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Brevican Knockdown Reduces Late-Stage Glioma Tumor Aggressiveness

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Abstract

Growing evidence supports the important role of the tumor microenvironment (TME) in cancer biology. A defining aspect of the glioma TME is the unique composition and structure of its extracellular matrix (ECM), which enables tumor cells to overcome the inhibitory barriers of the adult central nervous system (CNS). In this way, the TME plays a role in glioma invasion and the cellular heterogeneity that distinguishes these tumors. Brain Enriched Hyaluronan Binding (BEHAB)/brevican (B/b), is a CNS-specific ECM constituent and is upregulated in the glioma TME. Previous studies have shown B/b exerts a pro-invasive function, suggesting it may represent a target to reduce glioma pathogenesis. Herein, we also provide evidence that B/b expression is enriched in the glioma initiating cell (GIC) niche. We demonstrate B/b plays roles in the pathological progression, aggressiveness, and lethality of tumors derived from human GICs and traditional glioma cell lines. Interestingly, we found that B/b is not required to maintain the defining phenotypic properties of GICs and thereby acts primarily in late stages of glioma progression. This study suggests that the increased expression of B/b in the TME is a valuable therapeutic target for glioma.

Keywords

brevican; glioma; neural extracellular matrix; proteoglycan; central nervous system

INTRODUCTION

The tumor microenvironment (TME) plays an important role in cancer biology by facilitating interactions between tumor cells and their surrounding environment [1]. As in other cancers, the TME is important in the pathogenesis of glioma. High-grade gliomas (HGGs) are the most common and fatal form of primary intracranial tumors. Despite

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ETHICAL STATEMENTS

These experiments comply with the ethical standards and current laws of the country in which the research was performed. The authors declare they have no conflict of interest.

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significant progress in developing more effective adjuvant therapies for these tumors, patient mortality remains high [2]. Several aspects of glioma pathophysiology enable tumor cells to evade therapeutic treatments [3], including their ability to invade the normal surrounding brain tissue and their highly heterogeneous cellular composition.

The ability of glioma tumor cells to invade the normally inhibitory barriers of the CNS is unique, as even the most invasive peripheral tumors rarely invade the surrounding neural tissue [4]. This portends a unique interaction between glioma cells, the TME and the normal brain environment facilitates glioma invasion. The TME also provides a niche for establishing and maintaining the heterogeneity of cellular subpopulations within glioma tumors, including the treatment-resistant glioma initiating cell (GIC) population [5–7]. The broad functionality of the glioma TME in mediating aspects of glioma progression makes it an attractive therapeutic target [8].

The neural extracellular matrix (ECM) protein, Brain Enriched Hyaluronan Binding (BEHAB)/brevican (B/b), is a major constituent of the glioma TME. B/b is a CNS-specific member of the lectican family of chondroitin sulfate proteoglycans [9–11]. B/b expression is significantly increased in gliomas relative to normal brain [12]. B/b has been correlated with gliomas exhibiting proneural-like phenotypes [13–15], however B/b is overexpressed in the vast majority of HGGs and likely contributes to the progression of all molecular subtypes [12].

Several isoforms of B/b are increased in human high-grade gliomas compared to normal brain tissue, including glycosylated isoforms that are unique to gliomas [12,16–22]. Differentiated glioma cell lines implanted intracranially recapitulate the endogenous expression of B/b in clinical gliomas [12]. However, these cells lose B/b expression when implanted subcutaneously or grown *in vitro* [12], making it difficult to study the molecular mechanisms of the native protein and the role of B/b in a physiologically relevant environment. Forced overexpression of B/b through transfection and subsequent cleavage of the full-length protein to release bioactive fragments increases brain tumor aggressiveness in rodent models by enhancing tumor cell invasion [18,20,22,23]. A recent study demonstrated that knockdown of transfected B/b reduces the growth of subcutaneously implanted gliomas [24]. However, the effect of suppressing endogenous levels of B/b, which is naturally upregulated in gliomas, has not been previously investigated. Moreover, the effects of suppressing B/b in the critical GIC population still remain unknown.

The purpose of this study was to investigate the effects of B/b knockdown in intracranial invasive glioma and its potential impact in the GIC population to reduce tumor formation and prolong survival. Our results suggest that B/b may be a passenger gene during initial stages of tumor formation but may have significant impact in the late, more invasive stages of glioma progression as GICs become differentiated in the tumor parenchyma.

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MATERIALS AND METHODS

Cell Culture

CNS-1 cells were cultured as previously described [19]. GIC lines 0627 and 0913 were previously described [25]. GICs were cultured in a modified Sato's Medium [26] supplemented with 10ng/mL EGF and bFGF.

shRNA-mediated knockdown of B/b

siRNAs were transformed into shRNAs and cloned into the pRNAT-U6.1/Hygro vector (GenScript, Piscataway, NJ) for rodent models and the lentiviral delivery vector pLentiLox3.7 (pLL3.7) [27] (Addgene, Cambridge, MA, USA) for human GICs. Lentiviral production was performed as previously described [28]. To verify B/b knockdown, sample preparation and Western blotting was carried out as specified previously [21,29]. Isotopic *in situ* hybridization of B/b mRNA was performed as previously described [9], in all cases no signal was observed from sense probes.

Cell Viability Assays

For CNS1 cells, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit was used (Promega, Madison, WI). For GICs, a standard MTT assay was conducted on cells growing as non-adherent spheres. Absorbance levels were normalized to wells containing only media. Statistical comparisons were performed by two-way ANOVA.

Differentiation Assay

GICs were seeded on coverslips pre-coated with 5μg/mL laminin (Millipore). Media was changed to Sato's Medium Base without EGF and bFGF that was supplemented with 10% FBS and cells were collected at 12 days in vitro (DIV). Non-differentiated cells were plated on laminin-coated coverslips and collected at 3DIV.

Human Tumors

Paraffin-embedded human brain tumors classified as glioblastoma (GBMs) were fixed in 4% formalin and prepared at 5μM sections. Sections were rehydrated and heat-mediated antigen retrieval was completed with sodium citrate buffer.

Stereotaxic Tumor Implantation

For *in vivo* studies using CNS-1 cells or GICs, adult female Lewis rats or nude CD-1 mice were used, respectively (Charles River Laboratory, New Brunswick, NJ). Protocols were approved by Yale University and State University of New York Upstate Medical School animal care committees.

For intracranial CNS-1 cell grafts, 1.5×10^5 cells in 1.5μL was injected into the right thalamus of rats at 3.2mm posterior and 2.3mm to the right of Bregma at a depth of 5.5mm. Survival was plotted according to the method of Kaplan and Meier and statistical significance was determined using the Log-Rank (Mantel-Cox) test.

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For intracranial GIC grafts, 1.1×10^6 cells in 1.5μL was injected into the right striatum of CD-1 nude mice at 0.5mm anterior and 2.0mm to the right of Bregma at a depth of 3.5mm. Pairs of control and shB/b animals were established at the time of tumor implantation. Both animals in the pair were sacrificed when one was showing signs of neurological deterioration. To control for differences in survival times, analyses were carried out between animal pairs. Statistical analysis for survival was carried by Log-Rank (Mantel-Cox) test.

Tumor Analysis

Free-floating 40 μM coronal sections were collected and every sixth (CNS-1) or eighth (GICs) sections were analyzed for tumor volume reconstruction. GFP expression by implanted tumor cells was used to define tumor boundaries for histological analysis. Tumor volume was reconstructed based on the method of Cavalieri [30], using the formula $V= (0.24)[\Sigma_1^n(y)] - (0.04)y_{max}$. Anteroposterior spread (A/P spread) was determined by calculating the distance spanning sections with sparse tumor cells at the most rostral and caudal limits and was used as a measure of tumor cell dispersion. For CNS-1 tumors, statistical significance was determined by a standard two-tailed, equal variance *t*-test. Due to the paired nature of studies done with GICs, statistical significance was determined with the Wilcoxon matched-pairs signed rank test. The experimenter was blinded to the identity of all animals.

* The numbers of animals used for *in vivo* studies are specified in the text as (n= numerical value). In all studies, statistical significance was determined by a *p* value that was less than 0.05. See also Supplemental Methods. A list of shRNA and primer sequences and antibodies used in this study is detailed in Supplemental Table 1 and 2.

RESULTS

B/b knockdown in an invasive rodent model decreases tumor size and prolongs survival

The CNS-1 cell line is a reliable rodent model of invasive glioma, as the invasive characteristics and syngeneic nature of intracranial grafts created with this cell line are similar to human gliomas [17,18,31]. A fundamental question remains as to whether reducing B/b expression, which is naturally upregulated in glioma, could reduce the progression of these tumors. Therefore, we evaluated the effects of reducing B/b expression through shRNA-mediated knockdown on the pathophysiology of tumors created using the wildtype CNS-1 rodent model of invasive glioma.

To overcome the absence of endogenous B/b expression in CNS-1 cells *in vitro*, control and shRNA constructs designed against B/b (designated shB/b) were co-transfected with cDNA encoding rat full-length B/b. Western blot analysis confirmed the ability of shB/b constructs to reduce the expression of rat B/b in CNS-1 cells (Figure 1A). Stable transfection with these constructs showed no effect on cell proliferation *in vitro* by MTT assays (data not shown).

To analyze the functional consequences of reduced B/b we turned to an *in vivo* intracranial graft model, in which both the induction and importance of B/b has been previously detailed [12,18]. Wildtype CNS-1 cells were engineered to stably express either control or shB/b and

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were injected to the thalamus of adult rats to establish intracranial grafts. At 12 days post implantation (DPI) the brains of these animals were collected for histological examination (Figure 1B,C). Manual tumor volume reconstruction was derived using the Cavalieri estimate. Control tumors (n= 11) had a mean total tumor volume of 71.44 ± 9.595mm^3 while tumors expressing shB/b (n= 11) experienced a 34% reduction in mean volume, at 47.36 ± 2.130mm^3 (p = 0.024) (Figure 1D). Control tumors had a mean anteroposterior spread (A/P spread) of 5.415 ± 0.283mm while tumors expressing shB/b experienced a 27% reduction in A/P spread, at 3.9898 ± 0.07572mm (p= < 0.001) (Figure 1E).

To confirm that the reduction in tumor growth was due to reduced B/b expression, we performed in situ hybridization and verified that shB/b effectively reduced endogenous B/b expression in CNS-1 derived tumors (Figure 2A). We next evaluated animal survival. Rats with control gliomas (n= 9) reached their survival endpoint within 16.56 ± 2.79 DPI on average. Rats harboring gliomas with either of the two independent shB/b constructs (n= 10) survived 22% longer, with mean overall-survival of 20.6 ± 2.3 and 19.8 ± 0.87 DPI (p < 0.01) (Figure 2B).

B/b expression is enriched in the glioma initiating cell niche and expressed by GICs in vitro

We next evaluated the distribution of B/b protein in human HGGs. Interestingly, B/b was non-homogeneously distributed throughout the tumor core of human GBM tumors, wherein distinct sub-regions stained more intensely for B/b than others. We determined that sub-regions highly reactive for B/b preferentially surrounded cells that expressed Olig2 and CD133, markers of highly tumorigenic cell populations including the glioma initiating cell (GIC) subclass (Figure 3A–C).

To examine the role of B/b in the GIC population, we utilized two GIC cell lines 0627 and 0913 [25]. Although cultures of GICs have been documented to endogenously express the B/b gene (BCAN) *in vitro* [13], its expression has not been studied in detail. We first evaluated the expression of *BCAN* by RT-PCR. As expected RT-PCR yielded no band in U373MG cells, while 0627 and 0913 GICs yielded an intensely reactive band for the *BCAN* gene (Figure 3D). Western blot analysis showed that both 0627 and 0913 cell lysates expressed full-length B/b protein, which resolved at ~150kD for both cell lines. In conditioned media samples, full-length B/b and the ~80–90kD C-terminal proteolytic cleavage fragment were primarily detected in 0627 and not 0913 GICs (Figure 3E).

B/b knockdown has no obvious effects on GIC phenotype in vitro

We next investigated the function of B/b in GICs. We hypothesized that while the function of B/b in traditional serum cultured glioma cell lines is primarily associated with tumor cell invasion, B/b may have additional functions to support the defining characteristics of GICs.

Stable 0627 GICs expressing either the control plasmid or shRNA against human B/b (shB/b) were created. Western blot analysis confirmed that GICs expressing either independent shB/b construct significantly reduced all endogenous B/b protein isoforms (Figure 4A). Endogenous expression of B/b by GICs enabled us to investigate its

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physiological function using shRNA-mediated knockdown *in vitro*. We found that B/b knockdown had no effect on the growth potential of GICs *in vitro* (p= 0.6770) (Figure 4B). Likewise, the ability of GIC clones to form spheres between passages was not affected (Figure 4C). We further investigated the self-renewal capacity of GICs by analyzing the ability of single clones to form spheres and found no significant difference in the percentage of spheres formed (p= 0.4182) or their circumference (p= 0.1864) (Supplemental Figure 1). We found no differences in the expression of cell-fate markers in undifferentiated conditions or in the ability of GICs to differentiate in response to serum after B/b knockdown (Figure 4D, E). We performed Transwell-migration assays using GICs and found no consistent differences in the migration or invasion of control and shB/b shRNA expressing cells in these assays (data not shown).

Genes encoding transcription factors oligodendrocyte lineage transcription factor 2 (*OLIG2*), delta-like 3 (*DLL3*), and achaete-scute complex homolog 1 (*ASCL1*) are highly expressed with BCAN in the pro-neural molecular subtype [13,14]. Importantly, these genes play a significant role in maintaining the self-renewal and tumorigenic qualities of GICs [32,33]. Therefore our findings suggested B/b is not an extracellular regulator of pro-neural gene expression. We confirmed the enriched expression of pro-neural genes *OLIG2, ASCL1 and DLL3* in 0627 GICs relative to the traditional human cell line, U373MG (data not shown). We determined that a reduction in B/b expression could also be detected at the transcript level ($p<0.01$) and the expression of pro-neural transcription factors were not altered as a result of reduced B/b expression (Supplemental Figure 2).

B/b knockdown reduces aggressiveness in late stages of GIC-derived tumor pathogenesis

We reasoned that the function of B/b as a secreted protein may be best observed in the *in vivo* environment. Therefore, shRNA-expressing GICs were injected into the striatum of nude mice. Mice were evaluated until signs of neurological deterioration were detected. All animal pairs (n= 6) were sacrificed as a result of neurological deterioration that was observed in the control animal, but not in animals harboring shB/b-expressing tumors, with a median survival time of 62 DPI (p= 0.009). shB/b GIC-derived tumors had a 20% reduction in mean tumor volume relative to controls. The decrease encroached on statistical significance by two-tailed Wilcoxon matched-pairs signed rank test (p= 0.0625) and was statistically significant by a one-tailed test (p= 0.008) (Figure 5A). The A/P spread of shB/b tumors relative to controls was significantly decreased, as determined by one-tailed Wilcoxon matched-pairs signed rank test (Figure 5B, p= 0.011). However, both control GICs and those expressing shB/b formed large intracranial masses. While the tumor burden was reduced, the morphology of individual cells at the rostral and medial/lateral tumor/stromal boundaries were similar in both control and shB/b GIC-derived tumors (Figure 5C–F″).

To determine whether a reduction in tumor size *in vivo* was due to alterations in the ability of GICs to form intracranial grafts, we examined the histology of early-engrafted GICs. Control (n= 4) or shB/b (n= 4) GICs were injected into the striatum of nude mice and harvested at 19 or 20 DPI prior to formation of large neoplasms. We found that both control and B/b shRNA expressing tumors engrafted similarly (Supplemental Figure 3).

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DISCUSSION

The CNS-specific expression of B/b [9,10], its significant upregulation and processing in gliomas and GICs suggest that B/b may uniquely contribute to the pathogenesis of these tumors. While overexpression of B/b has an evident enhancing effect on tumor growth and invasion [18,20,22,23], results from loss-of-function experiments have not conclusively demonstrated the impact of B/b suppression in a clinically-relevant setting [24]. Therefore, we conducted shRNA-mediated knockdown studies in intracranial models of invasive glioma, including GIC-derived models. Our results indicate a significant reduction in the volume of intracranial tumors and reduced anteroposterior spread, resulting in increased animal survival. Given the well-known pro-invasive function of B/b, the observed reduction in A/P spread establishes that targeting endogenously expressed B/b reduces the invasive and tumorigenic potential of glioma cells, slowing disease progression.

Our results showing B/b is enriched in the GIC niche and in cultured GICs led us to hypothesize that B/b may be required for the defining characteristics of this unique cellular subclass – including the ability to self-renew, differentiate, and generate tumors [25,34,35]. However, our results suggest that while B/b is highly expressed by GICs, it does not critically contribute to their maintenance or defining properties *in vitro*. Similarly, B/b knockdown did not affect the expression of other pro-neural genes (O*LIG2*, *ASCL1* and *DLL3*). Since GICs harboring B/b shRNA were capable of forming intracranial grafts and giving rise to tumors, B/b appears as a passenger gene *in vitro* and during initial stages of tumor formation, but contributes to the end-stage progression of gliomas.

A potential ‘late’ gain-of-function of B/b in gliomas raises interesting questions about the progression of molecular mechanisms in the TME and remodeling of the ECM during tumor progression. Our previous work has shown B/b acts in gliomas to enhance tumor cell invasion only after cleavage of the full-length protein [20,23] and subsequent release of a bioactive N-terminal fragment that activates EGFR signaling and increases cell association with a fibronectin-rich matrix [23]. Interestingly, the GICs used in this study express very low levels of fibronectin compared to differentiated glioma cell lines and did not bind to fibronectin (data not shown), potentially explaining the lack of effect following B/b knockdown in GICs *in vitro*. Since fibronectin is a pro-invasive and pro-angiogenic molecule secreted by glioma cells as the tumor progresses, we suggest that the effect we have seen following B/b knockdown *in vivo* could be explained by a decreased ability of tumor cells to interact with their own fibronectin-rich matrix, resulting in slowed tumor progression. Together, our work and previous findings suggest that the pro-invasive function of B/b may coincide with ECM remodeling that likely manifest following phenotypic changes of GICs during tumor progression. Future work focused on the relationship between B/b and fibronectin co-expression during tumor progression will likely reveal particular modes of B/b-mediated invasion that are context specific and shed light on its role in the pathogenesis of glioma.

In sum, our study suggests that B/b knockdown decreases the pathological progression of glioma and the expression of the B/b gene, *BCAN*, represents a potentially valuable therapeutic target. The extracellular and CNS-specific nature of B/b expression, the glioma-

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specific expression of unique isoforms, its upregulation in the vast majority of gliomas, and its abundance in the GIC niche support its promise as a target for the directed delivery of therapeutic agents. Additionally, the correlation between the B/b gene, *BCAN*, and other tumorigenic pro-neural genes suggests that common upstream mechanisms may regulate their expressions at the level of gene transcription. Identifying these regulatory mechanisms could provide the initial steps to developing novel glioma therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

B/b	Brain Enriched Hyaluronan Binding/brevican
CNS	Central nervous system
DIV	Days in vitro
DPI	Days post injection
ECM	Extracellular Matrix
GIC	Glioma initiating cell
HGGs	High-grade gliomas
TME	Tumor microenvironment

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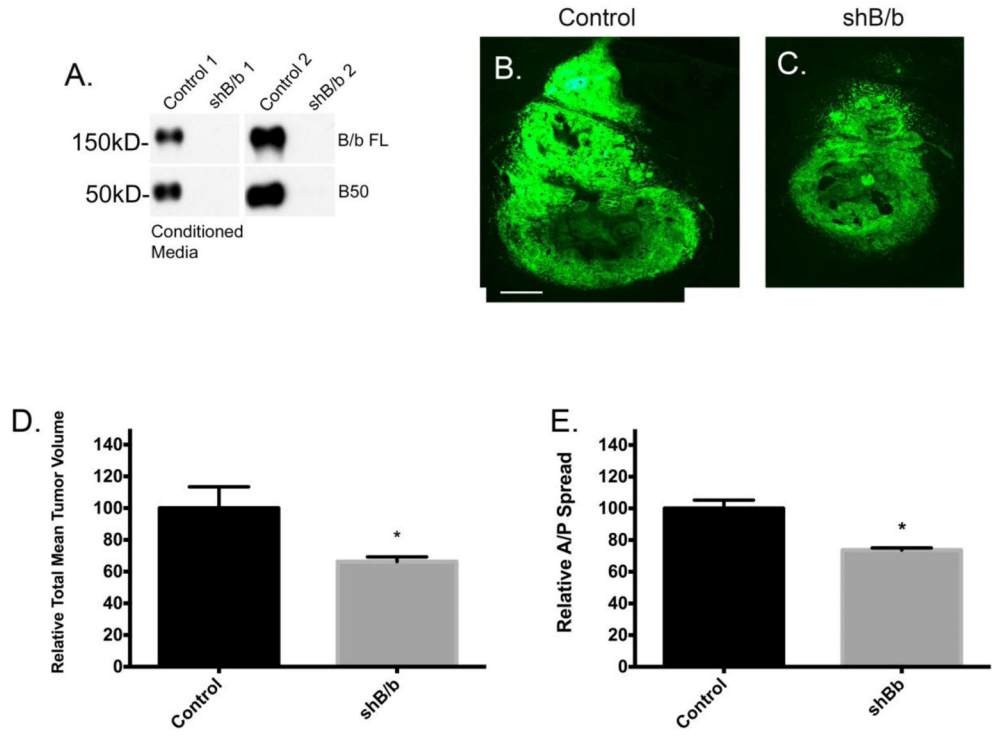

Figure 1. BEHAB/brevican knockdown reduces the tumor volume and anteroposterior spread (A/P spread) of tumors derived from a traditional rodent model of invasive glioma

A, Western blot analysis demonstrated that shRNA constructs designed against B/b (shB/b) effectively reduced the expression of exogenous full-length and proteolytic cleavage fragments of rat B/b induced by overexpressing cDNA encoding the full-length form of rat B/b in CNS-1 glioma cells. **B, C**, Wildtype CNS-1 glioma cells expressing either scrambled control (Control) or shRNA against B/b (shB/b) were injected into the thalamus of syngeneic adult rats. At 12 DPI, animals were anesthetized and perfused with PBS followed by 4% PB-PFA. Brains were post-fixed and processed for immunohistochemistry. Tumor cells are demarcated by the expression of GFP conferred by either control or shB/b expressing plasmids. Representative images showing a cross section of the resulting tumor core is presented, *scale* (*1mM*). **D**, Total mean tumor volumes were reconstructed using the Cavalieri Method. Two replicate groups of either control or shB/b tumors (utilizing two distinct brevican-directed and scrambled shRNAs respectively) were combined for analysis. Tumor volumes are presented relative to the average tumor volume for control tumors ± standard error of the mean (SEM), wherein control tumors were defined as 100. Results show that tumors created with shB/b CNS-1 glioma cells had significantly reduced tumor volumes relative to controls. (* indicates statistical significance $p = < 0.05$). **E**, The A/P spread of resulting tumors were determined by an estimation based on the appearance of tumor cells at the most rostral and caudal positions in collected serial sections. Values for A/P spread are presented relative to the average A/P spread from control tumors, wherein control tumors were defined as 100. Results show that tumors created with shB/b CNS-1 glioma cells had significantly reduced A/P spread relative to controls. (* indicates statistical significance $p = < 0.001$).

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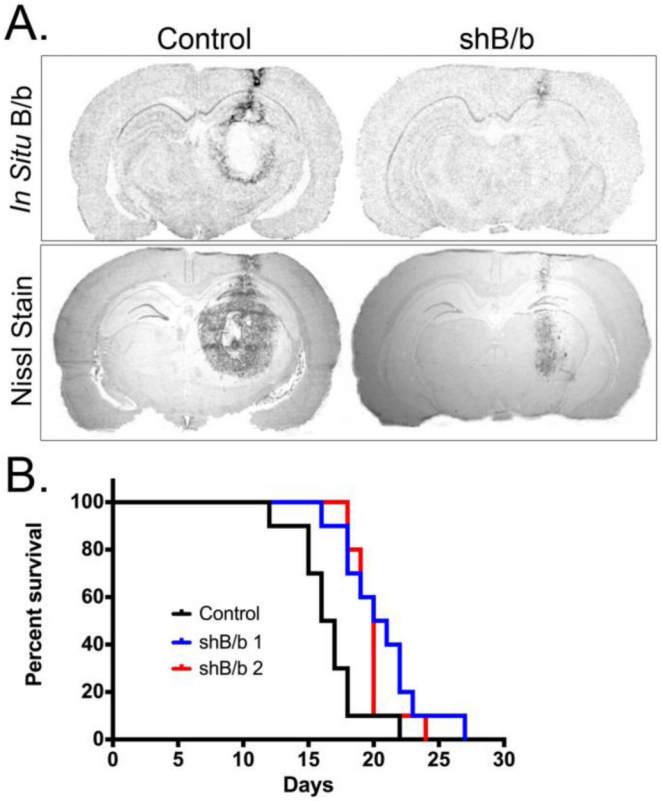

Figure 2. BEHAB/brevican knockdown in glioma increases animal survival

A, CNS-1 cells stably expressing scrambled control (Control) or B/b shRNA (shB/b) were implanted into the thalamus of rats. At 7 DPI, brains were collected, sectioned and processed for *in situ* hybridization with probes specific to B/b mRNA. Cresyl violet (Nissl) stain was used to histologically define the borders of the tumor mass. In control tumors, B/b mRNA was enhanced at the periphery of the tumor mass and nearly absent from the tumor core, consistent with its pro-invasive function. shB/b expressing tumors had significantly reduced levels of B/b transcript. **B,** Control or B/b knockdown (shB/b) CNS-1 glioma cells (harboring two independent shB/b constructs, shB/b 1 or shB/b 2) were injected into the thalamus of syngeneic adult rats and grown until significant neurological deterioration required euthanasia. Animals harboring shB/b CNS-1 glioma cells had significantly longer life expectancies than controls on log rank analysis of Kaplan-Meier survival curves ($p = < 0.05$).

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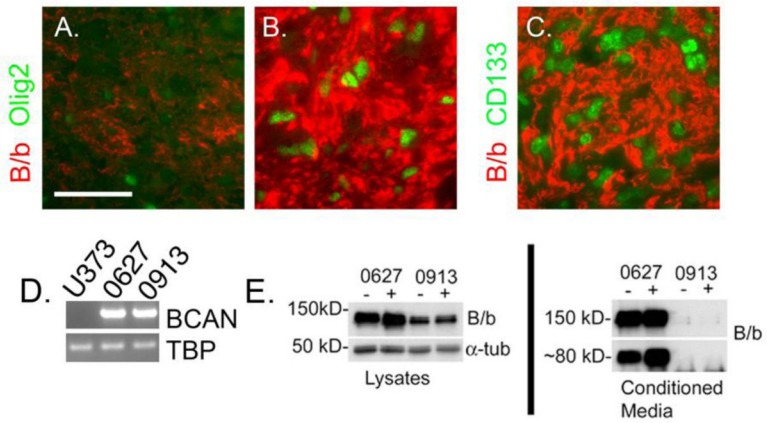

Figure 3. BEHAB/brevican expression is enriched in the glioma initiating cell extracellular niche in human glioblastoma tumors and is expressed by glioma initiating cells in vitro

A–C: Paraffin-embedded human GBM tumor core sections were immunostained with antibodies against B/b and markers enriched in highly tumorigenic cells (Olig2) and GICs (CD133). The expression of B/b was non-homogeneously distributed throughout the tumor core. Regions with high levels of reactivity for anti-B/b antibodies were enriched in regions containing cells positive for Olig2 and CD133. **A**, A representative region within the tumor core with low levels of B/b (red) contained limited population of cells positive for Olig2 (green). **B**, Regions within the tumor core with high levels of B/b (red) was enriched with cells positive for Olig2 (green) and **C**, CD133 (green). **D**, RT-PCR analysis of B/b (*BCAN*) mRNA expression in a traditional human glioma cell lines (U373MG) and cultures of GICs (0627 and 0913). U373MG cells lack endogenous *BCAN* expression, while GICs express high levels of this gene *in vitro*. TATA Box Binding Protein (TBP) served as a loading control. **E**, Western blot analysis for B/b expression in GICs show endogenous B/b expression in GICs *in vitro*, with the 0627 line having elevated levels of expression in both lysate and conditioned media samples. Samples were either treated with a control buffer (−) or chondroitinase (chABC) to remove CS-GAG chains (+). Full-length B/b protein was detected around 150kD in both lysate and conditioned media samples (CM), while C-terminal proteolytic cleavage fragments resolved at ~80 kD and were found primarily in conditioned media samples. The absence of an apparent molecular weight shift following chABC treatment (+) suggests B/b expressed by GICs is not extensively modified with CS-GAGs. α-tubulin was used as a loading control for lysate samples.

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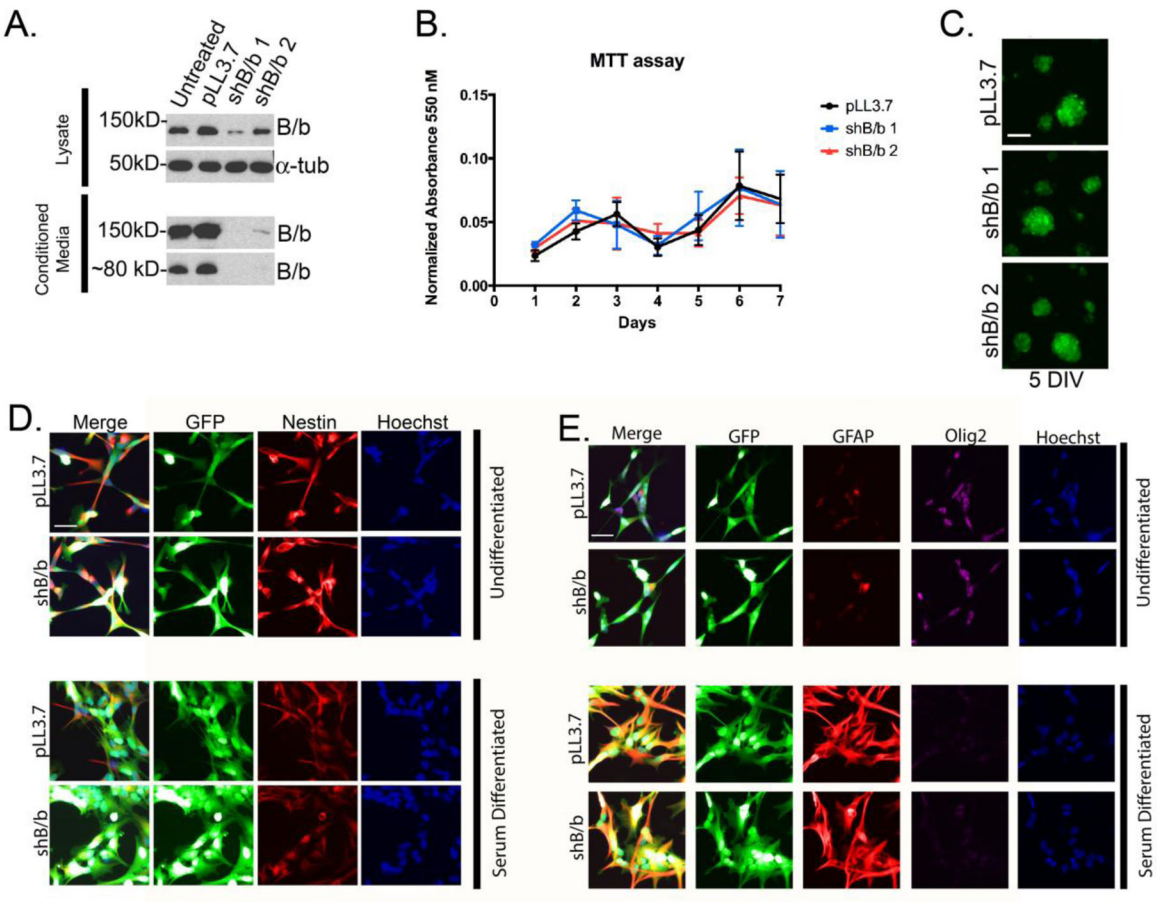

Figure 4. BEHAB/brevican does not alter the phenotypes of glioma initiating cells in vitro

A, Western blot analysis demonstrated that shRNA constructs designed against B/b (shB/b) effectively reduced the expression of full-length forms and proteolytic cleavage fragments in lysate and conditioned media samples relative to untreated and empty vector (pLL3.7) controls in 0627 GICs. α-tubulin was used as a loading control for lysate samples. **B**, A MTT assay was conducted to examine the *in vitro* proliferation profile of GICs expressing either control (pLL3.7) or two independent B/b knockdown lines (shB/b 1 and shB/b 2) over the course of 7 DIV. No significant difference in cell viability was detected between control and shB/b GICs for the duration of the assay. **C**, Sphere formation was examined at 5DIV between passages to evaluate the self-renewal and adhesive characteristics of control and B/b knockdown GICs. No differences were observed in their abilities to form spheres between passages, *scale (100μM)*. **D, E**, Control and shB/b expressing GICs were plated on laminin coated glass coverslips in undifferentiated conditions for 3 DIV or differentiated conditions for 12 DIV then analyzed by immunohistochemistry. **D**, Nestin immunoreactivity (red) was detected at similar levels in control and B/b knockdown GICs in undifferentiated conditions, while serum differentiation caused similar decreases in nestin immunoreactivity (red), *scale (50μM)*. Nuclei are counterstained with Hoechst (blue). **E**, Negligible GFAP immunoreactivity (red) and high levels of Olig2 immunoreactivity (violet) were detected at similar levels in control and B/b knockdown GICs in undifferentiated conditions, while differentiation caused similar increases and decreases in GFAP and Olig2 immunoreactivity,

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respectively, *scale (50μM)*. Nuclei are counterstained with Hoechst (blue) **C, D, E,** in all cases, GFP expression was driven by either control or shB/b expressing plasmids.

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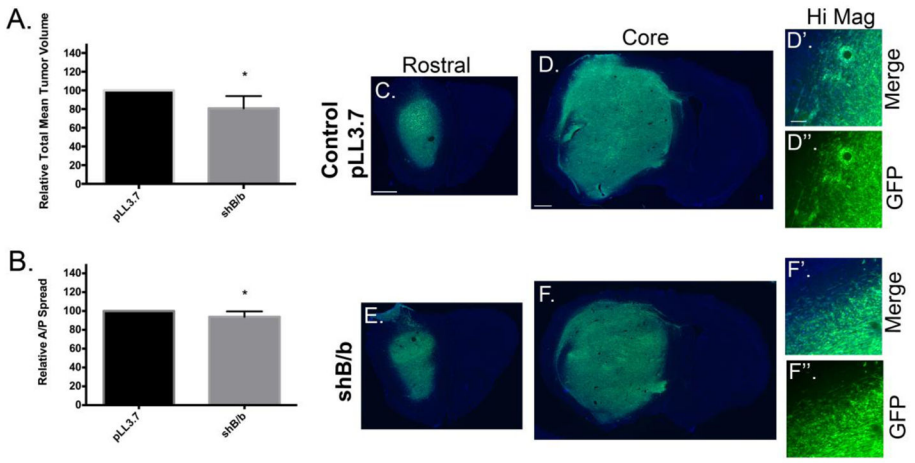

Figure 5. BEHAB/brevican reduces the tumor volume and anterior/posterior spread of glioma initiating cell-derived tumors

Control (pLL3.7) or B/b knockdown (shB/b) GICs were injected into the striatum of nude mice and collected in pairs at first sign of neurological deterioration. Animals were anesthetized and perfused with PBS followed by 4% PB-PFA. Brains were postfixed, cryo-protected in 30% phosphate-buffered sucrose and proccessed for immunohistochemistry. GFP expression by control or shB/b expressing plasmids was used to demarcate engrafted GICs. **A**, Total mean tumor volumes were reconstructed using the Cavalieri method. Tumor volumes are presented relative to the paired control, wherein control tumors were defined as 100. Results show that shB/b-GIC derived tumors had significantly reduced tumor volume relative to controls. (* indicates statistical significance, p =< 0.05). **B**, The A/P spread of resulting tumors were determined by an estimation based on the appearance of tumor cells at the most rostral and caudal positions in serially collected sections. Values for A/P spread are presented relative to the paired control, wherein control tumors were defined as 100. Results show that shB/b-GIC derived tumors had significantly reduced A/P spread relative to controls. (* indicates statistical significance, p =< 0.05). **C, D,** Low mag representative image shows control GIC-derived tumors at rostral (**C**) and central core (**D**) positions, *scale (1mM)*. Nuclei were counterstained with Hoechst (Blue). **D′, D″,** High mag image shows lateral invasion and diffusion of control GIC derived tumor cells into the surrounding brain tissue at the tumor/stromal border at core positions, *scale (100μM)*. **E, F**, Low mag representative image shows shB/b GIC-derived tumors have reduced tumor volumes, which can be observed at both rostral (**E**) and central core (**F**) positions relative to controls. However, shB/b GIC-derived tumors were still found to form large intracranial masses, *scale (1mM)*. Nuclei were counterstained with Hoechst (Blue). **F′, F″,** High mag image shows lateral invasion of shB/b GIC-derived tumor cells at the tumor/stromal border at core positions which are similar to controls, *scale (100μM)*.