

YB-1 Bridges Neural Stem Cells and Brain Tumor–Initiating Cells via Its Roles in Differentiation and Cell Growth

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Abstract

The Y-box binding protein 1 (YB-1) is upregulated in many human malignancies including glioblastoma (GBM). It is also essential for normal brain development, suggesting that YB-1 is part of a neural stem cell (NSC) network. Here, we show that YB-1 was highly expressed in the subventricular zone (SVZ) of mouse fetal brain tissues but not in terminally differentiated primary astrocytes. Conversely, YB-1 knockout mice had reduced Sox-2, nestin, and musashi-1 expression in the SVZ. Although primary murine neurospheres were rich in YB-1, its expression was lost during glial differentiation. Glial tumors often express NSC markers and tend to lose the cellular control that governs differentiation; therefore, we addressed whether YB-1 served a similar role in cancer cells. YB-1, Sox-2, musashi-1, Bmi-1, and nestin are coordinately expressed in SF188 cells and 9/9 GBM patient-derived primary brain tumor–initiating cells (BTIC). Silencing YB-1 with siRNA attenuated the expression of these NSC markers, reduced neurosphere growth, and triggered differentiation via coordinate loss of GSK3- β . Furthermore, differentiation of BTIC with 1% serum or bone morphogenetic protein-4 suppressed YB-1 protein expression. Likewise, YB-1 expression was lost during differentiation of normal human NSCs. Consistent with these observations, YB-1 expression increased with tumor grade ($n = 49$ cases). YB-1 was also coexpressed with Bmi-1 (Spearman's 0.80, $P > 0.001$) and Sox-2 (Spearman's 0.66, $P > 0.001$) based on the analysis of 282 cases of high-grade gliomas. These proteins were highly expressed in 10/15 (67%) of GBM patients that subsequently relapsed. In conclusion, YB-1 correlatively expresses with NSC markers where it functions to promote cell growth and inhibit differentiation. *Cancer Res*; 71(16); 5569–78. ©2011 AACR.

Introduction

Glioblastoma (GBM), the most common primary brain tumor in adults, is usually associated with a 2-year survival rate of only 10% to 25% (1). In children, primary brain tumors are the second most common type of cancer, following leukemia, with an incidence of 3.8 per 100,000 person-years (2, 3). Like adults, children who suffer from GBM have a low

chance of long-term survival, thus, a better molecular understanding of these tumors may lead to new therapeutic targets.

Y-box binding protein 1 (YB-1) is a transcription/translation factor involved in DNA repair (4) and multidrug resistance (5). Loss of YB-1 is embryonically lethal for mice where major developmental defects were reported in the brain (6, 7), yet the molecular mechanism underlying this is unknown. Although it is downregulated at postnatal life (8), it is highly expressed in cancer where its expression is associated with poor prognosis (4). YB-1 was highly expressed in gliomas when compared with surrounding normal brain tissues (9). We reported that YB-1 is essential for growth of adult and pediatric GBM cells by showing that silencing it with siRNA suppressed proliferation, invasion, and tumorigenesis (10). Furthermore, YB-1 conveyed resistance to temozolomide (10), a drug commonly used to treat GBM. Thus, YB-1 plays a role in normal and pathologic states of the brain. Whether these roles are related is not known.

Poor responses to conventional therapeutic approaches and frequent relapses are serious challenges for patients with brain tumors. There are various factors contributing to this therapeutic resistance and relapse. One of the potential culprits is the presence of brain tumor–initiating cells (BTIC) also referred to as repopulating cells which are multipotent, have the

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ability to self-renew, form neurospheres, and initiate tumor development (11, 12). This is a field that continues to rapidly evolve. There are notable similarities between BTICs and normal stem cells, the historical perspective of which is reviewed by several groups (13–15). The idea that cancer originates from stem cells is traced back to more than 150 years ago (14); however, we still do not know what the cell(s) of origin are for gliomas. Given this, we will refrain from using the term cancer stem cells. What we can say is that several groups have shown that cancer cells with characteristics of normal neural stem cells (NSC) are able to initiate tumor formation (16). TICs were first isolated from leukemia (17), followed by isolating from solid tumors including GBM (13, 18). Sox-2, Bmi-1, and musashi-1 are NSC markers that seem to track with brain BTICs (19). An intriguing feature of BTICs is that they maintain tumor cells in an undifferentiated state (19). For this reason, new therapeutic approaches that force BTICs to undergo differentiation and/or to cease proliferation are being sought to improve cancer treatment.

Prior studies from our laboratory forged a link between YB-1 and BTICs. This was originally discovered through genome-wide promoter occupancy studies where YB-1 binds to several genes associated with tumor-initiating cells such as CD44 and CD49f (20, 21). Furthermore, it was expressed in primary mammary progenitor cells from women who had undergone reduction mammoplasties suggesting that it played a role in normal mammary stem cells (20). Moreover, we reported that YB-1 induces breast cancer tumor-initiating cells to express CD44 and CD49f leading to enhanced cell growth and drug resistance (21). Given this, we questioned what role YB-1 may play in NSCs and/or BTICs derived from primary brain tumors.

Materials and Methods

Reagents and cells

Human recombinant platelet-derived growth factor-AA (PDGF-AA), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and ciliary neurotrophic factor (CNTF) were from PeproTech. Human recombinant insulin-like growth factor-1 (IGF-1) was from BioVision. Normal human astrocytes (AS), T98G, SF188, U251, and SK-MG-1, human glial cell lines and Daoy, medulloblastoma cell line, were from American Type Culture Collection. Kings-1 human glial cell line was from the Health Science Research Resources Bank.

Primary GBM cells isolation

L0, L1, L2, L3, FL17, and BT241 BTICs GBM cells were obtained from patients with primary GBM, as previously described (22, 23). Following surgical debulking, tissue samples were processed in a manner similar to that used to isolate human NSC (hNSC) from the adult nervous system (24–27). More specifically, GBM tissue samples were finely minced and placed in 0.05% trypsin/EDTA for 7 minutes at 37°C. An equal volume of DNase & trypsin inhibitor (Sigma) is added, mixed, and centrifuged at 700 × *g*, supernatant removed, and the pellet resuspended in 1 mL of NeuroCult (STEMCELL Tech-

nologies). The pellet is repeatedly pipetted, passed through a 40-μm mesh, and cells spun again (700 × *g*, 5 minutes). Dissociated cells are placed in NeuroCult containing EGF (20ng/mL) and plated in T25 flasks (Nunc) at a density of 200,000/mL. Resulting spheres are passage as per published and detailed protocols (24, 25, 27–31). After 2 to 3 passages, cells typically exhibit an arithmetic increase in the total number of cells generated, at which point they are considered a primary cell line and designated a line number. Cells are expanded and early passage aliquots cryopreserved for future use. They maintain differentiation potential, grow as secondary neurospheres, and express stem cell markers. The BT74 cells (originally referred to as GBM6) was isolated from a patient with GBM as previously described (32). The BTIC characteristics of BT74, GBM4, and GBM8 cells were reported by Wakimoto and colleagues (33) where they showed the cells are able to differentiate along the astrocyte and neuronal lineages, grow as secondary neurospheres, and give rise to secondary tumors (33). All BTICs were obtained through patient consent in accordance with the respective Institutional Review Board guidelines. In our laboratory, the primary GBM BTICs maintained attributes of NSC as confirmed by Sox-2, Bmi-1, and musashi-1 expression measured by immunocytochemistry and quantitative real-time PCR. The neurosphere can be serially passaged, and they have retained multipotent differentiation potential.

Immunohistochemistry and immunofluorescence analysis

Mouse samples were collected from YB-1^{+/+} and YB-1^{-/-} mouse embryos and prepared for immunohistochemistry as previously described (6). The animal experiments were carried out according to the Ethics guidelines at Kyushu University, Fukuoka, Japan. Then, specimens were incubated for 1 hour with YB-1 (1:200; JoyUp Biomedical), or glial fibrillary acidic protein (GFAP; 1:1,000; Sigma) antibodies. Signal detection was carried out by using Dako LSAB2 kit.

For fluorescence staining of mouse tissue, sections were fixed and prepared for immunohistochemistry as previously described (6). Primary antibodies used were: rabbit anti-YB-1 (1:200; JoyUp Biomedical), mouse anti-rat nestin (1:200; BD Pharmingen), and mouse anti-GFAP. Secondary antibodies used were Alexa 594 or Alexa 488 conjugated (Invitrogen). A series of WHO grades I to IV gliomas (*n* = 49 cases) were obtained from Kurume University, Kurume, Japan, and stained for YB-1 as described above. Subsequently, a tissue microarray consisting of 389 cores from 342 patients was obtained from Kings College Hospital, London, UK and stained for YB-1, Bmi-1, and Sox-2. Because of missing cores, 282 cases were scored for all 3 markers. This cohort consisted of 17 grade III anaplastic astrocytoma, 49 grade III anaplastic oligodendroglioma, and 275 grade IV GBM multiforme, with 19 patients having paired diagnostic and relapse samples available. Of these samples 15 were interpretable for YB-1, Sox-2, and Bmi-1. The median age of diagnosis was 58 years (range 26–83), and the median survival was 198 days (range: 2 days–5.6 years). There was a slight preponderance of males to females (1.4:1). All procedures were carried out in accordance with the

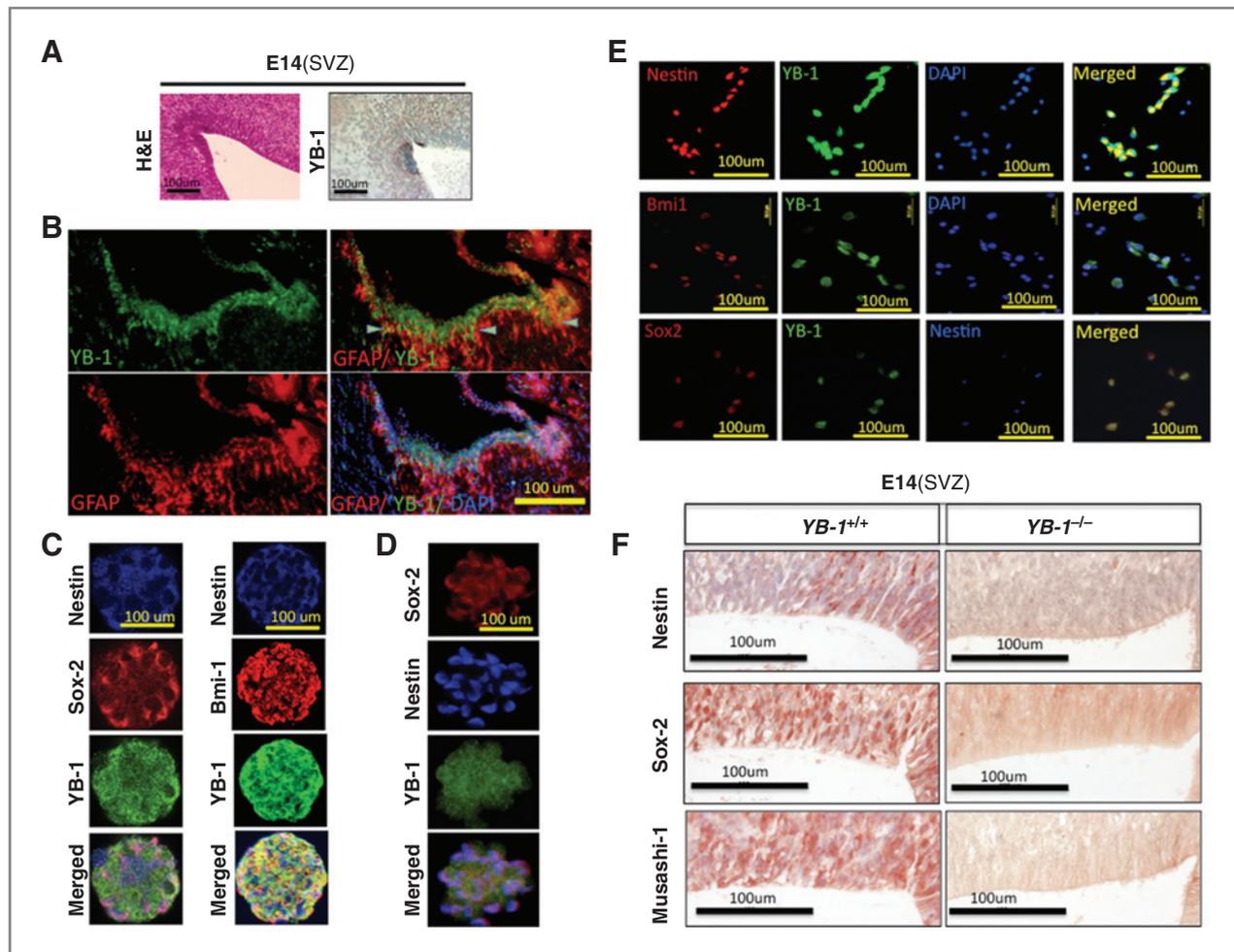


Figure 1. YB-1 is important in NSC development. A, SVZ of brain in mouse embryo (E14) is enriched in YB-1-positive cells. B, double staining with GFAP confirmed YB-1 expression in NSCs in E14 mouse embryos. C, neurospheres developed from E14 mouse embryos showed that nestin, Sox-2, and YB-1 were expressed together (left). Similarly nestin, Bmi-1, and YB-1 were expressed in the neurospheres (right) based on whole mount staining. D, this was confirmed by showing that Sox-2, nestin, and YB-1 were readily detectable in neurospheres from frozen sections. E, mouse neurospheres were again isolated from E16 mice, dissociated, and seeded on PDLO/gelatin-coated coverslips for 2 days in neural culture medium, supplemented with 20 ng/mL bFGF and 20 ng/mL EGF. Nestin, Bmi-1, Sox-2, and YB-1 were coexpressed in primary neural progenitor cells. DAPI was used for nuclear staining. F, the SVZ was sectioned from E14 wild type and YB-1^{-/-} mice. The cells were immunostained for nestin (top), Sox-2 (middle), or musashi-1 (bottom) antibodies. Nestin, Sox-2, and musashi-1-positive cells were expressed in the wild-type SVZ as expected (left). The expression of these NSC markers was lost in YB-1^{-/-} SVZ (right).

principles of the Helsinki Declaration and were approved by the Ethical Committee of each institution. The data were analyzed by using JMP 8.0.2 (SAS Institute Inc.).

Neurosphere growth, differentiation, and immunolabeling

Mouse neurosphere growth. Neurosphere growth, differentiation, and immunolabeling were carried out as previously described (34). Briefly, pregnant female C57BL/6 dams were anesthetized, and the gravid uterus was excised. Animal care and use followed the guidelines of the University of British Columbia. Primary neural progenitor cells (from neurospheres) and oligodendrocyte progenitor cells (OPC; from oligospheres) were grown on poly-DL-ornithine (PDLO)/gela-

tin-coated or PDLO-coated coverslips, respectively, and stimulated to undergo differentiation. The cells were fixed with 4% paraformaldehyde and stained for lineage-specific markers namely nestin, Sox-2, Bmi-1, A2B5, O4, CNPase (2',3'-cyclic-nucleotide 3'-phosphodiesterase), and GFAP (Millipore). Alexa Fluor 488 or 594 were used as secondary antibodies. The percentage of YB-1-positive cells relative to each of the differentiation markers was assessed by counting 5 fields.

To study cancer-derived neurospheres, SF188 cells and the primary BTICs were grown in NeuroCult media supplemented with NS-A proliferation supplements, EGF (20 ng/mL), bFGF (10 ng/mL), and heparin (2 mg/mL) in ultralow attachment plate. After 4 to 6 days of incubation, neurospheres were collected, washed in PBS, and fixed with acetone/methanol

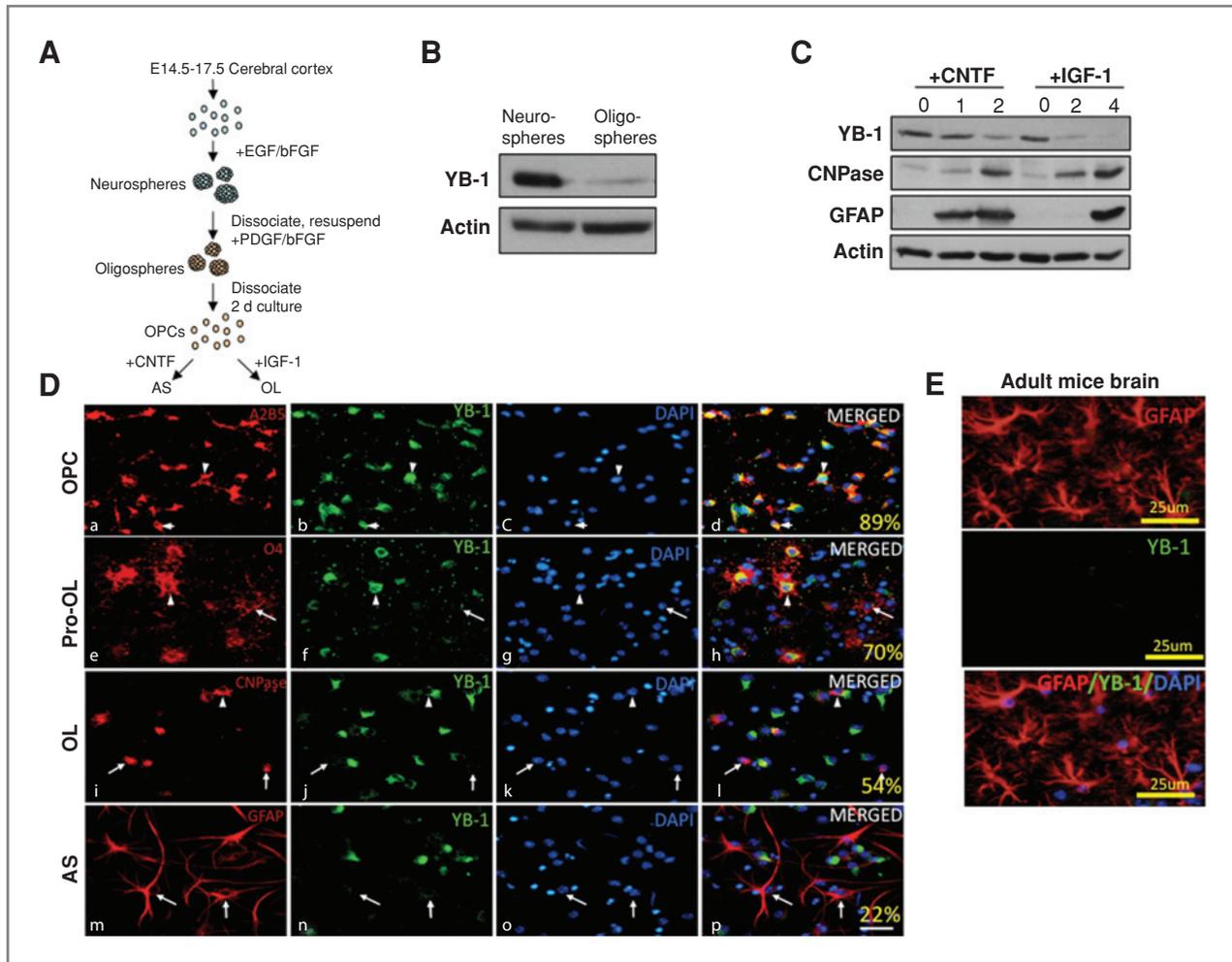


Figure 2. Downregulation of YB-1 upon glial differentiation. **A**, murine neurospheres were isolated from E14.5-17.5 mouse embryos as outlined. The cells were dissociated and grown in PDGF- and bFGF-containing media to promote the growth of oligospheres. These were dissociated and cultured as OPCs for 2 days and then cultured in the absence of PDGF and FGF and treated with triiodothyronine and either CNTF or IGF-1 to promote the development of astrocytes or oligodendrocytes, respectively. **B**, mouse neurospheres and oligospheres lysates were probed for YB-1 and actin. **C**, mouse OPCs were cultured for 2 days in oligosphere medium followed by incubation for the indicated times in OPC differentiation medium with 10 ng/mL CNTF or 100 ng/mL IGF-1. Lysates were probed for YB-1, CNPase, GFAP, and actin. **D**, mouse oligospheres were dissociated and seeded on PDLO-coated coverslips for 2 days in oligosphere medium (OPCs; a-d) followed by incubation for 2 days in OPC differentiation medium plus 100 ng/mL IGF-1 (e-l) or 10 ng/mL CNTF (m-p). Cells were incubated with antibodies against YB-1 and A2B5 (a-d), O4 (e-h), CNPase (i-l), or GFAP (m-p). Arrowheads, cells with high expression of YB-1. Arrows, cells with low expression of YB-1. Scale bar, 25 μ m. **E**, YB-1 was not detected in GFAP-expressing differentiated astrocytes in sections of adult mouse brain.

(*v/v*) at -20°C . BTICs were differentiated with either 1% FBS for 5 days, bone morphogenetic protein-4 (BMP-4; ref. 22), or lithium chloride for 72 hours as previously described (35).

YB-1 was silenced in SF188 and L1 cells with increasing amounts of siRNA (siYB-1) for 48 hours as previously described (10). In addition, a second siYB-1 oligonucleotide, #5'-UUUGCUGGUAUUUGCUGGAGGACC-3' (sense) 5'-GGUCCUCCACGCAAUUACCAGCAAAA-3' (antisense), was used to confirm the results observed in SF188 cells as well as T98G cells. The immunoblottings were carried out by using antibodies to YB-1 (1:1,000), GSK3- β (1:1,000, CST), musashi-1 (1:500), Sox-2 (1:500), Bmi-1 (1:500), GFAP (1:1,000), or vinculin (1:2,000).

Results and Discussion

YB-1 is important for the maintenance of mouse NSCs

YB-1 was highly expressed in the subventricular zone (SVZ) of normal E14 mouse tissues based on immunohistochemistry (Fig. 1A, right, brown stain). We confirmed the expression of YB-1 in the SVZ of E14 mouse tissues by staining serial sagittal sections for GFAP (Fig. 1B) and nestin (Supplementary Fig. S1A), which reportedly mark NSCs (36). In the adult brain, NSCs are mostly limited to the SVZ and subgranular zone (SGZ) of the dentate gyrus (37-40). To determine if YB-1 is expressed in proliferating cells of the adult mouse brain, we stained fresh sections of brain harvested from BrdU-injected

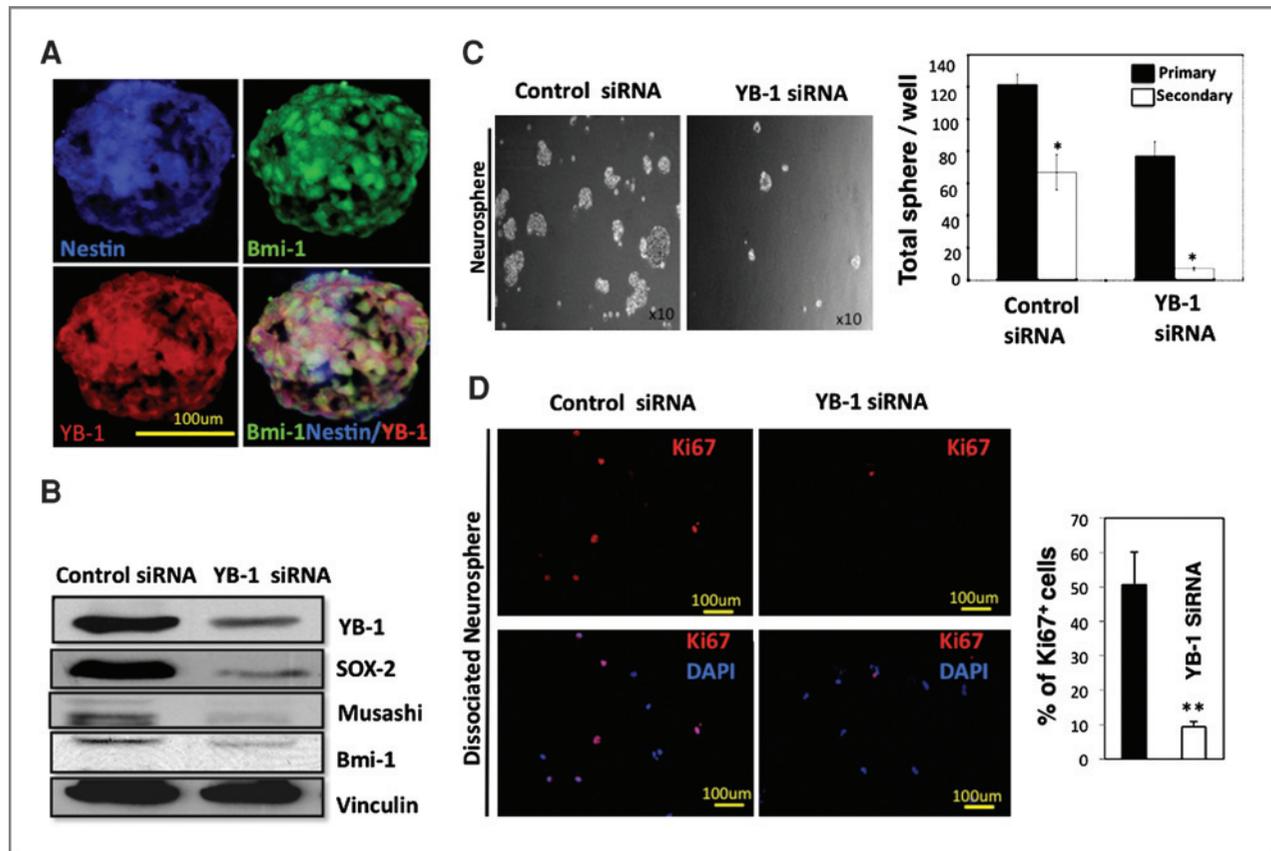


Figure 3. YB-1 is essential for GBM derived cells to form neurospheres. A, neurospheres were developed from SF188 GBM cells, and whole-mounted neurospheres were stained for nestin, YB-1, and Bmi-1. B, silencing YB-1 (5 nmol/L) for 48 hours reduced Sox-2, Bmi-1, and musashi-1 protein levels. C, loss of YB-1 led to suppressed primary and secondary neurosphere formation by approximately 90%. D, neurospheres taken from the first passage were isolated, dissociated, and plated on glass culture chambers. Loss of YB-1 in the replated neurospheres was associated with reduced cell proliferation based on Ki-67 staining as compared with cells treated with the scrambled control ($P < 0.001$, A). Scale bar: 100 μm ; D, bar: 100 μm . *, $P < 0.05$. **, $P < 0.05$.

mice with YB-1 and BrdU antibodies. There was a close association between YB-1 and BrdU staining in the SGZ (Supplementary Fig. S1B). Similar findings were observed in the SVZ (data not shown). Following these initial studies, primary neurospheres were isolated from E14 mice where we determined that YB-1 was expressed in conjunction with the NSC markers nestin, Sox-2, and Bmi-1 by staining whole mounts as well as frozen sectioned neurospheres (Fig. 1C and D). Given that neurospheres are comprised of a mixture of differentiated cells along with NSCs, we dissociated the spheres and examined YB-1 and stem cell marker expression at the cellular level. There was a close correlation between YB-1, nestin, Bmi-1, and Sox-2 (Fig. 1E). Furthermore, the population of nestin, Sox-2, and musashi-1-positive cells was significantly reduced in YB-1^{-/-} mice at E14 as compared with YB-1^{+/+} (Fig. 1F). Thus, YB-1 appears to be a constituent of normal NSCs.

YB-1 is silenced during glial differentiation

Neurospheres isolated from E14.5-17.5 mice were dissociated and induced to form oligospheres that in turn were dissociated and cultured as OPCs; Fig. 2A schematic). These

cells can be stimulated with CNTF or IGF-1 to promote differentiation into astrocytes or oligodendrocytes (OL; Fig. 2A). YB-1 was more highly expressed in the neurosphere cultures as compared with oligosphere cultures (Fig. 2B); the latter of which are more differentiated. Furthermore, YB-1 expression was lost during OPC differentiation following CNTF or IGF-1 treatment (Fig. 2C and D). Prior to further differentiation, the majority (89%) of the OPCs coexpressed the progenitor marker A2B5 and YB-1 (Fig. 2D, a-d). The OPCs were induced to differentiate into glial lineages by using CNTF for astrocytes or IGF-1 for oligodendrocytes. Interestingly, when the cells were prompted to differentiate *in vitro* they lost YB-1 expression. During differentiation to oligodendrocytes, most (70%) of prooligodendrocytes (Pro-OL) that expressed the early stage marker O4 also expressed YB-1 (Fig. 2D, e-h), whereas cells expressing the late stage marker CNPase were less likely (54%) to express YB-1 (Fig. 2D, i-l). Interestingly, differentiation along the astrocyte lineage marked by the increase in GFAP led to loss in YB-1 expression where only 22% of the cells still expressed YB-1 (Fig. 2D, m-p). Similarly, when primary neurospheres were differentiated along this lineage with 1% FBS, YB-1 was only expressed in

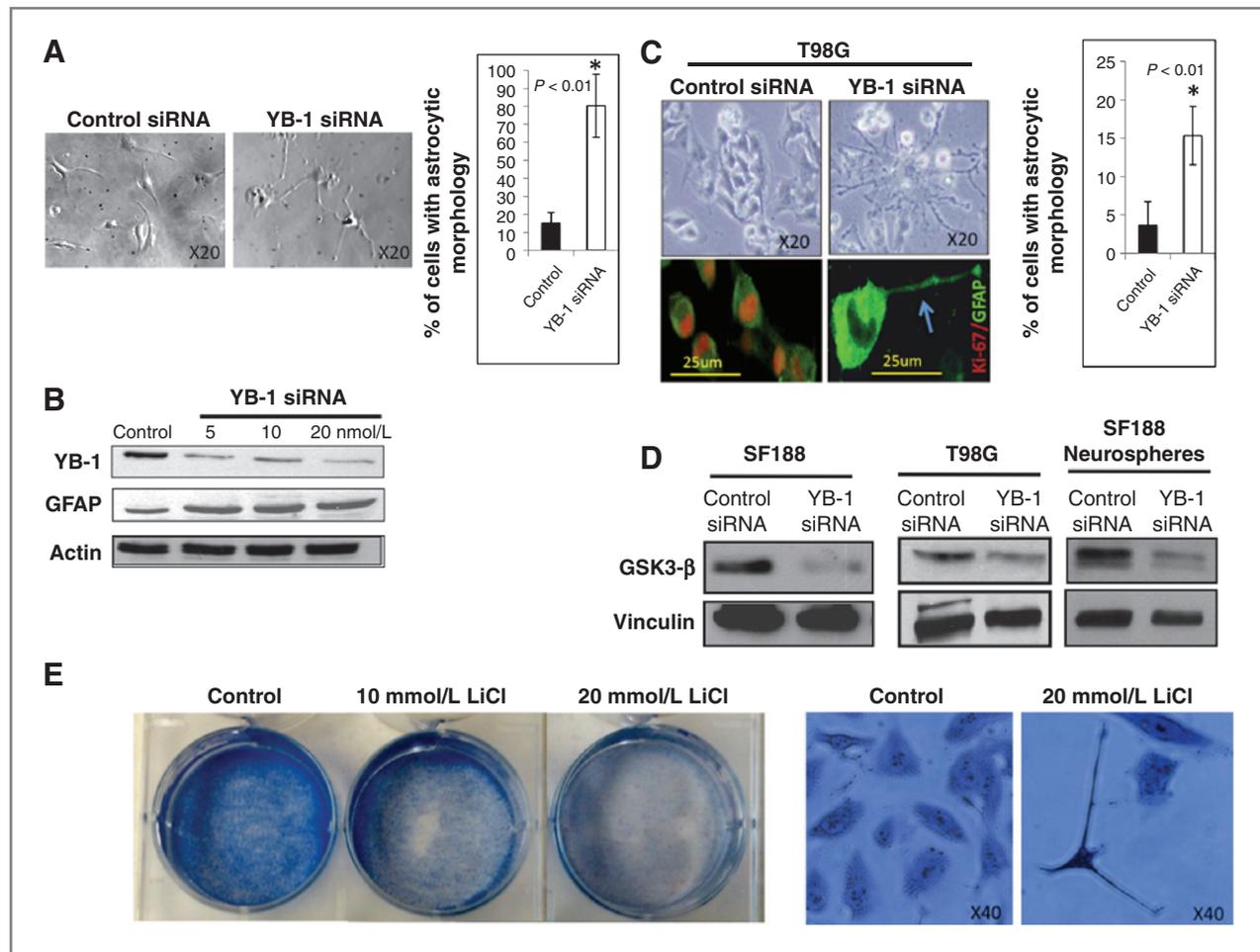


Figure 4. Loss of YB-1 leads to cellular differentiation in GBM cells. **A**, SF188 cells harvested from YB-1 knockdown neurospheres assumed an astrocyte-like morphology which was quantified by counting the average number in random fields. **B**, T98G cells transfected with increasing concentrations of a second YB-1 targeting siRNA (5–20 nmol/L) for 96 hours. Each of the siRNA's inhibited YB-1 expression by greater than 90%, therefore, 5 nmol/L was used for future experiments. Loss of YB-1 also corresponded with increased GFAP expression. **C**, loss of YB-1 after 5 days in monolayer prompted the development of astrocyte-like cells. There were approximately 3x more of these cells following siYB-1 ($P < 0.01$). The change in morphology corresponded with a reduction in Ki67 staining. The YB-1 siRNA treated cells also expressed higher levels of GFAP as compared with those similarly treated with the scramble control siRNA. **D**, loss of YB-1 consistently led to decreased GSK3- β expression in SF188 and T98G cells growth in monolayer. GSK3- β was also downregulated in primary neurospheres following YB-1 knockdown for 5 days. **E**, GSK3- β inhibition with LiCl suppressed SF188 growth and induced differentiation.

5% of mature astrocytes (Supplementary Fig. S1C). Likewise, mature astrocytes in adult mice do not express YB-1 (Fig. 2E). Thus, normal NSCs express YB-1 at high levels, however, as astrocytes develop its expression is silenced. How YB-1 is silenced is not known at this point. On the basis of previous studies, snail, hunchback, or Sox17 are candidates given their ability to transcriptionally repress gene expression, and they are predicted to bind to the YB-1 promoter (41).

YB-1 inhibition suppresses neurosphere growth and promotes differentiation

Glioma BTICs are considered poorly differentiated cells, sharing numerous features with NSCs (42). Here, we illustrate that SF188 GBM cells express the NSC markers nestin, Sox-2, and musashi-1 when cultured as neurospheres (Sup-

plementary Fig. S2A), and YB-1 is coordinately expressed along with Nestin and Bmi-1 (Fig. 3A) or Sox-2 (Supplementary Fig. S2B). Additionally, proliferating cells (Ki67-positive) within the neurospheres expressed considerable YB-1 (Supplementary Fig. S2C). In contrast, YB-1 inhibition by using siRNA led to a significant reduction in the expression of Sox-2, musashi-1, and Bmi-1 based on immunoblotting (Fig. 3B). However, this was not because of loss of mRNA expression (Supplementary Fig. S2D). The loss of these proteins following YB-1 knockdown corresponded with a reduction in primary and secondary neurosphere formation (Fig. 3C). Notably, the size of the secondary spheres was significantly reduced in the cells treated with YB-1 siRNA (Supplementary Fig. S2E). The few neurospheres that did form during the first pass from the siYB-1 treatment were dissociated and

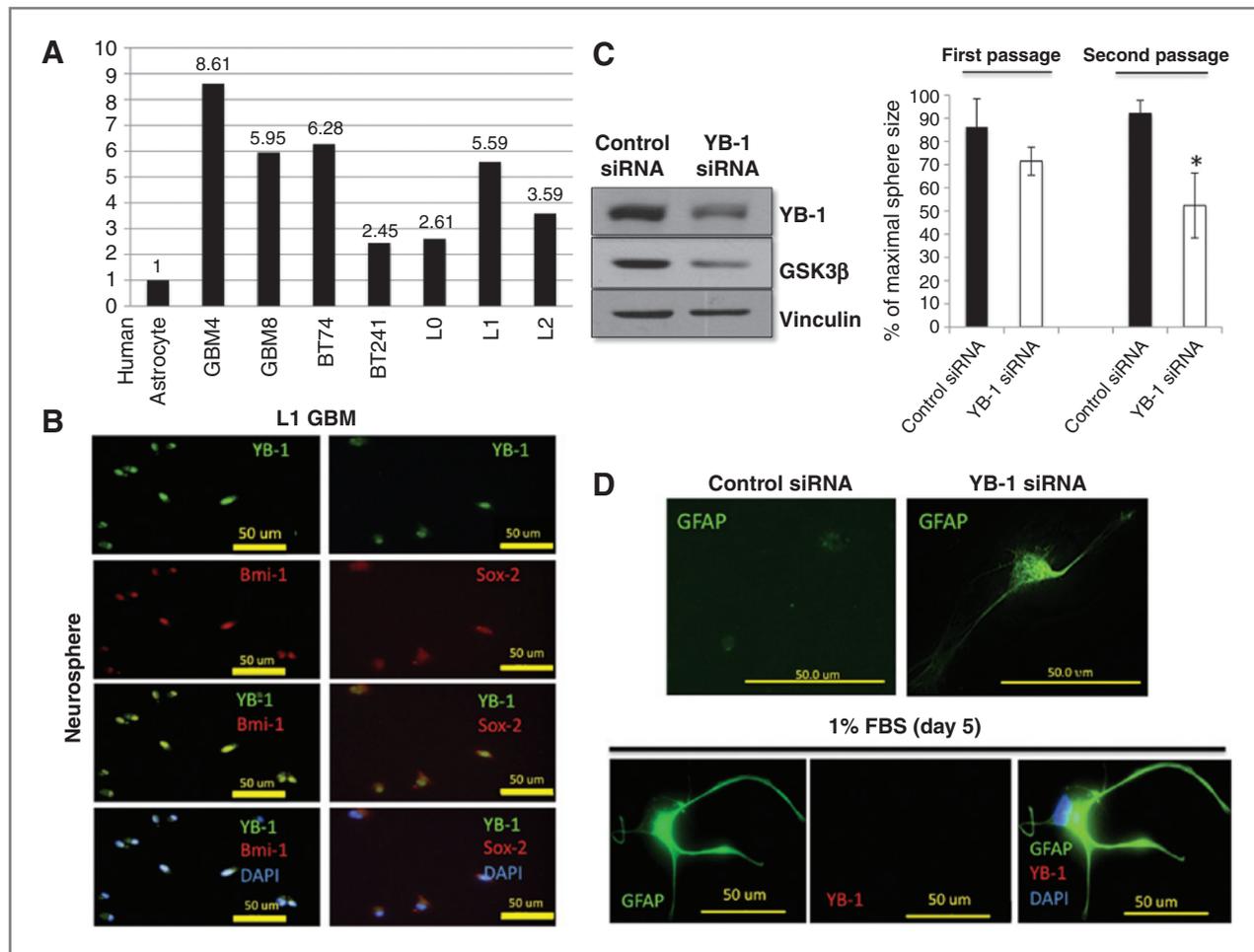


Figure 5. Loss of YB-1 leads to cellular differentiation in BTICs. **A**, YB-1 mRNA was consistently expressed in primary BTICs established from patients with GBM. Levels were also higher than what was found in human astrocytes. **B**, L1 cells were characterized for YB-1, Sox-2, and Bmi-1. **C**, loss of YB-1 expression by using siRNA inhibited GSK3- β expression and the ability of the L1 cells to grow as neurospheres. **D**, further YB-1 siRNA led to the induction of a differentiated phenotype. Similarly, 1% FBS stimulated differentiation of L1 cells (over 5 days) and repressed YB-1 expression.

plated in glass chambers to examine changes in proliferation and differentiation compared with the control siRNA treated spheres. Loss of YB-1 led to a marked reduction in Ki67 staining (Fig. 3D).

Following YB-1 knockdown, we observed a remarkable change in cellular morphology where some of the siYB-1-treated GBM cells became stellate in appearance and looked like astrocytes (Fig. 4A). To further confirm these findings, YB-1 was silenced in a second GBM cell line model (T98G), and again siYB-1 treatment stimulated changes in cellular morphology resembling normal astrocytes (Fig. 4B and C, additional examples Supplementary Fig. S3A). This was commensurate with increased expression of GFAP (Fig. 4B and C). At the cellular level, loss of YB-1 intensified GFAP expression and as such the cells became elongated and ceased to proliferate based on loss of Ki67 staining as compared with the control cells (Fig. 4C, bottom). Loss of YB-1 increased the percentage of differentiated cells by approximately 3-fold (Fig. 4C). Although YB-1 ablation

stimulated cellular differentiation of GBM cells and slowed cell proliferation, it did not induce cell death (Supplementary Fig. S3B). In a similar light, the transient expression of Flag:YB-1D102, (an active mutant) caused normal human astrocytes to lose their star-like appearance (Supplementary Fig S3C and D).

The observed morphologic changes described above are reminiscent of the way in which Bmi-1 and GSK3- β promote the undifferentiated state of GBM cells (35). It was recently reported that the GSK3- β pathway is highly expressed in GBM where it sustains dedifferentiation (35). Inhibition of GSK3- β by siRNA or lithium chloride (LiCl) induced morphologic differentiation (35) similar to the glial differentiation observed after YB-1 inhibition. Because we found that loss of YB-1 caused a reduction in Bmi-1, we examined SF188 and T98G cells for changes in GSK3- β . Total GSK3- β levels were reduced in both cell lines following YB-1 knockdown (Fig. 4D). Neurospheres from YB-1 siRNA-treated SF188 cells also expressed less GSK3- β (Fig. 4D). Likewise, inhibition of

GSK3- β with LiCl suppressed their growth and induced differentiation (Fig. 4E).

We also obtained 7 primary BTIC isolates from patients with GBM and showed that each of them expresses YB-1, and the levels are significantly higher than those found in normal human astrocytes (Fig. 5A). Each of these BTICs are maintained in three-dimensional cultures, therefore, we dissociated the neurospheres and plated the cells in monolayer to show that YB-1 colocalizes with Sox-2 and Bmi-1 (Fig. 5B, ex. L1 cells) and nestin (Supplementary Fig. S4A and B, ex. BT241 and GBM8 cells, respectively). YB-1 was then silenced in the L1, which reduced GSK3- β expression, suppressed neurosphere growth (Fig. 5C), and induced differentiation (Fig. 5D, top). Alternatively, differentiation of L1 cells with 1% FBS prompted the loss of YB-1 expression (Fig. 5D, bottom). To extend this further, we examined the possibility that BMP-4 might be a way to inhibit YB-1 expression in BTICs as it is known to stimulate differentiation of BTICs from GBM patients (22). YB-1 expression was determined in a panel of BTICs (L0, L1, L2,

L3, and GBM FL17) along with hNSC derived from the brain and astrocytes (Supplementary Fig. S4C). BMP-4 reduced levels of YB-1 in L0 cells (Fig. 6A, Supplementary Fig. S4D; ex. L0, and L2). Similarly, differentiation of hNSC caused a loss of YB-1 expression (Fig. 6B, Supplementary Fig. S4E). As expected, YB-1 was expressed in more than 96% of the hNSCs but was only found in 0.67% of human astrocytes (Fig. 6B). The study was expanded to include a panel of 5 BTICs (hGBM; L0-3, GBM FL17) where again YB-1 was reduced by BMP-4 (Fig. 6B) commensurate with differentiation (Fig. 6C, ex L0 cells). Thus, YB-1 is commonly expressed in primary BTICs and remains expressed as long as cells keep their undifferentiated status.

We then questioned whether YB-1 was associated with more aggressive types of glial tumors by using the WHO grading system as a guide. Glial tumors classified as being WHO I are indolent, slow growing lesions associated with favorable prognoses. In contrast, tumors that are WHO IV (GBM) are rapidly proliferating, and often resistant to

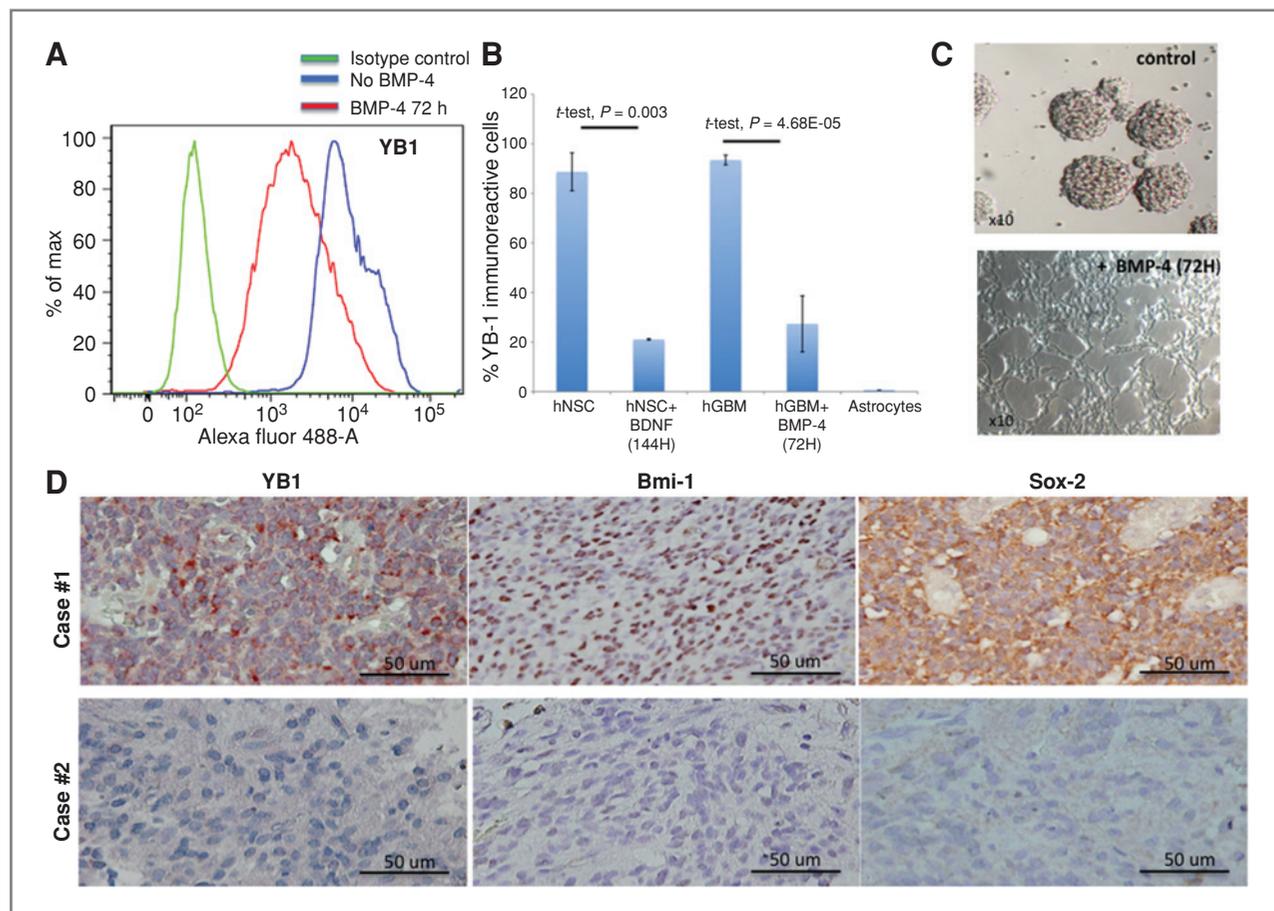


Figure 6. Primary human NSCs and BTICs from GBM express YB-1. A, BMP-4 (100 μ g/mL) stimulated differentiation of L0 cells after 72 hours leading to loss of YB-1 protein expression. B, differentiation of normal hNSCs suppressed YB-1 expression by approximately 80%. To the same degree, BMP-4 caused loss of YB-1 in a panel of BTICs (L0, L1, L2, L3, and GBM FL17 cells). Human astrocytes expressed only 0.67% YB-1-positive cells as compared with hNSCs or BTICs that expressed 95%. C, BMP-4 stimulated differentiation as shown by the change in morphology. D, YB-1, Bmi-1, and Sox-2 were profiled by immunostaining a tumor tissue microarray of 282 cases of grades III and IV gliomas. Examples of coordinate expression of these stem cell markers is shown for 2 cases.

chemotherapy as well as radiation. GBM represent the deadliest form of the disease where survival is only 12 to 17 months despite multimodal therapies (1). Here, we report that a glioma cell line isolated from a WHO grade I tumor expressed trace levels of YB-1, whereas those from WHO IV had higher levels (Supplementary Fig. S5A and B). Consistent with this observation, low-grade primary glial tumors (pilocytic astrocytomas) have little or no YB-1, whereas the high grade tumors (i.e., GBM) express abundant levels (Supplementary Fig. S5C and Table S1). In fact, analysis of these 49 patients showed a significant trend in YB-1 expression as the tumor grade increased ($P = 0.0008$, Supplementary Table S1). The pattern of YB-1 expression in the GBMs was scattered and rather infrequent in many instances, further supporting our data indicating that it is associated with a selected population of cells (Supplementary Fig. S5D).

To assess the specific relationship between YB-1, Bmi-1, and Sox-2, we turned to a larger collection of high-grade gliomas. The expression of YB-1, Bmi-1, and Sox-2 was interrogated in a cohort of 342 cases of high-grade gliomas of which 282 tumors were interpretable for the expression of all of these proteins (representative views; Fig. 6D). Interestingly, there was a strong positive correlation between YB-1 and Bmi-1 in these tumors (Spearman's 0.802, $P > 0.0001$; Supplementary Table S2). Similarly, YB-1 and Sox-2 were expressed to the same degree in the majority of patients (Spearman's 0.6608, $P > 0.0001$; Supplementary Table S2). As expected, Bmi-1 and Sox-1 were similarly expressed (Spearman's 0.7468, $P > 0.0001$; Supplementary Table S2). Within this cohort, there were 15 cases where tumor tissues were available before and after relapse and for which YB-1, Sox-2, and Bmi-1 staining was interpretable. It was noted that YB-1, Sox-2, and Bmi-1 were highly expressed in 10/15 (67%) of the cases that subsequently relapsed. The expression of these proteins was maintained in the relapse samples to varying degrees (data not shown). The coordinate expression of YB-1 along with Sox-2 and Bmi-1 in patient tumors further supports their collective role in glial tumors.

Modulating pathways involved in differentiation is considered as a novel strategy to treat brain tumors (43). One approach has been to identify kinases by screening a library of siRNAs (42). By using this approach, a number of interesting kinases were identified such as the transformation/transcrip-

tion domain-associated protein (TRRAP) that was shown to be essential for sustaining BTICs in an undifferentiated state (42). Similar to our study, they showed that inhibiting TRRAP induced differentiation and markedly suppressed BTIC proliferation based on Ki67 labeling. It is conceivable that stimulating the differentiation of BTICs will lead to new opportunities for therapeutic intervention in the future. Particularly as there are several pathways reported to be involved in chemoresistance in BTICs including increased expression of ATP-binding cassette transporters, conferring a high drug efflux capacity (44) and activation of DNA damage repair mechanisms (45). Of note, these pathways are reportedly regulated by YB-1, which then leads to chemotherapy resistance (46). Therefore, the relationship between YB-1 and glioma BTICs, that we report here, could explain some of the major findings regarding its involvement in multidrug resistance.

To conclude, YB-1 resides both in normal NSCs and BTICs of the brain, where its expression is associated with undifferentiated state. In primary tumors, YB-1 is associated with WHO IV grade tumors that also express high levels of Bmi-1 and Sox-2 suggesting they may contribute to treatment failure. The results of this study support targeting YB-1 as a novel approach in the management of aggressive GBM because this stimulates the BTICs to undergo differentiation and/or suppresses their proliferative capacity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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YB-1 Bridges Neural Stem Cells and Brain Tumor–Initiating Cells via Its Roles in Differentiation and Cell Growth

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