Id-1 is a Key Transcriptional Regulator of Glioblastoma Aggressiveness and a Novel Therapeutic Target

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ABSTRACT

Glioblastoma (GBM) is the most common form of primary adult brain tumors. A majority of GBMs grow invasively into distant brain tissue, leading to tumor recurrence, which is ultimately incurable. It is, therefore, essential to discover master regulators that control GBM invasiveness and target them therapeutically. We demonstrate here that the transcriptional regulator Id-1 plays a critical role in modulating the invasiveness of GBM cell lines and primary GBM cells. Id-1 expression levels positively correlate with glioma cell invasiveness in culture and with histopathological grades in patient biopsies. Id-1 knockdown dramatically reduces GBM cell invasion that is accompanied by profound morphological changes and robust reduction in expression levels of “mesenchymal” markers, as well as inhibition of self-renewal potential and down-regulation of glioma stem cell markers. Importantly, genetic knockdown of Id-1 leads to a significant increase in survival in an orthotopic model of human GBM. Furthermore, we show that a non-toxic compound, cannabidiol, significantly down-regulates Id-1 gene expression and associated glioma cell invasiveness and self-renewal. Additionally, cannabidiol significantly inhibits the invasion of GBM cells through an organotypic brain slice and glioma progression in vivo. Our results suggest that Id-1 regulates multiple tumor-promoting pathways in GBM, and that drugs targeting Id-1 represent a novel and promising strategy for improving the therapy and outcome of GBM patients.
INTRODUCTION

Approximately 20,000 new primary central nervous system tumors are diagnosed each year in the United States. These cancers, known as gliomas, represent the fourth most frequent cause of cancer–related death in younger patients (35–45 years). Moreover, the incidence of the most malignant type of tumor, glioblastoma (GBM), seems to be rising (1, 2). Currently available therapies are only modestly improving the median survival of glioma patients, which is about 14 months. It is hypothesized that certain cells within a heterogeneous primary tumor population evolve through sequential environmental pressure to become highly aggressive, resulting in effective migration and invasion (dispersal) into distant brain tissue (3), processes regulated by specific gene products.

Basic helix-loop-helix (bHLH) proteins are key regulators of lineage- and tissue-specific gene expression in a number of mammalian and non-mammalian organisms, and constitutive expression of Id (Inhibitor of DNA binding) proteins has been shown to inhibit the differentiation of various tissues (4, 5). Id proteins dimerize with bHLH proteins, but, because they lack basic domains, Id-bHLH heterodimers fail to bind DNA (6). Thus, Id proteins are dominant negative regulators of bHLH function. Consistent with de-differentiation of adult tissues being intricately connected to oncogenesis, our group and others have shown that the Id proteins are involved in the pathogenesis of human cancers (7, 8).

We previously showed a strong correlation between Id-1 expression and the invasive and metastatic behavior of breast cancer cells (9, 10). Moreover, aberrant expression of Id-1 protein represented a strong independent prognostic marker in node negative breast cancer (11). Using in vivo selection, transcriptome analysis, functional verification, and clinical validation, a set of genes that marks and mediates breast cancer metastasis to the lungs was identified (12). Among these genes corresponding to the lung metastasis signature, Id-1 was identified as one of the most active
at forming lung metastases and its specific knockdown resulted in a significant reduction in metastatic ability.

Higher levels of Id-1 gene expression have been detected in many different types of aggressive tumor cells, when compared to normal cells of the same tissue origin (7, 8) and several studies have suggested that Id proteins are involved in the development of brain tumors (13-15). Expression analysis of Id proteins in human astrocytic tumors documented increased Id-1, -2, and -3 levels in vascular endothelial cells of the high grade tumors (15), however tumor cells were not examined in this study. Interestingly, two recent studies identified Id-1 as a marker of stem-like tumor-initiating cells in patient-derived primary GBM cells (16) and a transgenic mouse model of disease (17), suggesting Id-1 as a potential therapeutic target.

In this report, we show that Id-1 is a key regulator of brain tumor cell invasiveness and neurosphere growth, and that Id-1 expression is specifically up-regulated in tissues from patients with high-grade gliomas. Importantly, we demonstrate that targeting Id-1 expression using either genetic approaches or the non-toxic cannabinoid, cannabidiol (CBD), leads to a significant reduction in the invasion of both GBM cell lines and patient-derived primary GBM cultures. CBD also significantly inhibits GBM dispersal ex vivo, and reduces tumor growth and Id-1 expression in vivo.
MATERIALS AND METHODS

Primary GBM tissue sample and neurosphere growth assays
Tissue samples were obtained during surgery from patients diagnosed with GBM using an IRB-approved protocol. They were then subjected to enzymatic digest, mechanically dissociated, and cultured as neurospheres as previously described by our group (18). In the neurosphere assays, GBM primary cells as well as GBM cell lines were cultured in neurosphere media (Neural Basal Media+ EGF/FGF2) at 100 and 10 cells/well in 96-well plates. Cells were fed every other day, and neurosphere formation was monitored daily for 7 days.

RNA interference
The following siRNAs purchased from Santa Cruz Biotechnology were used in this study: control siRNA (sc-37007), a non-targeting 20-25 nt siRNA designed as a negative control and Id-1 siRNA (sc-29356), a pool of 3 target-specific 20-25 nt siRNAs designed to knock down gene expression. For optimal siRNA transfection efficiency, Santa Cruz Biotechnology’s siRNA transfection reagent (sc-29528) and siRNA transfection medium (sc-36868) were used. For experiments using primary cells, we used the “smart pool” of 4 oligonucleotides targeting Id-1 (catalog #L-005051) and the control non-targeting pool (catalog # D-001810) from Dharmacon, as previously described by our group (19). Primary GBM cells were plated at 2x10^5 cells per well in six-well plates, and treated with 60 pmols control or Id-1 siRNA. 5 hrs after starting the incubation, the media containing siRNA were replaced with low serum-containing media, and cells cultured for an extra 72 hrs prior to functional assays.

Immunohistochemistry
Tissue microarray slides containing 143 cores, corresponding to 73 cases, were obtained from US Biomax. The cases included glioma grades II-IV, as well as normal brain tissues. Slides were
baked and processed for Id-1 using immunohistochemistry as described (9, 10). Briefly, slides were de-parafinized through a series of xylenes and ethanol, followed by antigen retrieval using Citra Plus solution (Biogenex). Slides were incubated overnight (4°C, in a humidified chamber) with anti-Id-1 antibody (1 µg/ml, Santa Cruz Biotechnology) or the mixture of antibody and blocking peptide. Signal was detected using the “Super Sensitive Polymer-HRP” detection system (Biogenex), according to the manufacturer’s instructions. Slides were counterstained with hematoxylin and de-hydrated using ethanol/xylenes. The percentage of Id-1-positive cells was rated as follows: 2 points, 10-50% positive cells; 3 points, 51-80% positive cells; and 4 points, over 80% positive. The staining intensity was rated as follows: 1 point, weak staining; 2 points, moderate intensity; and 3 points, strong intensity. Points were added to generate overall scores (negative expression, <10% of cells stained positive, regardless of intensity; low expression, 2-3 points; moderate expression, 4-5 points; and high expression, 6-7 points).

Xenograft intracranial model of GBM

Tumors were generated in female athymic nu/nu mice by the intracranial injection of 0.3x10^6 parental U251 cells (used for the drug treatment experiments) or U251 cells expressing ctl- or Id-1-shRNA in 4 µl of RPMI. Survival studies were carried out in accordance with NIH guidelines involving experimental neoplasia and our approved IACUC protocol. Animals were removed from the study when they demonstrated any single sign indicative of significant tumor burden development, including hunched back, sustained decreased general activity, or a significant decrease in weight.

For drug treatment studies (5 mice per group), CBD was dissolved in a mixture of 2% ethanol, 2% Tween 80 and 96% saline and treatment (intraperitoneal injection with 15 mg/kg CBD given 5 days a week for 28 days) was initiated 7 days after the injection of the cells. When vehicle-treated mice first demonstrated signs of significant disease progression (hunched posture and reduced mobility) 35 days after injection of the tumor cell line, mice in all groups were euthanized.
Whole brain was fixed in 4% formaldehyde for 24 hrs. Starting from the frontal lobe, the brains were sliced consecutively into 2 mm coronal sections using a mouse brain slicer matrix (Zivic Instruments) and were paraffin imbedded.

**Statistical analyses**

Significant differences were determined using ANOVA or the unpaired Student’s t-test, where suitable. Bonferroni-Dunn post-hoc analyses were conducted when appropriate. Survival between groups was compared using Kaplan-Meier analysis. P values <0.05 defined statistical significance.

*Additional methods are described in the supplementary information section available on line.*
RESULTS

Id-1 expression correlates with GBM cell invasiveness.

To determine whether there was a correlation between Id-1 expression and GBM cell invasiveness, we evaluated four GBM cell lines. In SF210 and U87 cells, Id-1 protein was not detected whereas significant levels of Id-1 were expressed in SF126 and U251 cells (Figure 1A). We next determined whether there was a correlation between the expression of Id-1 and the magnitude of GBM cell invasion (Figure 1B). In the two cell lines expressing Id-1 (SF126 and U251), there was a substantial increase (5- to 7-fold) in cell invasion in comparison to cell lines where Id-1 expression was below the level of detection using Western blot analysis.

Id-1 is expressed in multiple primary GBM cultures grown in neurosphere conditions.

Primary GBM-derived cells were evaluated for Id-1 expression using immunofluorescence or Western blotting within 48 hrs from initial culturing in neurosphere medium (Figure 1C-D). Using immunofluorescence, we detected Id-1 in several primary GBM-derived cultures (examples shown in Figure 1C). Moreover, of the 23 primary GBM-derived cultures analyzed, 70% expressed Id-1 protein as determined by Western blot analysis (Supplementary Table 1). Representative examples of two Id-1-negative primary GBM cultures (GBM 3 and 4) and two cultures that expressed high Id-1 levels (GBM 5 and 6) are shown in Figure 1D. All cultures tested were derived from patients diagnosed with grade IV GBM.

Id-1 expression correlates with higher tumor grades in a GBM tissue microarray (TMA).

Subsequent studies using immunohistochemical (IHC) analyses of TMAs containing a variety of different grade gliomas documented that Id-1 expression levels correlate with tumor grade (Figure 2 and Table 1). The Id-1 expression levels in the samples were determined by both percentages of positive cells and staining intensities (as described in the Materials and Methods). A gradual
An increase of Id-1-positive samples was observed from normal (0% with 4+ points), pilocytic astrocytoma (25% with 4-5 points and 12% with 6-7 points), astrocytoma II (55% with 4-5 points and 10% with 6-7 points), astrocytoma III (68% with 4-5 points and 20% with 6-7 points), and grade IV astrocytoma (GBM, 24% with 4-5 points and 70% with 6-7 points). These data suggest that Id-1 expression levels are highest in GBMs, the most invasive and malignant phenotype of human gliomas.

**Id-1 controls the invasive and the “mesenchymal”-like phenotype of GBM cells.**

To knockdown Id-1 gene expression, we used the pLXSN-control and pLXSN-Id-1 antisense retroviral vectors (9). We found that inhibition of Id-1 gene expression (Figure 3A) prevented cell invasion (Figure 3B) and that, overall, Id-1 knockdown could reverse the mesenchymal phenotype (as previously defined in primary human GBMs (20, 21) of U251 cells (Figure 3C). Specifically, expression of the vimentin and alpha-tubulin proteins was inhibited and the expression of the key EMT regulator, Snail, was significantly down-regulated. Conversely, Id-2 expression was up-regulated in Id-1 knockdown cells, reminiscent of a more differentiated phenotype since Id-2 overexpression has been previously shown to promote lineage-specific differentiation of GBM neurospheres (22). Moreover, the Id-1 knockdown cells lost expression of phospho-FAK (Figure 3D) as well as secretion of pro-MMP2 and its active isoform (Figure 3E), and lost the active forms of MT1-MMP (Figure 3C). Finally, Id-1 down-regulation triggered dramatic changes in cell morphology (Figure 3F), but only reduced glioma cell proliferation by 36% (p=0.20, Student’s t-test).

**Id-1 regulates the growth of primary GBM neurospheres.**

Double immunofluorescence analysis of primary GBM-derived cultures demonstrated that Id-1 co-localized with one of the markers of stemness, Sox2 (Figure 4A). Using real-time PCR, we detected a preferential expression of Id-1 in glioma stem-like cell fractions (CD133+) where Id-1
levels were three-fold increased as compared to the CD133- fractions (Supplementary Figure 1). Primary GBM cells treated with Id-1 siRNA were subjected to proteomic analysis using a stem cell factor antibody array (Figure 4B). Id-1 knockdown reduced expression of several transcription factors that regulate GBM stem cell self-renewal, including Sox2, Oct-3/4, and Nanog. These data were confirmed using Western blot analysis (Figure 4C). Sox2 is a critical determinant of glioma tumor initiating cell growth in vivo (23), suggesting that Id-1 inhibition may profoundly interfere with GBM self-renewal capacity. Using the same primary GBM cells transiently expressing Id-1 siRNA, we also detected a decrease in Snail expression and FAK phosphorylation, although not at strong as the decrease presented in Figure 3 using U251 cells stably-infected with pLXSN-Id-1 antisense.

We then measured the effects of Id-1 on the growth of primary GBM cultures grown in neurosphere conditions. As shown in Figure 4D-E, Id-1 knockdown using siRNA resulted in a significant reduction in the neurosphere growth of two primary-derived GBM cultures. A reduction in Sox2 levels paralleled the decrease in neurosphere number and size of the Id-1 knockdown cells (Figure 4F). In addition to primary GBM cells, we used U251 cells stably expressing Id-1 antisense and grown in neurosphere media to assess the effects of Id-1 knockdown on neurosphere formation. As shown in Supplementary Figure 2, U251 Id-1-antisense (AS) cells formed significantly fewer neurospheres compared to the U251 control (LXSN) cells. Taken together, these data strongly suggest that Id-1 expression promotes a stem-like phenotype in human GBM.

**Inhibiting Id-1 expression leads to modulation of multiple genes associated with aggressiveness and resistance to chemotherapy, and enhances survival in an orthotopic mouse model of GBM.**

Using proteins extracted from cells infected with pLXSN-control or pLXSN-Id-1 antisense, we performed a phospho-kinase array to screen for proteins whose phosphorylation either increased or decreased upon Id-1 knockdown. As presented in Figure 5A-B, we determined that Id-1 knockdown modulated activity of critical pathways promoting GBM aggressiveness by down-
regulating phosphorylation of ERK1/2 and AKT. Conversely, Id-1 knockdown increased phosphorylation of p38 and CHK2. The modulation of the activity of these four kinases by Id-1 was confirmed by Western blot (Figure 5C). Since glioma stem-like cells are primarily responsible for resistance to both radiation and chemotherapy (24), targeting Id-1 could add a significant therapeutic benefit for glioma patients because it is enriched in the stem-like fraction of GBM cells.

We established stable pooled populations of U251 cells expressing control shRNA (ctl-shRNA) or Id-1-shRNA to determine whether reduction of Id-1 expression in GBM cells alters disease progression in vivo (Supplementary Figure 3). Using Western analysis, we first confirmed the reduction of Id-1 expression by Id-1 shRNA, and determined that this down-regulation led to a significant decrease in the invasion rates of the U251 cells. Finally, athymic nu/nu mice were injected intracranially with U251 cells expressing ctl- or Id-1-shRNA and closely monitored for signs of disease progression. Knockdown of Id-1 led to a median increase in survival of 20 days (p=0.04), and 50% of the mice in the Id-1 knockdown group were still alive 140 days after tumor implantation.

**Cannabidiol (CBD) inhibits Id-1 gene expression and corresponding brain cancer cell invasiveness of U251 cells and primary GBM cells.**

We have recently shown in culture that CBD was an effective inhibitor of Id-1 expression and corresponding breast cancer cell aggressiveness, i.e., invasion and proliferation (25, 26). To determine whether CBD could inhibit Id-1 expression in aggressive brain cancers, U251 cells were treated with CBD for three days and analyzed for Id-1 protein using Western analysis. In U251 cells, CBD produced a concentration-dependent down-regulation of Id-1 (Figure 6A). Additionally, the down-regulation of Id-1 expression correlated with a concentration-dependent inhibition of U251 cell invasion (Figure 6B). Similar activity was observed in primary GBM cells that express Id-1 (Figure 6C-D). Moreover, CBD modulated the phosphorylation of several phospho-kinases in U251 cells including ERK and AKT (Supplementary Figure 4).
CBD inhibits neurosphere formation and Sox2 levels in primary GBM cells.
We next tested whether CBD-induced down-regulation of Id-1 would be as effective as Id-1 knockdown in controlling the stem-like potential of GBM cells. Primary glioblastoma-derived cultures grown in neurosphere conditions were dissociated to a single cell suspension and plated in 12-well plates (100 cells/well) in the presence or absence of CBD. Time-lapse microscopy revealed that treatment with CBD produced a 60%±10 (n=3) reduction in neurosphere formation in primary GBM cultures over a 48-hr period (Figure 6E-F). Importantly, we further determined that Id-1 and Sox2 expression decreased in the neurospheres upon CBD treatment (Figure 6G), similar to what was observed using Id-1 knockdown (see Figure 4).

CBD significantly inhibits GBM dispersal ex vivo and reduces tumorigenicity and Id-1 expression in vivo.
To determine whether CBD could inhibit GBM cell invasion through intact brain tissue, we used an organotypic brain slice assay (27). GFP-labeled U251 cells were treated with vehicle or CBD, and cells that successfully invaded through the slice were visualized using an inverted microscope. We found that CBD was highly effective at inhibiting invasion of U251 cells (Figure 7A).

Although CBD was highly effective at reducing Id-1 expression in cultured cancer cells, it had yet to be determined whether the compound could effectively down-regulate Id-1 expression in vivo. Additionally, no cannabinoid has been shown to inhibit human GBM progression in an orthotopic model. Therefore, tumors were generated in athymic nu/nu mice by intracranial injection of U251 GBM cells. 7 days after tumor implantation, mice were injected systemically (intraperitoneal) with 15 mg/kg CBD 5 days a week for 28 days until vehicle-treated animals demonstrated signs of significant disease progression, when all mice in the study were euthanized in order to compare tumor growth. CBD produced a robust reduction of GBM progression, decreasing the tumor area by 95% (Figure 7B-C). In one of the five mice treated with CBD, no
tumor cells were observed in any of the brain regions analyzed. Target validation showed that, in tumors responding to treatment, CBD produced a significant down-regulation of Id-1 expression (Figure 7D) (the number of Ki67-positive nuclei was also significantly decreased).

In addition to the intracranial model, we performed a longitudinal assessment of the efficacy of CBD in a subcutaneous model of GBM (Supplementary Figure 5). Again, CBD significantly reduced tumor progression and also inhibited the expression of Id-1 and Ki67. Similar to the intracranial model, CBD eradicated the tumor in one of the five mice treated. Overall, CBD was highly effective at reducing Id-1 expression and aggressiveness in cancer cells in culture as well as down-regulating Id-1 expression and tumorigenesis in vivo.
DISCUSSION

Although previous studies suggested a role for Id proteins in the biology of glial cells (13, 15), there is a paucity of data defining the functional role that Id-1 plays in glioma progression. We demonstrate here for the first time that, in GBM cell lines and primary cultures of GBM tissue, Id-1 expression is associated with a significant increase in invasiveness of cells. Whereas the expression of Id-1 protein was not detected in normal human brain tissue, its expression highly correlated with increased brain tumor grade and it was detected in 70% of the primary GBM tissues analyzed (Supplementary Table 1). Furthermore, functional studies demonstrate that Id-1 expression directly impacts GBM cell invasion and self-renewal, as measured by culture and ex vivo invasion assays and neurosphere growth assays, respectively.

In U251 cells, Id-1 knockdown led to almost complete inhibition of cell invasion but only a modest reduction in cell growth. Even more striking was the profound change in morphology produced in this cell population where the cells rounded up into grape-like cluster. These changes suggested induction of a cellular differentiated state. In agreement with this observation, we observed a significant reduction in expression of markers associated with EMT (vimentin and snail) and invasion (MT1-MMP, MMP-2, and p-FAK). While experiments assessing GBM cell invasion in culture and in an organotypic brain slice assay provide strong evidence for a role of Id-1 in controlling GBM dispersal, future studies including imaging of GBM cells in orthotopic models will help to further clarify the importance of Id-1 on the regulation of the invasive phenotype during tumor progression. Using a phospho-kinase array, we also found multiple proteins associated with tumor aggressiveness were down-regulated upon knockdown of Id-1, notably p-ERK and p-AKT pathways. In addition, phosphorylation of specific proteins (p38 MAPK and CHK2) associated with chemosensitivity was modulated in a fashion that would resensitize the cells to first-line agents. This is in agreement with previous studies focusing on the role of Id-1 in bladder and prostate cancer cells (28, 29).
We also studied the impact of reducing Id-1 expression on primary GBM-derived cultures grown in neurosphere conditions (30). We found that Id-1 was preferentially expressed in the CD133+ subpopulation of primary GBM cells. Id-1 knockdown significantly reduced primary GBM neurosphere growth and expression levels of Sox2, which has been shown to drive the tumorigenicity of glioma initiating cells (23); therefore, targeting Id-1 may in turn down-regulate Sox2 expression and thus reduce tumor recurrence, which is believed to be driven by glioma stem-like cells. The above-mentioned results suggest that Id-1 promotes the maintenance of an undifferentiated, stem-like phenotype in brain cancer cells.

It has previously been reported that the self-renewing capacity of hematopoietic stem cells is severely compromised in the absence of Id-1 (31). Interestingly, recently published results showed that glioma initiating cells characterized by high CD44 and Id-1 levels were responsible for tumor growth and recurrence in vivo and could be targeted using TGFβ inhibitors (16). Furthermore, high levels of Id-1 specifically labeled a subpopulation of highly tumorigenic glioma cells isolated from a transgenic mouse model of the disease (32, 33).

Consistent with the breast cancer study (25), we found that the non-psychoactive cannabinoid CBD significantly down-regulated Id-1 expression in serum-derived and primary GBM cells. As expected, we observed robust inhibition of glioma cell invasiveness similar to that observed using genetic knockdown of Id-1. In GBM cells, CBD was able to inhibit p-ERK and p-AKT. In primary GBM cultures, CBD inhibited neurosphere growth and the expression levels of Sox2. These effects of CBD were similar to those observed with genetic silencing of Id-1 expression in GBM cells. CBD was also effective at inhibiting tumor dispersal through an organotypic brain slice where neuro-glial cell morphology, anatomical components, and network connections are preserved.

To determine whether CBD was effective at down-regulating Id-1 expression in vivo, we used an intracranial U251 glioma xenograft model. Treatment of mice with CBD significantly reduced Id-1 expression within the tumor tissue, demonstrating that the drug can effectively...
modulate this target in an orthotopic mouse model of the disease. Importantly, treatment with CBD also produced a robust inhibition of tumor progression. Since the knockdown of Id-1 produced only a modest effect on cell growth in culture, we expect that CBD may have additional antitumor properties not related to the down-regulation of Id-1, i.e., this additional activity of CBD may be related to the production of reactive oxygen species (ROS), and modulation of lipoxygenase and fatty acid amide hydrolase (34-36).

In conclusion, our results establish Id-1 as a key regulator of both invasion and stemness in GBM cells and demonstrate that the non-toxic cannabinoid compound CBD down-regulates Id-1 expression and tumor aggressiveness in culture and \textit{in vivo}. The data also shed light on some of the key pathways that control GBM cell dispersal and progression. A greater understanding of these pathways may lead to more effective therapies for cancer patients including the additional refinement of cannabinoid analogs targeting Id-1. We expect our efforts to ultimately translate to the development of future clinical trials with nontoxic compounds that target the expression of Id-1, a master regulator of GBM aggressiveness. With its lack of systemic toxicity and psychoactivity, CBD is an ideal candidate agent in this regard and may prove useful in combination with front-line agents for the treatment of patients with aggressive and high-grade GBM tumors.
ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. Id-1 is expressed in GBM cell lines and primary cultures and its expression levels correlate with increased invasiveness.  **A)** GBM cells were analyzed for expression of Id-1 by Western blot analysis.  Loading controls were carried out by stripping the blots and re-probing with an anti-actin antibody.  **B)** Boyden chamber invasion assay was used to compare the invasiveness of GBM cell lines.  Assays were performed in modified Boyden Chambers with Matrigel coated 8 µm pore filter inserts.  Data are presented as mean number (#) of cells (in triplicate wells) that invaded through the Matrigel.  The experiment was repeated twice with similar results; bars ± SE.  Data were compared using a one-way ANOVA with a corresponding Bonferroni’s post-hoc test.  (*) indicates statistically significant differences from SF210 and U87 cell invasiveness (p<0.001).  **C)** Primary GBM cultures designated as GBM 1 (upper panels) and GBM 2 (lower panels) were processed for Id-1 immunofluorescence.  The left panels show cells that were pretreated with the Id-1 blocking peptide.  The lack of signal in the left panels demonstrates the specificity of Id-1 staining illustrated in the middle panels (arrows).  The right panels show immunostaining for Nestin.  Nuclei were counterstained with DAPI.  Bar = 100 µm.  **D)** Primary GBM cultures were processed for Id-1 by Western blotting.  Panel shows an example of two GBM cultures (GBM 3 and 4) that are negative for Id-1 and two primary cultures that express Id-1 (GBM 5 and 6).

Figure 2. Id-1 expression levels, measured by IHC, correlate with brain tumor grade.  Tissue microarray (TMA) slides corresponding to 46 cases (92 cores) were obtained from Biomax.  The cases included glioma grades II-IV, as well as normal brain tissues.  Slides were processed for Id-1 immunostaining.  Panels show a representative example of a grade IV (GBM) positive for Id-1, and illustrate a low-grade astrocytoma (LGA) with lower Id-1 expression levels, while the control/normal
brain (NB) tissue does not show any specific Id-1 immunostaining. Bar = 50 μm, Inset magnification 40X.

**Figure 3. Inhibition of Id-1 expression leads to a marked decrease in U251 cell invasiveness and profound morphological changes.** A) Pooled U251 cells infected with pLXSN-control or pLXSN-Id-1 antisense were analyzed for expression of Id-1 by Western blot analysis. B) Mean number of cells (from triplicate wells) which invaded Matrigel are shown. The experiment was repeated twice. Bars ± SE; * p<0.007 by Student’s t-test. C) Expression of vimentin, alpha-tubulin, Id-2, Snail and MT1-MMP in the two cell populations was analyzed by Western blotting. D) The expression of phospho-FAK and total FAK was compared. E) The expression of gelatinases was determined using zymography. F) The morphology of pooled U251 cells infected with pLXSN-control or pLXSN-Id-1 antisense (AS) was compared by phase microscopy (upper panels) and cells were processed for vinculin (green) and phalloidin (red) immunofluorescence (lower panels). Bar = 100 μm.

**Figure 4. Id-1 knockdown in primary human GBMs reduces neurosphere growth and Sox2 levels.** A) Primary GBM cells were processed for Id-1 immunofluorescence (left panel) and double immunofluorescence Id-1/Sox2 (right panel). Propidium iodide (PI) was used to counterstain cell nuclei. Bar = 100 μm. B) Primary GBM cells were treated with control scrambled (ctl) or Id-1 siRNA (“smart pool” of 4 targeting oligonucleotides) and cell lysates were used to hybridize with a stem cell antibody array. The spots corresponding to Sox2 (1), Oct-3/4 (2) and Nanog (3) are indicated. C) A portion of the cell lysates was used to confirm Id-1 knockdown in GBM #1 and down-regulation of Sox2, Oct-3/4, Nanog, Snail and p-FAK levels by Western analysis. D) Primary GBM cells treated with control (ctl) or Id-1 siRNA were observed in a neurosphere formation assay. Microphotographs were captured 120 h following initial cell plating. Bar = 100 μm. E) Average
sphere numbers in triplicate wells from a representative experiment in two separate primary-derived GBM cultures are shown. The experiment was repeated three times. ** p< 0.001, Student’s t-test. F) Cell lysates were prepared from GBM #2 cultured in neurosphere conditions in the presence of Id-1 siRNA and subjected to Western blot analysis for Sox2 and Id-1.

Figure 5. Id-1 knockdown modulates activity levels of phosphoproteins that control glioma cell survival and aggressiveness. A) U251 control and stable Id-1 AS U251 cells were grown in the absence of serum for 48 hrs and cell lysates were subjected to phopho-kinase array profiling. Blots illustrate phosphorylation levels of several cellular kinases in duplicate. Numbered spots are as follows: (1) p-p38; (2) p-CHK2; (3) p-ERK1/2; (4) p-AKT. B) Films were scanned and quantified, following manufacturer’s instructions. Relative phosphorylation levels for several cellular kinases are shown. C) A portion of the cell lysates was used to confirm phosphorylation levels of several kinases using Western blot analysis. Total protein levels of the indicated phospho-kinases were not changed.

Figure 6. CBD inhibits Id-1 expression and corresponding GBM cell invasiveness, and reduces primary GBM neurosphere growth. U251 and primary GBM cells were treated for 3 days with CBD (1 or 1.5 µM). A and C) Proteins were extracted from U251 and primary GBM cells and analyzed for Id-1 using Western blot analysis. B and D) Relative invasiveness of U251 and primary GBM cells was calculated as the invasion of CBD-treated versus vehicle control-treated cells. Mean cell numbers in triplicate wells were compared using a Student’s t-test or a one-way ANOVA with a corresponding Dunnett’s post-hoc test. (*) indicates statistically significant differences from control (p<0.01). The experiments were repeated twice and error bars represent the SE. E) Phase contrast images from time-lapse videos show inhibition of neurosphere formation of primary GBM cells by 1 µM CBD. Time is indicated in hours, Bar = 50 µM. F) Data are presented as mean number (#) of primary GBM neurospheres in triplicate wells treated with
vehicle control or with CBD. The experiment was repeated twice. Bars ± SE; *p< 0.05 by Student’s t-test. G) Western blotting was performed to compare Id-1 or Sox2 expression in neurospheres from vehicle control- versus CBD-treated primary GBM cells.

Figure 7. CBD inhibits GBM invasion through an organotypic brain slice, and reduces Id-1 expression and tumor progression in vivo. A) GFP-labeled U251 cells were treated with vehicle (control) or 1 µM CBD for 72h. Cells that migrated through the slice were counted using an inverted fluorescence microscope. Data is shown as mean number of cells in triplicate wells. Bars ± SE, *p<0.05 Student’s t-test. The experiment was repeated three times with similar results. Inset: representative samples of invading cells visualized from the bottom of the slice. B) Tumors were generated in a xenograft mouse model by intracranial injection of 0.3x10^6 U251 cells (5 mice/group). Daily treatments with 15 mg/kg CBD were initiated seven days after tumor implantation. C) Panoramic viewer software (3DHISTECH) was used to measure the area of the tumor in the brain. (*) indicates statistically significant differences from control (p<0.02). D) Representative sections demonstrate reduction in Id-1 (upper panel) and Ki67 (middle panel) expression in tumors responsive to CBD treatment. Bar = 100 µM. The insets (top panels) represent a 20-fold magnification. Negative IgG controls (NC) are also shown.
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Table 1. Scoring of Id-1 immunostaining of human brain tissues.
Figure 1

**A**

![Image of Western Blot](image1)

**B**

![Graph showing invasiveness](image2)

**C**

![Images of GBM samples](image3)

**D**

![Another Western Blot](image4)
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7
Id-1 is a Key Transcriptional Regulator of Glioblastoma Aggressiveness and a Novel Therapeutic Target

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