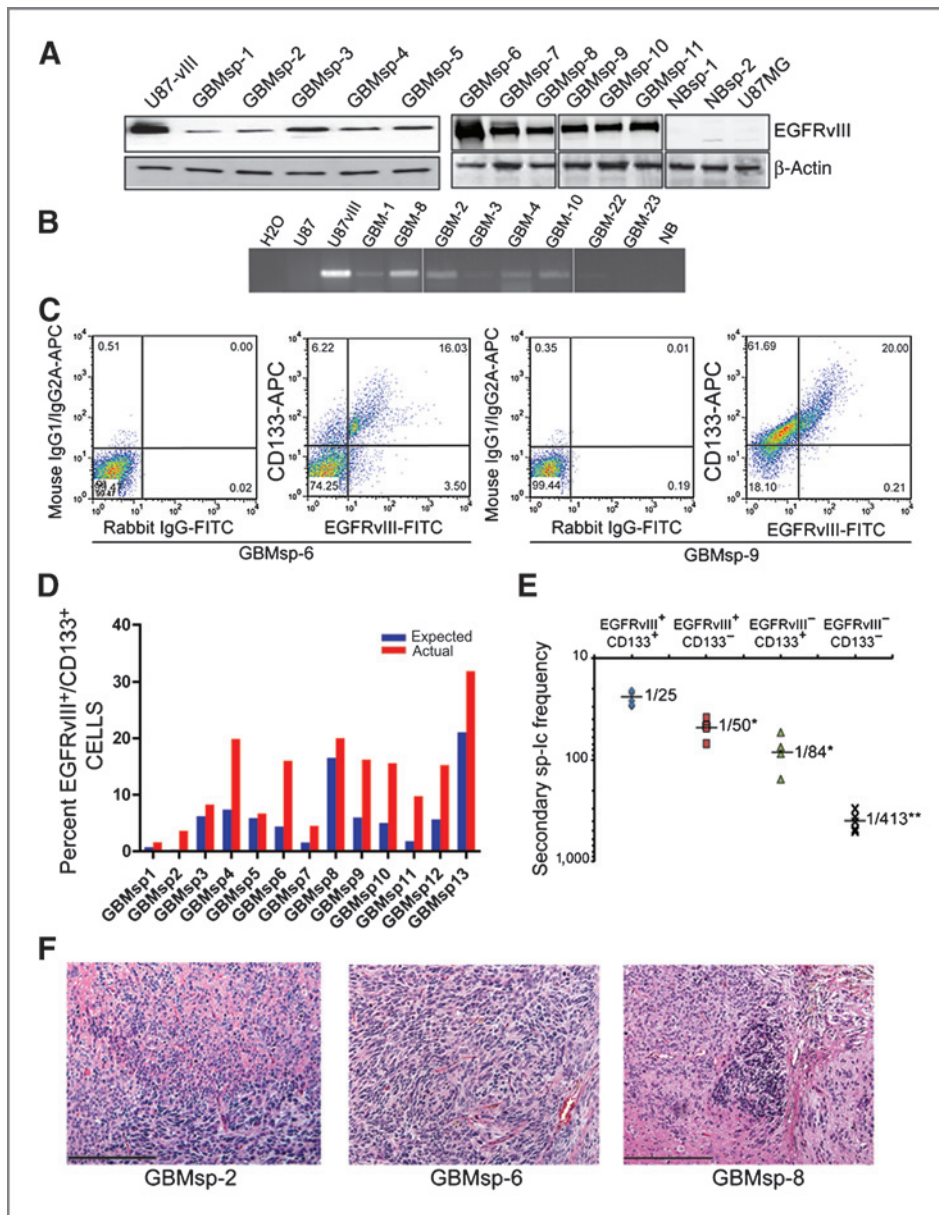


Figure 2. Expression of EGFRvIII in GBMsp. Tumor spheres derived from dissociated primary human glioblastomas were cultured in stem-cell media prior to analysis. A, immunoblot analysis of lysates from GBMsp, normal brain spheres (NBsp) from epileptic patients, and control U87MG and U87-vIII for EGFRvIII expression and β -actin. B, RT-PCR for EGFRvIII transcription from total RNA of cultured GBMsp and normal brain (NB) and U87MG and U87-vIII samples as controls. C, flow cytometry analysis of coexpression of EGFRvIII (x-axis) and CD133 (y-axis) in dissociated GBMsp. Left, isotype control background (mouse IgG1/IgG2B for CD133 and rabbit IgG for EGFRvIII); right, signal for anti-CD133 and anti-EGFRvIII. D, comparison of actual versus expected CD133/EGFRvIII coexpression in all cultured GBMsp samples tested. E, secondary sphere initiating (sp-ic) frequency of six tumor sphere lines from cells sorted into four populations EGFRvIII⁺/CD133⁺ (◆), EGFRvIII⁺/CD133⁻ (■), EGFRvIII⁻/CD133⁺ (▲), EGFRvIII⁻/CD133⁻ (x). *, $P < 0.01$; **, $P < 0.001$. F, cultured GBMsp cells were intracranially injected into NOD/SCID mice and assayed for tumor formation. Shown are micrographs of hematoxylin and eosin staining of three sphere lines; Scale bar, 500 μ m.

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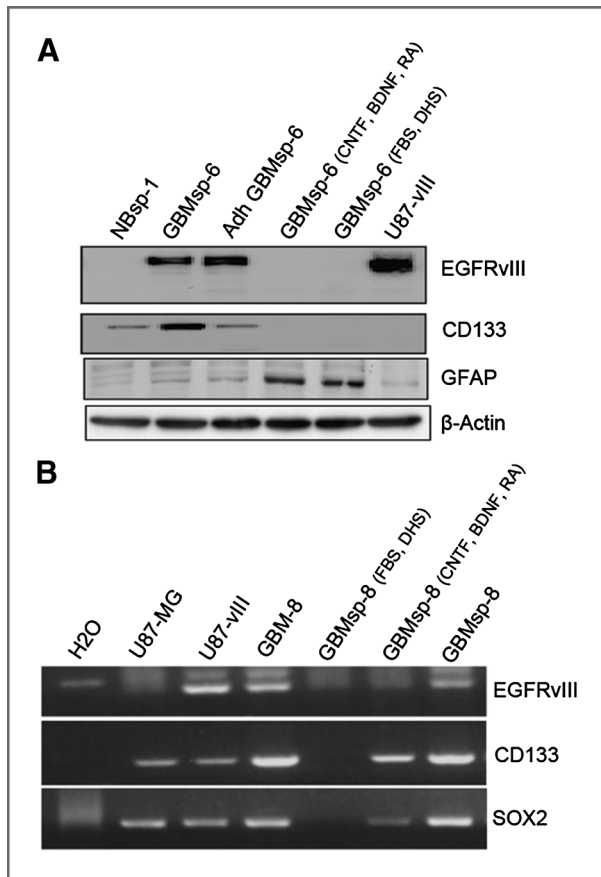


Figure 3. Effects of differentiation on EGFRvIII and marker expression. GBMsp were grown either as a suspension culture or adherent culture in tumor stem media or differentiated by withdrawing EGF and FGF and exposing to two separate differentiating conditions; CNTF, BDNF, and retinoic acid (CNTF, BDNF, RA) or 5% FBS + 5% donor horse serum (FBS, DHS). A, immunoblot analysis with anti-EGFRvIII, anti-CD133, anti-GFAP, and anti-β-actin as control. Four separate cell lines were tested; representative blots of GBMsp-6 are shown. B, whole-cell RNA was used in RT-PCR analysis with primers specific for EGFRvIII, CD133, and SOX2 gene products. Each experiment was performed in two different glioblastoma sphere lines. Representative gels of GBMsp-8 are shown.

tumorigenicity. Overall, the data from Figs. 1 and 2 support our hypothesis that EGFRvIII identifies a putative CSC population. The fact that the EGFRvIII⁺/CD133⁺ population showed the highest tumor formation and self-renewal capacity suggests that the use of these two markers further refines the detection of the CSC population.

EGFRvIII association with stem-cell markers

Much like neural stem cells, during differentiation, glioblastoma CSCs lose expression of stem-cell markers and express markers for neural and astrocytic lineages (40, 41). Immunoblot and RT-PCR analysis comparing undifferentiated versus differentiated tumor sphere samples showed that loss of EGFRvIII expression upon differentiation correlates with the loss or reduction of expression of the stem/progenitor cell markers CD133 and SOX2. Conversely, differentiation resulted

in the expression of the glial lineage marker glial fibrillary acidic protein (GFAP; Fig. 3A and B). To better understand on a cell-by-cell basis the effects of differentiation on EGFRvIII and other markers, we performed immunofluorescence microscopy. In cultured tumor spheres, EGFRvIII showed no coexpression with vimentin, and only very low coexpression with GFAP or Tuj1, but showed a approximately 4- to 17-fold greater coexpression with nestin and stage specific embryonic antigen 1 (SSEA-1; Fig. 4 and Supplementary Table S3). GBMsp cells were differentiated by culture in 10% serum, and similar to Fig. 3, all sphere lines examined lost all EGFRvIII expression, as well as SSEA-1-expression, yet maintained GFAP, vimentin, and Tuj1 (Fig. 4, differentiated). Of note, wtEGFR expression was maintained upon differentiation (data not shown). These findings strongly support the notion that EGFRvIII expression can define a subpopulation of stem/progenitor-like cells in glioblastoma by showing that EGFRvIII is associated with markers of undifferentiated cells that is lost upon differentiation while markers of differentiation are maintained or gained.

Development of a bispecific EGFRvIII/CD133 antibody

If EGFRvIII defines a CSC population, then depleting EGFRvIII⁺ and more specifically EGFRvIII⁺/CD133⁺ cells should prevent tumor formation. A BsAb that recognizes EGFRvIII and CD133 would have the highest affinity toward a cell expressing both the proteins but lower affinity toward cells expressing either antigen alone. scFvs against CD133 and EGFRvIII were cloned. The scFv against EGFRvIII has been previously described (42) and the scFv against CD133 was generated as described in Methods. These scFvs were combined to create the anti-EGFRvIII/CD133 chimera named BsAb, and bivalent constructs for EGFRvIII (di-EGFRvIII) and CD133 (di-CD133). Each construct contains a human IgG1-Fc domain for engaging NK cells (Fig. 5A). We confirmed that the BsAb has the highest binding for cells expressing both epitopes, whereas di-EGFRvIII and di-CD133 have a high affinity for cells expressing only high levels of each protein, respectively (Figs. 5B and Supplementary Fig. S3A and S3B). EGFRvIII⁺/CD133⁺ cells bound BsAb at concentrations as low as 200 nmol/L, whereas mixtures of cells expressing each antigen independently showed low affinity toward the BsAb even at 2,000 nmol/L (Fig. 5B). The BsAb does not cross-link cells independently expressing each protein as pools of U87-EGFRvIII and U87-CD133 cells do not bind (Figs. 5B and Supplementary Fig. S3C). To be effective, the BsAb must be able to identify a rare stem-cell population among the more abundant differentiated cell population. Comparison of an EGFRvIII-specific antibody with the BsAb by IHC demonstrates that the BsAb recognizes only a subset of cells recognized by the EGFRvIII antibody in glioblastoma samples (Supplementary Fig. S3D), and a competitive sandwich ELISA using EGFRvIII⁺/CD133⁺ cells showed that the BsAb bound to dual epitope-expressing cells even in the presence of a vast excess of cells expressing individual proteins (Fig. 5C). Furthermore, using four different neurosphere lines (3 tumor, 1 nonneoplastic), we found specific binding of the BsAb correlated with EGFRvIII⁺/CD133⁺ expression (Fig. 5D). These results demonstrate that the BsAb preferentially binds to individual cells coexpressing both proteins.

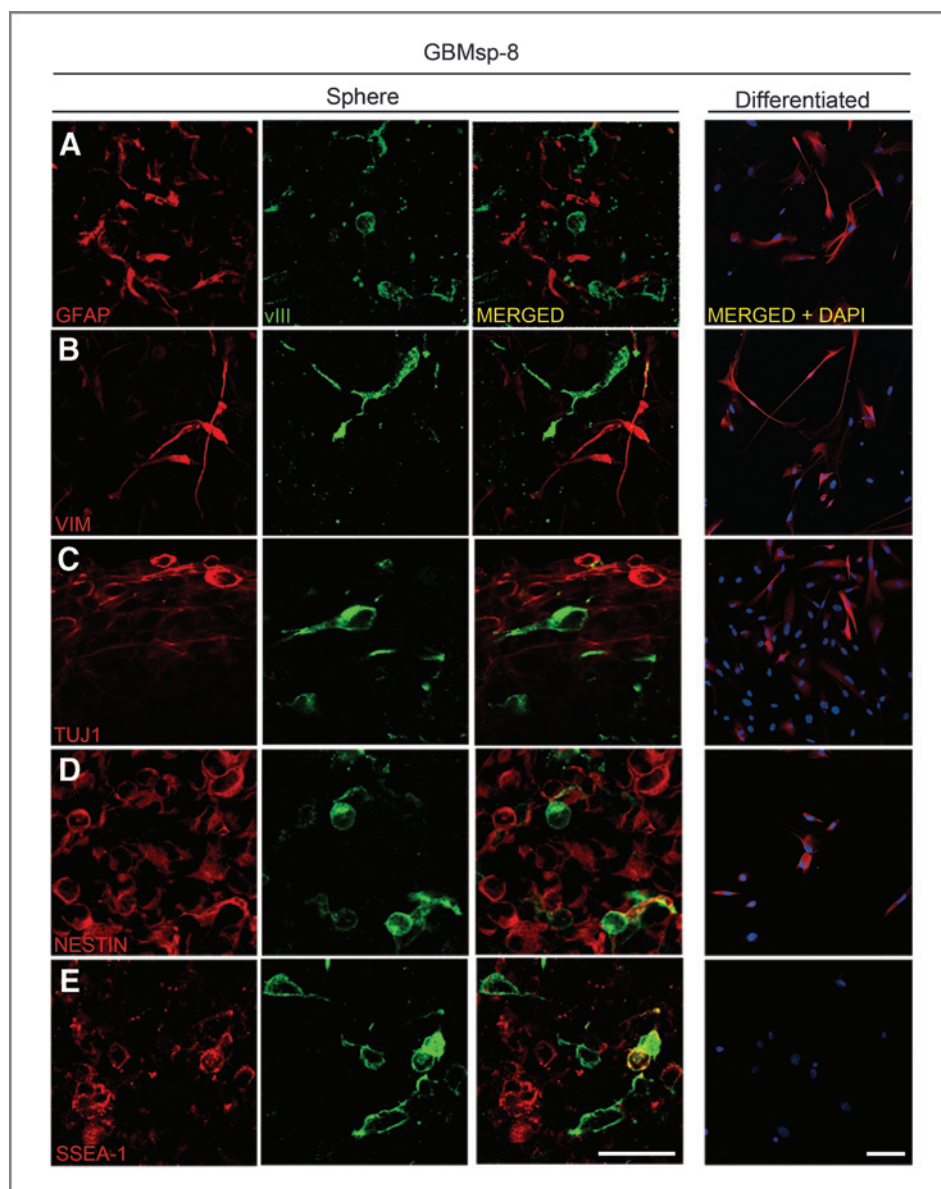


Figure 4. EGFRvIII is not colocalized with markers of differentiation in cultured GBMsp. EGFRvIII⁺ neurospheres were maintained in stem-cell media (Sphere) or dissociated and differentiated with media containing 10% serum (Differentiated). Cells were immunostained with antibodies against EGFRvIII (vIII) and GFAP (A); vimentin (VIM; B); β -III tubulin (TUJ1; C); nestin (NESTIN; D); and SSEA-1 (E), and imaged by confocal microscopy. Each staining combination was performed on three separate glioblastoma neurosphere lines with similar results. Images are from GBMsp-8 (0.16- μ m sections). Scales, 37.5 μ m in A–E (Sphere), and 75 μ m in all differentiated micrographs. See also Supplementary Table S3. DAPI, 4',6-diamidino-2-phenylindole.

The BsAb is cytotoxic to human glioblastoma cells

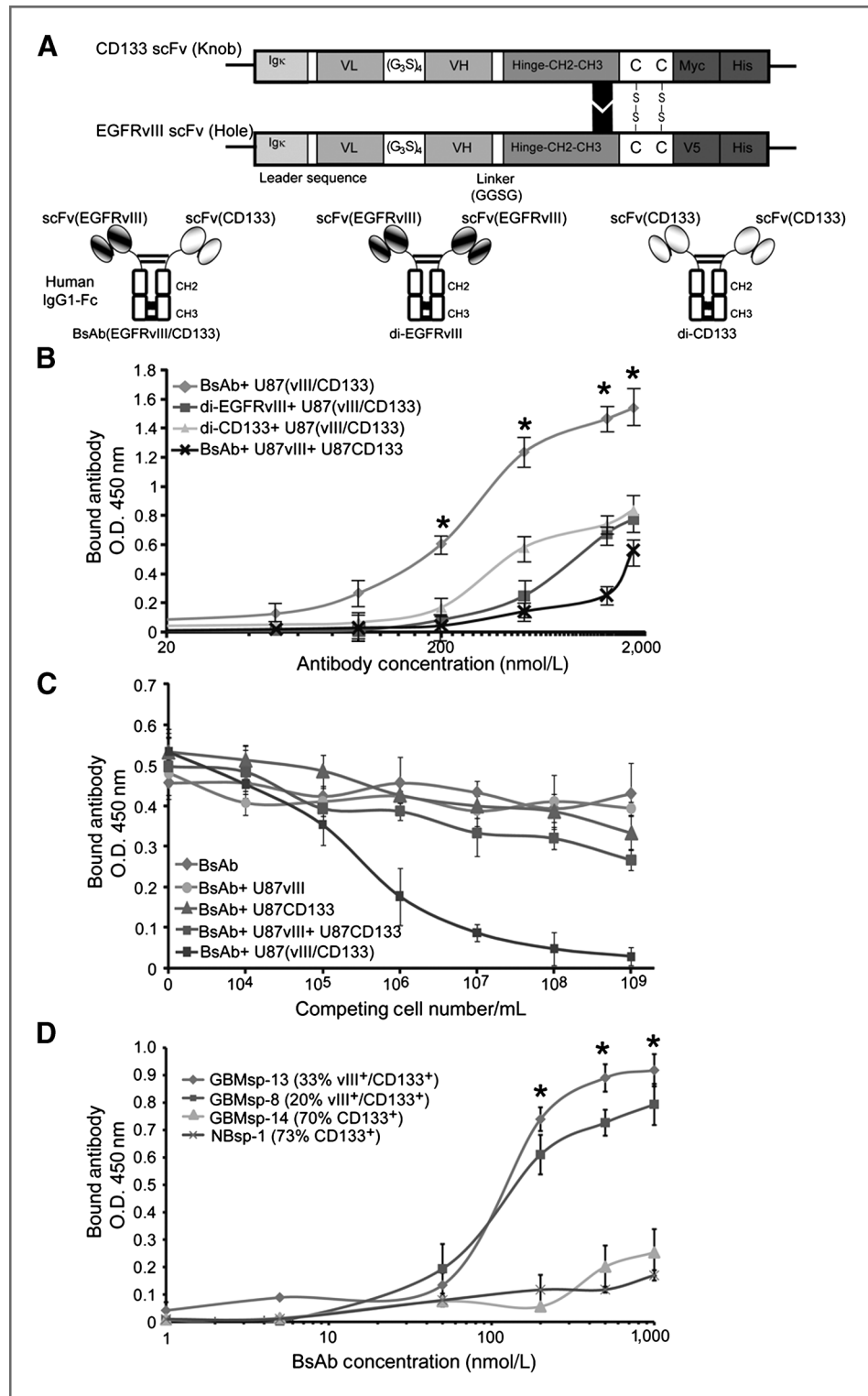
We established that di-EGFRvIII and BsAb exhibited significant NK cell-induced toxicity toward U87-EGFRvIII/CD133 cells (Supplementary Fig. S4A). Then, using a effector to target (E:T) ratio of 10:1, we found that as little as 0.1 μ g/mL (8.3 nmol/L) of BsAb was sufficient to induce cytotoxicity in EGFRvIII⁺/CD133⁺ cells (Supplementary Fig. S4B). Mirroring the binding results, the BsAb induced the greatest cytotoxicity in EGFRvIII⁺/CD133⁺ cells, whereas the divalent constructs worked best in cells expressing only the respective epitope (Supplementary Fig. S4C). Finally, we tested freshly resected glioblastoma tumor (GBM-8) or dissociated GBMsp to assay cytotoxicity toward cells from human patients. In GBM-8 and GBMsp lines that had high EGFRvIII⁺/CD133⁺ cells (GBMsp-4 and GBMsp-6), the BsAb induced higher cytotoxicity compared with GBMsp lines that expressed either CD133 (GBMsp-14 and

GBMsp-15) or EGFRvIII (GBMsp-17) alone (Fig. 6A). Importantly, we also found low toxicity toward normal neural stem cells (NBsp-1 and NBsp-2).

The BsAb decreases self-renewal and tumorigenicity

LDA of GBMsp cells pre and post cytotoxicity assay showed a significantly decreased renewal capability from BsAb treatment, whereas di-EGFRvIII was effective to a lesser degree, and a human IgG1-Fc fragment had no effect. No reagent significantly affected normal brain neurospheres (Figs. 6B and Supplementary Fig. S5). For tumor prevention analysis, the antibody constructs (all at 0.01 μ g/10⁵ cells) were combined with glioblastoma or GBMsp cells and injected into the cortex of NOD/SCID mice, which were sacrificed after 26 weeks and analyzed for tumor formation (Fig. 6C and D). The BsAb severely inhibited tumor

Figure 5. Characterization of the anti-CD133/anti-EGFRvIII BsAb. **A**, schematic of the BsAb showing scFv chains of anti-CD133 and anti-EGFRvIII. Ig κ , leader sequence from κ chain of human IgG1; VL, variable light chain; (G₃S)₄, hinge peptide; V_H, variable heavy chain; CH2-CH3, Fc domain from human IgG1; Myc, V5, and His, tags for purification and identification. Divalent anti-CD133 (di-CD133) and anti-EGFRvIII (di-EGFRvIII) antibodies were also created. **B**, ELISA dose-response analysis of the BsAb (◆), di-EGFRvIII (■), and di-CD133 (▲), reagents on U87 cells cotransfected with EGFRvIII and CD133, and analysis of the BsAb reagent on an equal mix of cells transfected with either EGFRvIII or CD133 (x). *, *P* < 0.001. **C**, competitive sandwich ELISA to identify specificity of BsAb toward EGFRvIII⁺/CD133⁺ cells. BsAb was not preincubated (◆) or preincubated with U87 cells expressing either CD133 (▲), EGFRvIII (●), or both proteins expressed in the same cell (EGFRvIII/CD133; ■), or a pool of cells expressing CD133 or EGFRvIII (■) before incubation with membrane fractions from U87-EGFRvIII/CD133 cells (10⁵ cells per well). **D**, live cell immunoprecipitation and ELISA analysis of BsAb binding to glioblastoma tumor spheres in cultures with variable EGFRvIII and CD133 expression. GBMsp-13 (◆), GBMsp-8 (■), GBMsp-14 (▲), NBsp-1 (x). *, *P* < 0.001. Data points are represented as mean ± SEM of three individual experiments. See also Supplementary Fig. S3.



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formation (26 total mice, 1 of 22 mice showed tumor formation; *P* < 0.001) as compared with Hu-IgG1-Fc that showed no reduction (12 injected, 8 of 8 analyzed showed tumors). Di-EGFRvIII and di-CD133 did not statistically

significantly reduce tumor formation (Fig. 6D). Furthermore, the survival probability of mice receiving the BsAb is significantly increased compared with the monospecific antibody recipients, strongly suggesting that targeting of the

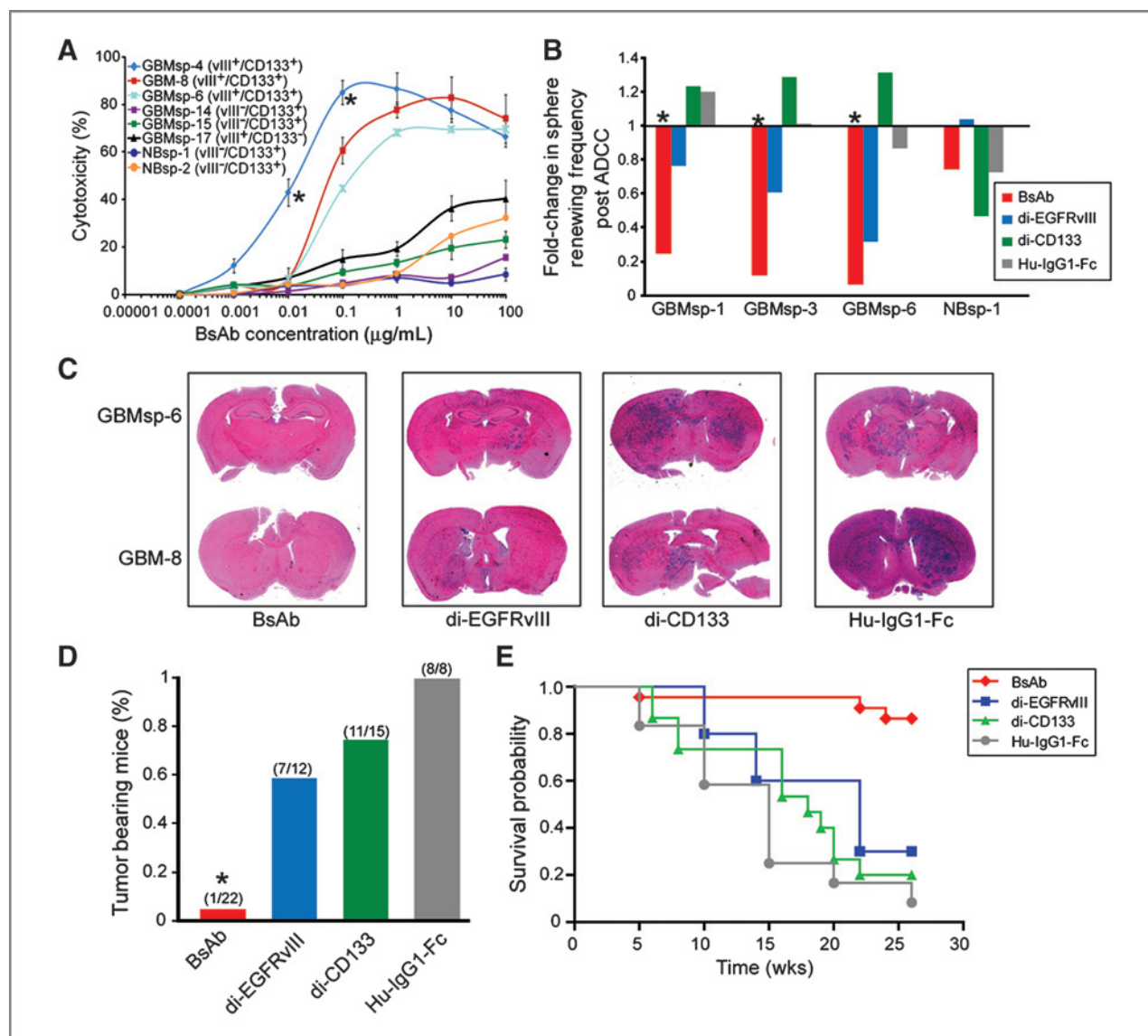


Figure 6. BsAb eliminates EGFRvIII⁺/CD133⁺ cells from culture, enhances cytotoxicity, impairs GBMsp tumorigenicity, and enhances survival. **A**, BsAb ADCC on primary glioblastoma cells (GBM-8), glioblastoma tumor spheres (GBMsp), and normal neurospheres from epileptic patients (NBsp). All experiments were carried out in triplicate. Error bars, \pm SEM. *, $P < 0.001$. **B**, fold-change in the frequency of NBsp- and GBMsp-initiating cells pre- and post-ADCC. Data represented as change in sphere renewing frequency, with and without the presence of BsAb (red) di-EGFRvIII (blue), di-CD133 (green), and human-IgG1-Fc (gray). **C**, antibodies were mixed with unsorted cells (100,000 total) from EGFRvIII⁺/CD133⁺ tumors, injected into the brains of NOD/SCID mice, and assayed after 26 weeks. Coronal sections from mouse brain were stained with hematoxylin and eosin. **D**, percentage tumor-bearing mice from four human glioma samples (GBMsp-4, GBMsp-6, GBM-8, and GBM-12) coinjected with the various antibodies. Mice were analyzed after 26 weeks. **E**, Kaplan-Meier survival probability shows that BsAb significantly enhances survival ($P < 0.0001$) as compared with the monospecific antibodies. See also Supplementary Figs. S4 and S5.

EGFRvIII⁺/CD133⁺ CSC population effectively prevents glioblastoma formation (Fig. 6E).

Discussion

Several studies have shown that EGFRvIII is expressed in tumor spheres derived from glioblastoma samples, but whether this was incidental or significant to CSC biology has not been investigated (38, 39). We have found that EGFRvIII is associated with markers used to identify stem cells or CSCs, including

CD133, nestin, SOX2, and SSEA-1, but shows little to no association with markers of differentiation, including vimentin, Tuj-1, and GFAP. EGFRvIII expression, which is difficult to maintain under standard conditions, is preserved using tumor sphere conditions but the differentiation of tumor spheres results in the loss of EGFRvIII. Cell populations containing EGFRvIII have the highest degree of self-renewal and tumorigenicity. Collectively, our data suggest that the EGFRvIII oncogene can be used to define the CSC population.

Antibodies and small-molecule inhibitors against wtEGFR also bind to EGFRvIII, but are less potent against this variant (43–45). Thus, if EGFRvIII⁺ cells represent the tumor-initiating population, there may be a CSC basis for why anti-EGFR therapy has not been effective against tumors expressing EGFRvIII (46). Interestingly, a peptide vaccine against EGFRvIII has demonstrated improved overall survival for glioblastoma patients in phase II clinical trials (19). Our data also provide an explanation for why therapy directed against a minority of cells can be effective in increasing survival: it is the targeting of CSCs and not the bulk of the tumor that can indeed be therapeutically effective (19). The vaccine is administered after total resection and radio-/chemotherapy and serves to inhibit tumor recurrence. The finding that the anti-EGFRvIII and BsAb can effectively prevent intracranial tumor formation suggests that it can be used in a similar manner where patients are treated immediately postresection to prevent tumor recurrence. Because these antibodies are passive immunotherapy, it would have specificity against the cells responsible for recurrence and complement the effects of the vaccine if used in combination. Recent preliminary experiments show that the BsAb can be used to treat existing intracranial tumors in mice, indicating that this agent can penetrate the blood–brain barrier. Whether the BsAb studied here can ultimately be used in clinical trials awaits further preclinical work to confirm that it can induce the regression of preexisting tumors in animal models.

Although CD133 has been widely used to isolate CSC in many solid tumors, its use has been debated (29–31). Clearly, this marker can enrich for CSCs, but our work and other studies show that the true CSC may comprise only a fraction of the marker-positive pool. We have found that EGFRvIII⁺/CD133⁺ primary tumor cells are the most highly enriched population for self-renewal and tumorigenicity, strongly suggesting that the addition of EGFRvIII expression to CD133 expression more accurately defines a CSC population in glioblastoma. An important corollary of the CSC hypothesis is that elimination of the cancer stem-cell population is necessary and sufficient to eliminate a tumor. We tested this notion by developing the BsAb to precisely recognize EGFRvIII⁺/CD133⁺ cells. The specific lysis of the EGFRvIII⁺/CD133⁺ population significantly reduces the implantation of primary glioblastoma tumors in mice and prolongs survival. Because these mice were observed for 26 weeks, these data also suggest that the spontaneous acquisition of tumor-initiating properties by non-EGFRvIII⁺/CD133⁺ cells is likely to be very low. Thus, our findings provide support for the hypothesis that elimination of CSCs is sufficient to eradicate a tumor *in vivo*.

A dual-marker approach may prove more specific than the targeting of a single marker for several reasons. Nearly all studies isolating CSCs have relied on normal stem-cell markers, such as CD133 (3) or CD15/SSEA-1 (4, 47, 48), which would present a potential toxicity problem if used as a target. This is especially relevant in the case of brain tumors as the brain has a finite pool of stem cells and limited capacity for regeneration. Many markers have been pro-

posed to define glioblastoma CSCs (3–5) yet no single marker has definitively isolated these cells. The true CSC population may comprise only a fraction of any marker-positive pool. The addition of another marker provides an independent selection that enhances the identification of the CSC. This work represents another step toward the goal of precisely identifying the CSC population, which is likely to require a series of studies building upon this and other findings.

The BsAb could be useful in treating other tumor types such as ovarian, prostate, colon, and lung, in which CD133 (49) and EGFRvIII expression (11, 13) has been reported. However, our bispecific approach demonstrates a proof-of-principle that could be extended to other cell-surface protein and stem-cell marker combinations. It is likely that there are other oncogenic receptors that show CSC-specific expression. Amplification of growth factor receptors is a common event in aggressive malignancies and has been implicated in the genesis of tumors through expansion of the CSC population. For example, the overexpression of HER2 in breast cancer has been linked to an increase in the CSC population (50). As more receptors and CSC markers are identified, this unique approach can be used to develop strategies to identify tumor-initiating cancer stem cells in a wide variety of malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.S. Mitra, G. Li, A.J. Wong

Development of methodology: D.R. Emlet, P. Gupta, M. Holgado-Madruga, S.S. Mitra, S.-Y. Han, H. Vogel, S.S. Skirboll, A.J. Wong

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.R. Emlet, P. Gupta, C.A. Del Vecchio, S.S. Mitra, G. Li, S.S. Skirboll

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.R. Emlet, P. Gupta, C.A. Del Vecchio, S.S. Mitra, G. Li, K.C. Jensen, A.J. Wong

Writing, review, and/or revision of the manuscript: D.R. Emlet, P. Gupta, M. Holgado-Madruga, C.A. Del Vecchio, S.S. Mitra, G. Li, S.S. Skirboll, A.J. Wong

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.R. Emlet, P. Gupta, S.-Y. Han, L.W. Xu, A.J. Wong

Study supervision: S.S. Mitra, A.J. Wong

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