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

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Abstract

Glioblastoma is the most common form of primary adult brain tumors. A majority of glioblastomas grow invasively into distant brain tissue, leading to tumor recurrence, which is ultimately incurable. It is, therefore, essential to discover master regulators that control glioblastoma invasiveness and target them therapeutically. We show here that the transcriptional regulator Id-1 plays a critical role in modulating the invasiveness of glioblastoma cell lines and primary glioblastoma cells. Id-1 expression levels positively correlate with glioma cell invasiveness in culture and with histopathologic grades in patient biopsies. Id-1 knockdown dramatically reduces glioblastoma cell invasion that is accompanied by profound morphologic changes and robust reduction in expression levels of "mesenchymal" markers, as well as inhibition of self-renewal potential and downregulation of glioma stem cell markers. Importantly, genetic knockdown of Id-1 leads to a significant increase in survival in an orthotopic model of human glioblastoma. Furthermore, we show that a nontoxic compound, cannabidiol, significantly downregulates Id-1 gene expression and associated glioma cell invasiveness and self-renewal. In addition, cannabidiol significantly inhibits the invasion of glioblastoma cells through an organotypic brain slice and glioma progression *in vivo*. Our results suggest that Id-1 regulates multiple tumor-promoting pathways in glioblastoma and that drugs targeting Id-1 represent a novel and promising strategy for improving the therapy and outcome of patients with glioblastoma. *Cancer Res; 73(5); 1559–69. ©2012 AACR.*

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, seems to be increasing (1, 2). Currently available therapies are only modestly improving the median survival of patients with glioma, 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.

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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 dedifferentiation 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

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compared with 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). Interestingly, 2 recent studies identified Id-1 as a marker of stem-like tumor-initiating cells in patient-derived primary glioblastoma 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 upregulated in tissues from patients with high-grade gliomas. Importantly, we show that targeting Id-1 expression using either genetic approaches or the nontoxic cannabinoid, cannabidiol, leads to a significant reduction in the invasion of both glioblastoma cell lines and patient-derived primary glioblastoma cultures. Cannabidiol also significantly inhibits glioblastoma dispersal *ex vivo* and reduces tumor growth and Id-1 expression *in vivo*.

Materials and Methods

Primary glioblastoma tissue sample and neurosphere growth assays

Tissue samples were obtained during surgery from patients diagnosed with glioblastoma using an Institutional Review Board-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, glioblastoma primary cells as well as glioblastoma cell lines were cultured in neurosphere media [neural basal media + EGF/fibroblast growth factor (FGF)2] at 100 and 10 cells per 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 nontargeting 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 knockdown 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 nontargeting pool (catalog # D-001810) from Dharmacon, as previously described by our group (19). Primary glioblastoma cells were plated at 2×10^5 cells per well in 6-well plates and treated with 60 pmols control or Id-1 siRNA. Five hours after starting the incubation, the media containing siRNA were replaced with low serum-containing media, and cells cultured for an extra 72 hours before functional assays.

Immunohistochemistry

Tissue microarray (TMA) slides containing 143 cores, corresponding to 73 cases, were obtained from U.S. 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 deparafinized 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 dehydrated 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, >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 glioblastoma

Tumors were generated in female athymic *nu/nu* mice by the intracranial injection of 0.3×10^6 parental U251 cells (used for the drug treatment experiments) or U251 cells expressing control (ctl) or Id-1-short hairpin RNA (shRNA) in 4 μL of RPMI. Survival studies were conducted in accordance with NIH guidelines involving experimental neoplasia and our approved Institutional Animal Care and Use Committee protocol. Animals were removed from the study when they showed 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), cannabidiol was dissolved in a mixture of 2% ethanol, 2% Tween-80, and 96% saline, and treatment (intraperitoneal injection with 15 mg/kg cannabidiol given 5 d/wk for 28 days) was initiated 7 days after the injection of the cells. When vehicle-treated mice first showed 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 hours. 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-embedded.

Statistical analyses

Significant differences were determined using ANOVA or the unpaired Student *t* test, where suitable. Bonferroni–Dunn *post hoc* analyses were conducted when appropriate. Survival between groups was compared using Kaplan–Meier analysis. $P < 0.05$ defined statistical significance.

Additional methods are described in the Supplementary Information section available on line.

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Results

Id-1 expression correlates with glioblastoma cell invasiveness

To determine whether there was a correlation between Id-1 expression and glioblastoma cell invasiveness, we evaluated 4 glioblastoma 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 (Fig. 1A). We next determined whether there was a correlation between the expression of Id-1 and the magnitude of glioblastoma cell invasion (Fig. 1B). In the 2 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.

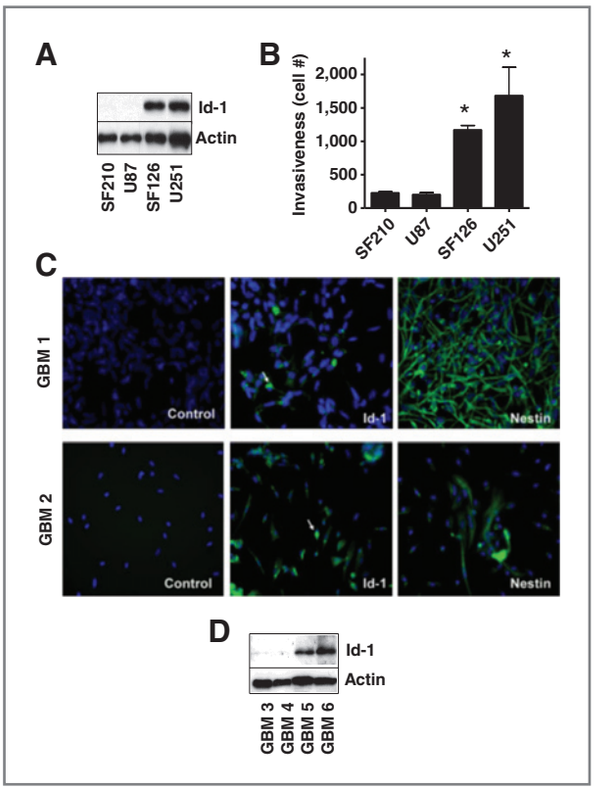

Figure 1. Id-1 is expressed in glioblastoma (GBM) cell lines and primary cultures and its expression levels correlate with increased invasiveness. A, GBM cells were analyzed for expression of Id-1 by Western blot analysis. Loading controls were carried out by stripping the blots and reprobing with an anti-actin antibody. B, Boyden chamber invasion assay was used to compare the invasiveness of GBM cell lines. Assays were conducted 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 *post hoc* test. *, statistically significant differences from SF210 and U87 cell invasiveness ($P < 0.001$). C, primary GBM cultures designated as GBM 1 (top) and GBM 2 (bottom) were processed for Id-1 immunofluorescence. Left, cells that were pretreated with the Id-1 blocking peptide. The lack of signal in the left shows the specificity of Id-1 staining illustrated in the middle (arrows). Right, immunostaining for Nestin. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. D, primary GBM cultures were processed for Id-1 by Western blotting. Panel shows an example of 2 GBM cultures (GBM 3 and 4) that are negative for Id-1 and 2 primary cultures that express Id-1 (GBM 5 and 6).

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

Primary glioblastoma-derived cells were evaluated for Id-1 expression using immunofluorescence or Western blotting within 48 hours from initial culturing in neurosphere medium (Fig. 1C and D). Using immunofluorescence, we detected Id-1 in several primary glioblastoma-derived cultures (examples shown in Fig. 1C). Moreover, of the 23 primary glioblastoma-derived cultures analyzed, 70% expressed Id-1 protein as determined by Western blot analysis (Supplementary Table S1). Representative examples of 2 Id-1–negative primary glioblastoma cultures (GBM 3 and 4) and 2 cultures that expressed high Id-1 levels (GBM 5 and 6) are shown in Fig. 1D. All cultures tested were derived from patients diagnosed with grade IV glioblastoma.

Id-1 expression correlates with higher tumor grades in a glioblastoma 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 (Fig. 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 Materials and Methods). A gradual 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 (glioblastoma, 24% with 4–5 points and 70% with 6–7 points). These data suggest that Id-1 expression levels are highest in glioblastomas, the most invasive and malignant phenotype of human gliomas.

Id-1 controls the invasive and the mesenchymal-like phenotype of glioblastoma 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 (Fig. 3A) prevented cell invasion (Fig. 3B) and that, overall, Id-1 knockdown could reverse the mesenchymal phenotype (as previously defined in primary human glioblastomas; refs. 20, 21) of U251 cells (Fig. 3C). Specifically, expression of the vimentin and α-tubulin proteins was inhibited, and the expression of the key epithelial–mesenchymal transition (EMT) regulator, Snail, was significantly downregulated. Conversely, Id-2 expression was upregulated in Id-1 knockdown cells, reminiscent of a more differentiated phenotype as Id-2 overexpression has been previously shown to promote lineage-specific differentiation of glioblastoma neurospheres (22). Moreover, the Id-1 knockdown cells lost expression of phospho-focal adhesion kinase (FAK; Fig. 3D) as well as secretion of pro-MMP2 and its active isoform (Fig. 3E) and lost the active forms of MT1-MMP (Fig. 3C). Finally, Id-1

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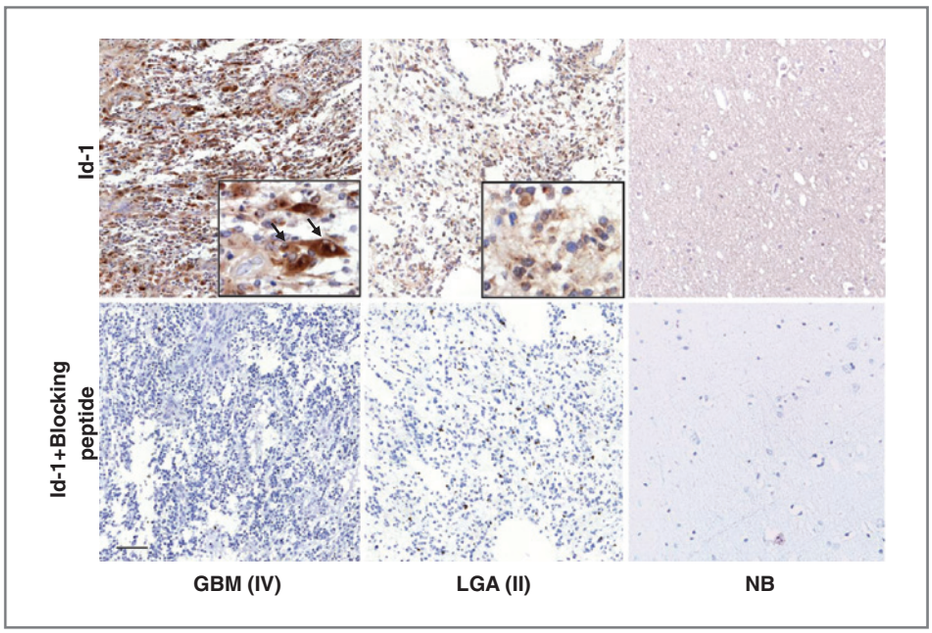

Figure 2. Id-1 expression levels, measured by IHC, correlate with brain tumor grade. TMA slides corresponding to 46 cases (92 cores) were obtained from Biomax. The cases included glioma grades II–IV, as well as normal brain tissues. Slides were processed for Id-1 immunostaining. Panels show a representative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

downregulation triggered dramatic changes in cell morphology (Fig. 3F), but only reduced glioma cell proliferation by 36% ($P = 0.20$; Student t test).

Id-1 regulates the growth of primary glioblastoma neurospheres

Double immunofluorescence analysis of primary glioblastoma-derived cultures showed that Id-1 colocalized with one of the markers of stemness, Sox2 (Fig. 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 3-fold increased as compared with the CD133- fractions (Supplementary Fig. S1). Primary glioblastoma cells treated with Id-1 siRNA were subjected to proteomic analysis using a stem cell factor antibody array (Fig. 4B). Id-1 knockdown reduced expression of several transcription factors that regulate glioblastoma stem cell self-renewal, including Sox2, Oct-3/4, and Nanog. These data were confirmed using Western blot analysis (Fig. 4C). Sox2 is a critical determinant of glioma tumor-initiating cell growth *in vivo* (23), suggesting that Id-1 inhibition may profoundly interfere with glioblastoma self-renewal capacity. Using the same primary glioblastoma cells transiently expressing Id-1 siRNA, we also detected a decrease in Snail expression and FAK phosphorylation, although not as strong as the decrease presented in Fig. 3 using U251 cells stably infected with pLXSN-Id-1 antisense.

We then measured the effects of Id-1 on the growth of primary glioblastoma cultures grown in neurosphere conditions. As shown in Fig. 4D and E, Id-1 knockdown using siRNA resulted in a significant reduction in the neurosphere growth of 2 primary-derived glioblastoma cultures. A reduction in Sox2 levels paralleled the decrease in neurosphere number and size of the Id-1 knockdown cells (Fig. 4F). In addition to primary glioblastoma 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 Fig. S2, U251 Id-1 antisense (AS) cells formed significantly fewer neurospheres compared with the U251 control (LXSN) cells. Taken together, these data strongly suggest that Id-1 expression promotes a stem-like phenotype in human glioblastoma.

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 glioblastoma

Using proteins extracted from cells infected with pLXSN-control or pLXSN-Id-1 antisense, we conducted a phosphokinase array to screen for proteins whose phosphorylation either increased or decreased upon Id-1 knockdown. As presented in Fig. 5A and B, we determined that Id-1 knockdown modulated activity of critical pathways promoting

Table 1. Scoring of Id-1 immunostaining of human brain tissues.

Diagnosis/ score	Adult normal brain (14 cases)	Astrocytoma I (Pilocytic - 8 cases)	Astrocytoma II (16 cases)	Astrocytoma III (13 cases)	Glioblastoma (28 cases)
4–5 points	0%	25%	55%	68%	24%
6–7 points	0%	12%	10%	20%	70%

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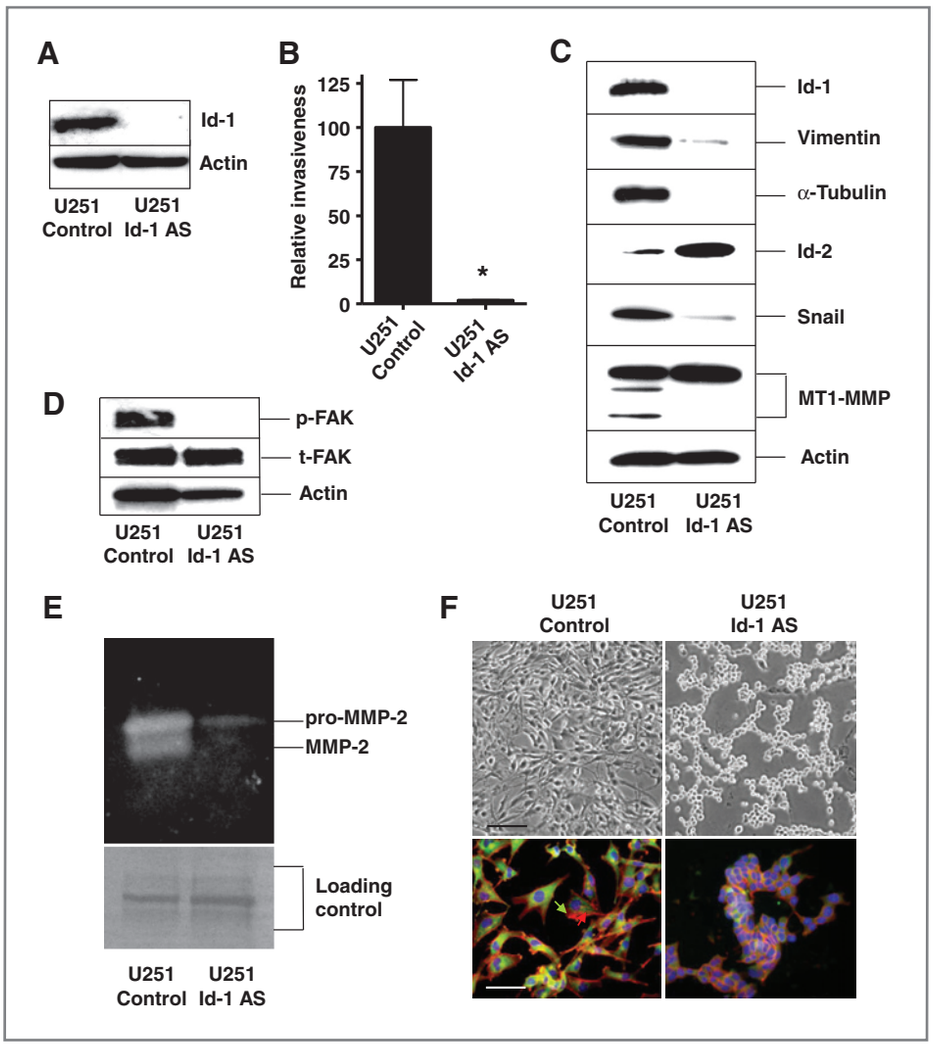

Figure 3. Inhibition of Id-1 expression leads to a marked decrease in U251 cell invasiveness and profound morphologic changes. A, pooled U251 cells infected with pLXSN-control or pLXSN-Id-1 antisense were analyzed for expression of Id-1 by Western blot analysis. B, mean number of cells (from triplicate wells) that invaded Matrigel are shown. The experiment was repeated twice. Bars ± SE. *, $P < 0.007$ by Student t test. C, expression of vimentin, α-tubulin, Id-2, Snail, and MT1-MMP in the 2 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 contrast microscopy (top) and cells were processed for vinculin (green) and phalloidin (red) immunofluorescence (bottom). Bar, 100 μm.

glioblastoma aggressiveness by downregulating phosphorylation of extracellular signal–regulated kinase (ERK)1/2 and AKT. Conversely, Id-1 knockdown increased phosphorylation of p38 and CHK2. The modulation of the activity of these 4 kinases by Id-1 was confirmed by Western blotting (Fig. 5C). As 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 patients with glioma because it is enriched in the stem-like fraction of glioblastoma cells.

We established stable pooled populations of U251 cells expressing ctl-shRNA or Id-1-shRNA to determine whether reduction of Id-1 expression in glioblastoma cells alters disease progression *in vivo* (Supplementary Fig. S3). Using Western blot analysis, we first confirmed the reduction of Id-1 expression by Id-1 shRNA and determined that this downregulation 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 inhibits Id-1 gene expression and corresponding brain cancer cell invasiveness of U251 cells and primary glioblastoma cells

We have recently shown in culture that cannabidiol was an effective inhibitor of Id-1 expression and corresponding breast cancer cell aggressiveness, that is, invasion and proliferation (25, 26). To determine whether cannabidiol could inhibit Id-1 expression in aggressive brain cancers, U251 cells were treated with cannabidiol for 3 days and analyzed for Id-1 protein using Western blot analysis. In U251 cells, cannabidiol produced a concentration-dependent downregulation of Id-1 (Fig. 6A). In addition, the downregulation of Id-1 expression correlated with a concentration-dependent inhibition of U251 cell invasion (Fig. 6B). Similar activity was observed in primary glioblastoma cells that express Id-1 (Fig. 6C and D). Moreover, cannabidiol modulated the phosphorylation of several

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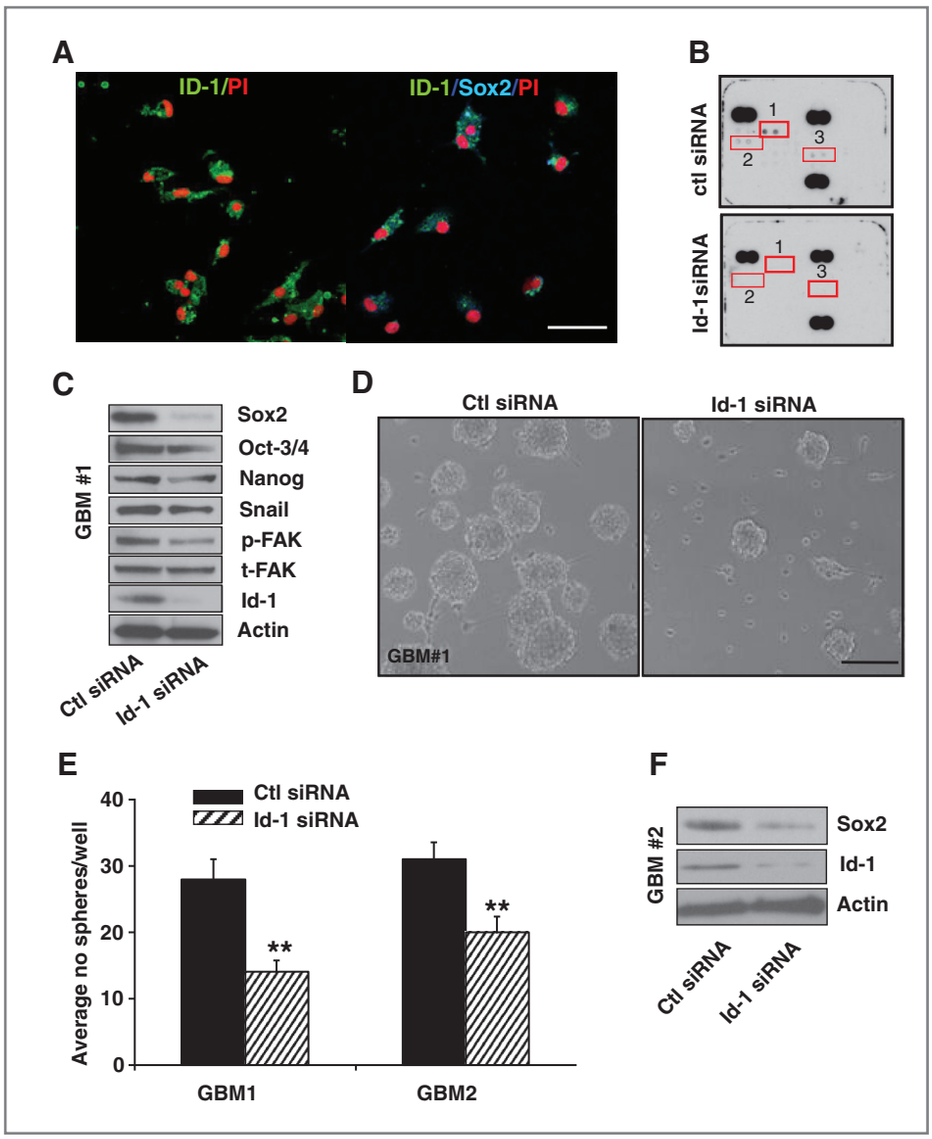

Figure 4. Id-1 knockdown in primary human glioblastomas (GBM) reduces neurosphere growth and Sox2 levels. A, primary GBM cells were processed for Id-1 immunofluorescence (left) and double immunofluorescence Id-1/Sox2 (right). Propidium iodide was used to counterstain cell nuclei. Bar, 100 μm. B, primary 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 downregulation of Sox2, Oct-3/4, Nanog, Snail, and p-FAK levels by Western blot analysis. D, primary GBM cells treated with control (ctl) or Id-1 siRNA were observed in a neurosphere formation assay. Microphotographs were captured 120 hours following initial cell plating. Bar, 100 μm. E, average sphere numbers in triplicate wells from a representative experiment in 2 separate primary-derived GBM cultures are shown. The experiment was repeated 3 times. **, $P < 0.001$, Student 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.

phospho-kinases in U251 cells, including AKT (Supplementary Fig. S4).

Cannabidiol inhibits neurosphere formation and Sox2 levels in primary glioblastoma cells

We next tested whether cannabidiol-induced downregulation of Id-1 would be as effective as Id-1 knockdown in controlling the stem-like potential of glioblastoma 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 cannabidiol. Time-lapse microscopy revealed that treatment with cannabidiol produced a 60% ± 10% ($n = 3$) reduction in neurosphere formation in primary glioblastoma cultures over a 48-hour period (Fig. 6E and F). Importantly, we further determined that Id-1 and Sox2 expression decreased in the neurospheres upon cannabidiol treatment (Fig. 6G), similar to what was observed using Id-1 knockdown (see Fig. 4).

Cannabidiol significantly inhibits glioblastoma dispersal *ex vivo* and reduces tumorigenicity and Id-1 expression *in vivo*

To determine whether cannabidiol could inhibit glioblastoma cell invasion through intact brain tissue, we used an organotypic brain slice assay (27). GFP-labeled U251 cells were treated with vehicle or cannabidiol and cells that successfully invaded through the slice were visualized using an inverted microscope. We found that cannabidiol was highly effective at inhibiting invasion of U251 cells (Fig. 7A).

Although cannabidiol was highly effective at reducing Id-1 expression in cultured cancer cells, it had yet to be determined whether the compound could effectively downregulate Id-1 expression *in vivo*. In addition, no cannabinoid has been shown

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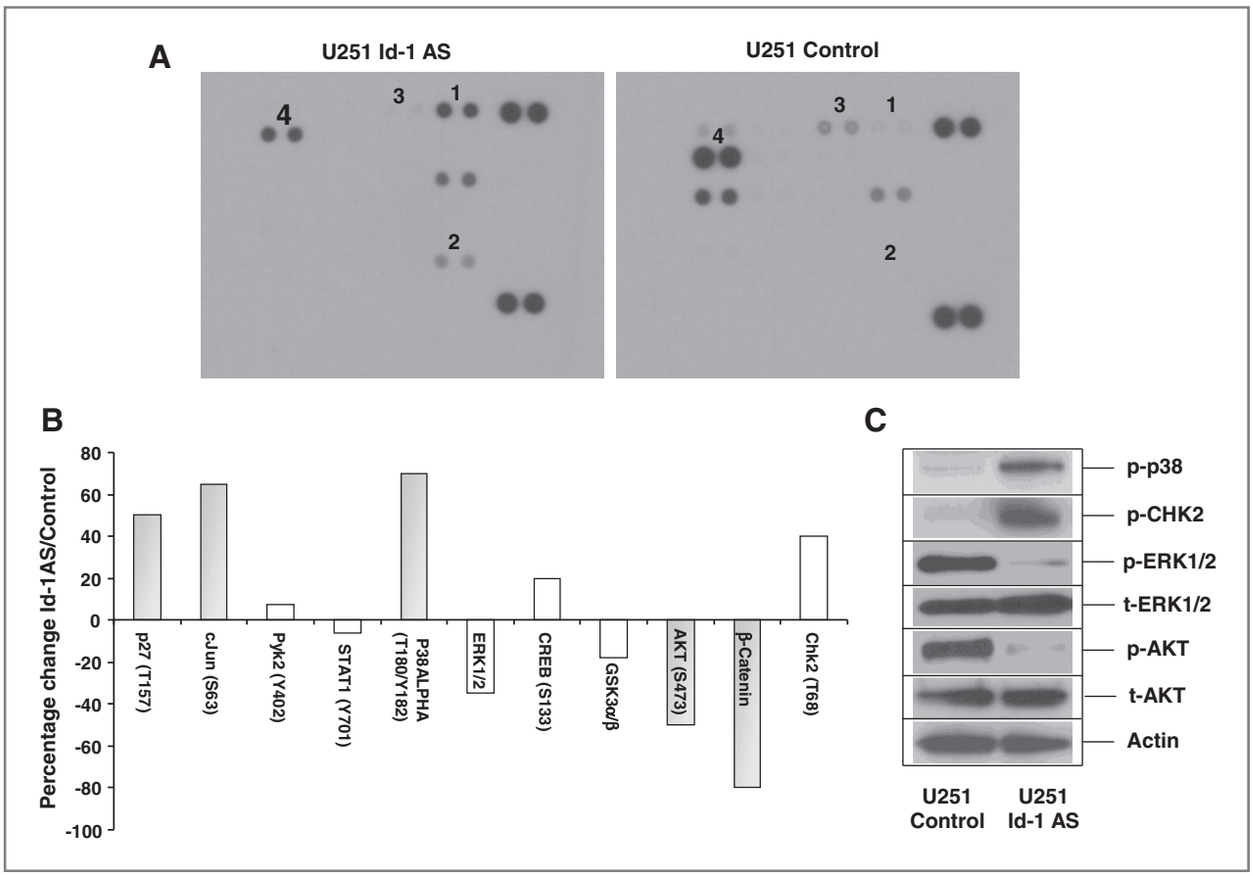

Figure 5. Id-1 knockdown modulates activity levels of phosphoproteins that control glioma cell survival and aggressiveness. A, U251 control and stable Id-1 AS U251 cells were grown in the absence of serum for 48 hours, and cell lysates were subjected to phopho-kinase array profiling. Blots illustrate phosphorylation levels of several cellular kinases in duplicate. Numbered spots are as follows: p-p38 (1), p-CHK2 (2), p-ERK1/2 (3), and p-AKT (4). 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.

to inhibit human glioblastoma progression in an orthotopic model. Therefore, tumors were generated in athymic *nu/nu* mice by intracranial injection of U251 glioblastoma cells. Seven days after tumor implantation, mice were injected systemically (intraperitoneal) with 15 mg/kg cannabidiol 5 d/wk for 28 days until vehicle-treated animals showed signs of significant disease progression, when all mice in the study were euthanized to compare tumor growth. Cannabidiol produced a robust reduction of glioblastoma progression, decreasing the tumor area by 95% (Fig. 7B and C). In 1 of the 5 mice treated with cannabidiol, no tumor cells were observed in any of the brain regions analyzed. Target validation showed that in tumors responding to treatment, cannabidiol produced a significant downregulation of Id-1 expression (Fig. 7D; the number of Ki67-positive nuclei was also significantly decreased).

In addition to the intracranial model, we conducted a longitudinal assessment of the efficacy of cannabidiol in a subcutaneous model of glioblastoma (Supplementary Fig. S5). Again, cannabidiol significantly reduced tumor progression and also inhibited the expression of Id-1 and Ki67. Similar to the intracranial model, cannabidiol eradicated the tumor in 1 of the 5 mice treated. Overall, cannabidiol was highly effective at reducing Id-1 expression and aggressiveness in cancer cells in culture as well as downregulating 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 show here for the first time that in glioblastoma cell lines and primary cultures of glioblastoma 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 glioblastoma tissues analyzed (Supplementary Table S1). Furthermore, functional studies show that Id-1 expression directly impacts glioblastoma cell invasion and self-renewal, as measured by culture

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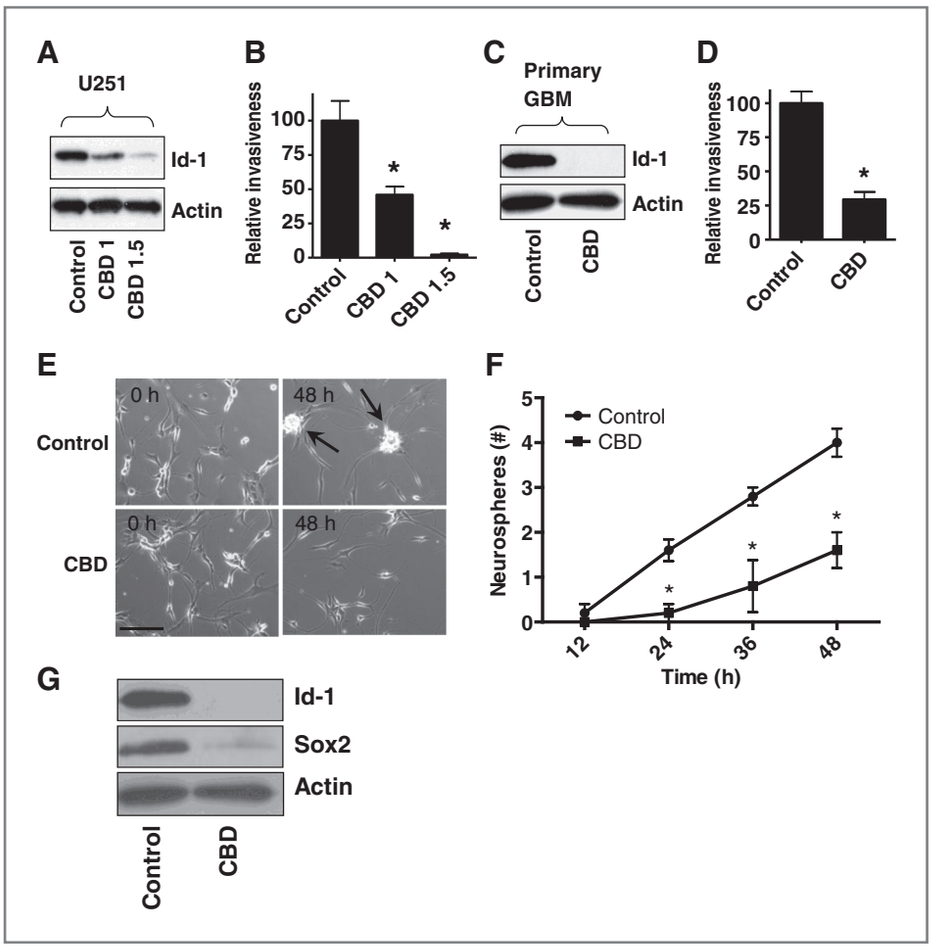

Figure 6. Cannabidiol (CBD) inhibits Id-1 expression and corresponding glioblastoma (GBM) cell invasiveness and reduces primary GBM neurosphere growth. U251 and primary GBM cells were treated for 3 days with CBD (1 or 1.5 μmol/L). A and C, proteins were extracted from U251 and primary GBM cells and analyzed 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 the Student *t* test or a one-way ANOVA with a corresponding Dunnett *post hoc* test. *,statistically significant differences from control ($P < 0.01$). The experiments were repeated twice and error bars represent SE. E, phase contrast images from time-lapse videos show inhibition of neurosphere formation of primary GBM cells by 1 μmol/L 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 *t* test. G, Western blotting was conducted to compare Id-1 or Sox2 expression in neurospheres from vehicle control- versus CBD-treated primary GBM cells.

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 glioblastoma cell invasion in culture and in an organotypic brain slice assay provide strong evidence for a role of Id-1 in controlling glioblastoma dispersal, future studies including imaging of glioblastoma 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 that multiple proteins associated with tumor aggressiveness were downregulated 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 glioblastoma-derived cultures grown in neurosphere conditions (30). We found that Id-1 was preferentially expressed in the $CD133^+$ subpopulation of primary glioblastoma cells. Id-1 knockdown significantly reduced primary

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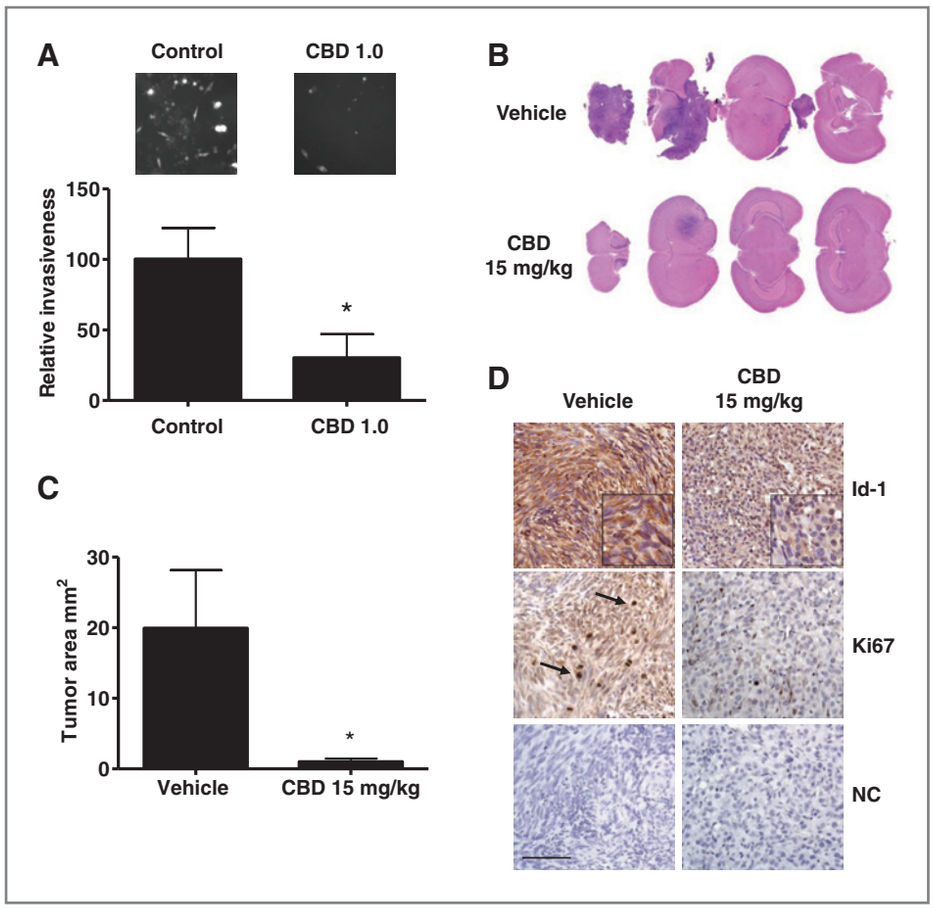

Figure 7. Cannabidiol (CBD) inhibits glioblastoma (GBM) invasion through an organotypic brain slice and reduces Id-1 expression and tumor progression *in vivo*. A, GFP-labeled U251 cells were treated with vehicle (control) or 1 μmol/L cannabidiol for 72 hours. Cells that migrated through the slice were counted using an inverted fluorescence microscope. Data are shown as mean number of cells in triplicate wells. Bars ± SE. *, $P < 0.05$, Student *t* test. The experiment was repeated 3 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 0.3×10^6 U251 cells (5 mice/group). Daily treatments with 15 mg/kg CBD were initiated 7 days after tumor implantation. C, panoramic viewer software (3DHISTECH) was used to measure the area of the tumor in the brain. *, statistically significant differences from control ($P < 0.02$). D, representative sections show reduction in Id-1 (top) and Ki67 (middle) expression in tumors responsive to CBD treatment. Bar, 100 μm. The insets (top) represent a 20-fold magnification. Negative IgG controls (NC) are also shown.

glioblastoma 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 downregulate Sox2 expression and thus reduce tumor recurrence, which is believed to be driven by glioma stem-like cells. The abovementioned 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 nonpsychoactive cannabinoid cannabidiol significantly downregulated Id-1 expression in serum-derived and primary glioblastoma cells. As expected, we observed robust inhibition of glioma cell invasiveness similar to that observed using genetic knockdown of Id-1. In glioblastoma cells, cannabidiol was able to inhibit p-ERK and p-AKT. In primary glioblastoma cultures, cannabidiol inhibited neurosphere growth and the expression levels of Sox2. These effects of cannabidiol were similar to those observed with genetic silencing of Id-1 expression in glioblastoma cells. Cannabidiol 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 cannabidiol was effective at downregulating Id-1 expression *in vivo*, we used an intracranial U251 glioma xenograft model. Treatment of mice with cannabidiol significantly reduced Id-1 expression within the tumor tissue, showing that the drug can effectively modulate this target in an orthotopic mouse model of the disease. Importantly, treatment with cannabidiol also produced a robust inhibition of tumor progression. As the knockdown of Id-1 produced only a modest effect on cell growth in culture, we expect that cannabidiol may have additional antitumor properties not related to the downregulation of Id-1, that is, this additional activity of cannabidiol 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 glioblastoma cells and show that the nontoxic cannabinoid compound cannabidiol downregulates Id-1 expression and tumor aggressiveness in culture

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and *in vivo*. The data also shed light on some of the key pathways that control glioblastoma cell dispersal and progression. A greater understanding of these pathways may lead to more effective therapies for patients with cancer, 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 glioblastoma aggressiveness. With its lack of systemic toxicity and psychoactivity, cannabidiol 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 glioblastoma tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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