

Cannabinoids Induce Glioma Stem-like Cell Differentiation and Inhibit Gliomagenesis^{*S}

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Glioma stem-like cells constitute one of the potential origins of gliomas, and therefore, their elimination is an essential factor for the development of efficient therapeutic strategies. Cannabinoids are known to exert an antitumoral action on gliomas that relies on at least two mechanisms: induction of apoptosis of transformed cells and inhibition of tumor angiogenesis. However, whether cannabinoids target human glioma stem cells and their potential impact in gliomagenesis are unknown. Here, we show that glioma stem-like cells derived from glioblastoma multiforme biopsies and the glioma cell lines U87MG and U373MG express cannabinoid type 1 (CB₁) and type 2 (CB₂) receptors and other elements of the endocannabinoid system. In gene array experiments, CB receptor activation altered the expression of genes involved in the regulation of stem cell proliferation and differentiation. The cannabinoid agonists HU-210 and JWH-133 promoted glial differentiation in a CB receptor-dependent manner as shown by the increased number of S-100 β - and glial fibrillary acidic protein-expressing cells. In parallel, cannabinoids decreased the cell population expressing the neuroepithelial progenitor marker nestin. Moreover, cannabinoid challenge decreased the efficiency of glioma stem-like cells to initiate glioma formation *in vivo*, a finding that correlated with decreased neurosphere formation and cell proliferation in secondary xenografts. Gliomas derived from cannabinoid-treated cancer stem-like cells were characterized with a panel of neural markers and evidenced a more differentiated phenotype and a concomitant decrease in nestin expression. Overall, our results demonstrate that cannabinoids target glioma stem-like cells, promote their differentiation, and inhibit gliomagenesis, thus giving further support to their potential use in the management of malignant gliomas.

Malignant gliomas remain the most deadly human brain tumors, with poor prognosis despite years of research in anti-tumoral therapeutic strategies. A hallmark characteristic of gliomas is their molecular and cellular heterogeneity (1, 2), which is considered one of the reasons for their high malignancy and recurrence. Moreover, even morphologically or histologically related tumors may behave very differently. Neoplastic transformation of differentiated glial cells was for many years the most accepted hypothesis to explain the origin of gliomas (1, 2). However, recent findings support the existence of a stem cell-derived origin for different types of cancers such as gliomas and hematopoietic, breast, and prostate tumors (2, 3). In particular, glioma-derived stem-like cells (GSCs)⁴ have been isolated from both human brain tumors (4–8) and several glioma cell lines (6, 9, 10). GSCs are crucial for the malignancy of gliomas (8–10) and may represent the consequence of transformation of the normal neural stem cell compartment (11). These findings are in line with the observation that gliomagenesis is frequently associated with adult brain germinal zones, in particular the subventricular zone (2, 3). It is therefore imperative that the development of new therapeutic strategies for the management of gliomas takes into account their cellular diversity and origin. Among those strategies, cannabinoid-based drugs may represent an alternative to other established chemotherapeutics (12).

The discovery of an endogenous cannabinoid system (13), together with the great improvement in our understanding of the signaling mechanisms responsible for cannabinoid actions (12, 13), has fostered the interest in the potential therapeutic applications of cannabinoids (14). Several studies have demonstrated a significant antitumoral action of cannabinoid ligands in animal models (12). Thus, cannabinoid administration to nude mice curbs the growth of different tumors, including gliomas, lung adenocarcinomas, thyroid epitheliomas, lymphomas, and skin carcinomas (12). The antitumoral action on gliomas relies on at least two mechanisms: induction of apoptosis of tumor cells (15, 16) and inhibition of tumor angiogenesis (17). Besides their wide distribution in tumor cells, cannabinoid receptors are expressed and functionally active in neural progenitors, in which they regulate cell proliferation and differentiation (18, 19). This background prompted us to investigate the

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⁴ The abbreviations used are: GSCs, glioma-derived stem-like cells; CB₁, cannabinoid receptor type 1; CB₂, cannabinoid receptor type 2; EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; GFAP, glial fibrillary acidic protein; GBM, glioblastoma multiforme; eCB, endocannabinoid.

actions of cannabinoids on human GSCs and their impact in gliomagenesis. Our results show that GSCs express cannabinoid receptors and that cannabinoid stimulation reduces glioma initiation *in vivo*, a finding that correlates with increased cell differentiation. These findings provide further support for cannabinoid-based antitumoral therapies that are able to target the brain tumor stem cell compartment.

EXPERIMENTAL PROCEDURES

Materials—The following materials were kindly donated as indicated: SR141716 and SR144528 (Sanofi-Aventis, Montpellier, France), JWH-133 (J. Huffman), anti-mouse cannabinoid type 1 (CB₁) receptor polyclonal antibody (K. Mackie), and anti-trimethyl-histone H3 (Lys⁹) polyclonal antibody (Upstate; I. Flores). Mouse anti-human nestin monoclonal antibody and anti-Musashi-1 polyclonal antibody were from Chemicon, and rabbit anti-Ki-67 monoclonal antibody (clone SP6) was from Lab Vision Corp. Anti-cannabinoid type 2 (CB₂) receptor polyclonal antibody was from Affinity BioReagents, and anti-S-100 β antibody was from Swant. Recombinant human epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) were from R&D Systems. Mouse anti- β -tubulin III monoclonal antibody was from Covance Inc., and anti-gial fibrillary acidic protein (GFAP), anti-MAP2 (microtubule-associated protein 2), and anti- α -tubulin antibodies were from Sigma. Glioblastoma multiforme (World Health Organization grade IV astrocytoma) samples were provided by the Tissue Bank Network of the Molecular Pathology Program of the Spanish National Cancer Centre.

GSC Culture and Gliomagenesis *in Vivo*—GSCs were obtained from human brain tumor biopsies digested with collagenase (type Ia, Sigma) in Dulbecco's modified Eagle's medium at 37 °C for 90 min (17) and grown under non-adherent conditions (19) in neural stem cell culture medium composed of Dulbecco's modified Eagle's and Ham's F-12 media supplemented with B-27 (Invitrogen), 50 mM Hepes, 2 μ g/ml heparin, 20 ng/ml EGF, 20 ng/ml FGF-2, and 20 ng/ml leukemia inhibitory factor. Of eight biopsies employed, five rendered cells that fulfilled stem-like cell criteria (3). Representative results are shown for one of these GSC lines and were also extended to GSCs derived from classical human glioma U87MG and U373MG cell lines. Cell lines were grown as described (15, 17) and inoculated *in vivo*, and after tumor digestion, GSCs were cultured. Clonal neurospheres were grown at 1000 cells/ml and analyzed by flow cytometry for the expression of different stem cell markers, including CD133, the stem cell factor receptor c-Kit, and their ability to exclude Hoechst 33342 (side population analysis). Differentiation experiments were performed in polyornithine-coated plates, and adherent GSCs were grown in neural stem cell culture medium without growth factors. GSCs were cultured in the presence of the indicated stimuli after overnight growth factor deprivation.

Differentiation experiments with at least three independent cultures were performed by quantification of the percentage of total cells that expressed the indicated neural antigens or that were highly positive for histone H3 trimethylated at Lys⁹. A minimum of 10 fields were scored in a double-blinded manner to minimize subjective interpretations. Quantified fields were

selected randomly by visualizing total cells with a microscope Hoechst filter. Stock solutions of cellular effectors were prepared in Me₂SO, and the concentrations employed were selected based on previous studies on cannabinoid regulation of neural progenitors (19). No significant influence of Me₂SO on any of the parameters determined was observed at the final concentration used (0.1%, v/v). Control incubations included the corresponding vehicle content. Gliomagenesis was induced by subcutaneous flank inoculation into athymic nude mice of U87MG-GSCs or glioblastoma multiforme (GBM) GSCs in 100 μ l of phosphate-buffered saline supplemented with 0.1% glucose (16). In some experiments, 1.5 mg/kg JWH-133 or the corresponding vehicle was administered daily to subcutaneous gliomas. Tumor growth was measured with an external caliper, and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$.

Fluorescence-activated Cell Sorter Analysis—GSCs were dissociated with Accutase (Innovative Cell Technologies Inc., San Diego, CA) following the manufacturer's instructions; cells (0.5×10^6 /condition) were fixed in 2% paraformaldehyde at 4 °C in phosphate-buffered saline; and nonspecific binding was blocked using 2% goat serum in phosphate-buffered saline. Antibodies and their corresponding controls were incubated for 30 min at 4 °C in 2% goat serum/phosphate-buffered saline, and cells (10,000/recording) were analyzed using a FACSCalibur flow cytometer. Phycoerythrin-conjugated anti-CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) and phycoerythrin/Cy5-conjugated anti-CD117 (c-Kit; BD Biosciences) antibodies were employed following the manufacturer's instructions.

mRNA Detection and Quantification—mRNA was obtained with an RNeasy Protect kit (Qiagen Inc., Valencia, CA) using an RNase-free DNase kit. cDNA was subsequently obtained using a SuperScript first-strand cDNA synthesis kit (Roche Applied Science), and amplification of cDNA was performed with the following primers: human CB₁, CGT GGG CAG CCT GTT CCT CA (sense) and CAT GCG GGC TTG GTC TGG (antisense; 403-bp product); human CB₂, CGC CGG AAG CCC TCA TAC C (sense) and CCT CAT TCG GGC CAT TCC TG (antisense; 502-bp product); human fatty acid amide hydrolase, TGG GAA AGG CCT GGG AAG TGA ACA (sense) and GCC GCA GAT GCC GCA GAA GGA G (antisense; 458-bp product); human monoacylglycerol lipase, ACC CTG GGC TTC CTG TCT TCC TTC (sense) and TTC CTG CCG TGG CTG TCC TTT GAG (antisense; 564-bp product); human TRPV1 (transient receptor potential cation channel, subfamily V, member 1), CGC CGC CAG CAC CGA GAA (sense) and ACC GAG TCC CTG GCG CTG ATG TC (antisense; 546-bp product); human glyceraldehyde-3-phosphate dehydrogenase, GGG AAG CTC ACT GGC ATG GCC TTC C (sense) and CAT GTG GGC CAT GAG GTC CAC CAC (antisense; 318-bp product); human Musashi-1, GAT GGT CAC TCG GAC GAA GAA (sense) and CAA ACC CTC TGT GCC TGT TG (antisense; 149-bp product); human nestin, GAG AGG GAG GAC AAA GTC CC (sense) and TCC CTC AGA GAC TAG CGC AT (antisense; 128-bp product); human NOTCH1, GCC GCC TTT GTG CTT CTG TTC (sense) and CCG GTG GTC TGT CTG GTC GTC (antisense; 251-bp product); human OCT4, GAC AAC AAT GAA AAT CTT CAG GAG A (sense) and TTC

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TGG CGC CGG TTA CAG AAC CA (antisense; 217-bp product); and human SOX2, GCA CAT GAA CGG CTG GAG CAA CG (sense) and TGC TGC GAG TAG GAC ATG CTG TAG G (antisense; 206-bp product). CB₁ and CB₂ receptor PCR amplifications were performed under the following conditions: 93 °C for 1 min; two rounds at 59 °C for 30 s, 72 °C for 1 min, and 93 °C for 30 s; two rounds at 57 °C for 30 s, 72 °C for 1 min, and 93 °C for 30 s; and 35 cycles at 55 °C for 30 s, 72 °C for 1 min, and 93 °C for 30 s. Finally, after a final extension step at 72 °C for 5 min, PCR products were separated on 1.5% agarose gels. Real-time quantitative PCR was performed with TaqMan probes (Applied Biosystems, Foster City, CA). Amplifications were run in a 7700 real-time PCR system, and the values obtained were adjusted using 18 S RNA levels as a reference.

cDNA Arrays—Total RNA was extracted from vehicle- or HU-210-treated GBM-GSCs cells, and poly(A)⁺ RNA was isolated with Oligotex resin (Qiagen Inc.) and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of 50 μCi of [α -³³P]dATP for the generation of radiolabeled cDNA probes. Purified radiolabeled probes were hybridized to stem gene array membranes (GEArray Q series, SuperArray Bioscience Corp., Frederick, MD) according to the manufacturer's instructions.⁵ Hybridization signals were detected using a PhosphorImager and analyzed using Phoretix housekeeping genes on the blots as internal controls for normalization. The selection criteria were set conservatively throughout the process, and the genes selected were required to exhibit at least a 50% change in expression.

Western Blotting—Western blot analysis was performed as described previously (19). Cleared cell extracts were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Following incubation with primary antibodies, blots were developed with horseradish peroxidase-coupled secondary antibodies using an enhanced chemiluminescence detection kit. Loading controls were performed with anti- α -tubulin antibody. Densitometric quantification of the lumino-grams was performed using a GS-700 imaging densitometer (Bio-Rad) and MultiAnalyst software.

Identification of Endocannabinoids in GSCs—Samples were dissolved in 1 volume of high pressure liquid chromatography-grade methanol and precipitated with 1 volume of acetone, and non-miscible material was filtered. The supernatant was evaporated, and the residue was partitioned between chloroform and water. A preparative TLC plate (Silica Gel 60 F₂₅₄, 1 mm) was pre-developed with chloroform/methanol (1:1, v/v). Then, the residue of the chloroform layer was redissolved and applied to a Finnigan LCQ MS detector. Detection was performed using the electrospray ionization technique with the full-scan mass spectrometric mode, providing a full spectrum of samples between *m/z* 100 and 550. The rest of the mixture was loaded onto a TLC plate (40:6:1 (v/v) chloroform/petroleum ether/methanol) with synthetic anandamide and 2-arachidonoylglycerol as references. The plate was scrapped, and after methanol extraction, filtrates were analyzed by gas chromatography-

mass spectrometry with an electron impact detector (Hewlett-Packard G1800 GCD HP-5971) after derivatization with the silylating agent *N,O*-bis(trimethylsilyl)trifluoroacetamide to form trimethylsilyl ethers at free hydroxyl groups.

Immunofluorescence and Confocal Microscopy—Immunofluorescence was performed in 10-μm tumor sections or in cultured cells as described previously (17, 18). Samples were incubated with the indicated antibodies and their corresponding secondary antibodies, either anti-rabbit or anti-mouse antibody highly cross-adsorbed with Alexa Fluor 488 (Molecular Probes). CB receptor expression was determined with anti-rabbit secondary antibody highly cross-adsorbed with Alexa Fluor 594. The number of positive cells was normalized to the total cell number identified by counterstaining with TOTO-3 iodide or Hoechst 33342. GSC differentiation was determined in a minimum of five tumor sections. The human origin of the cells immunostained with the different neural markers was confirmed by double labeling with anti-human nucleus antibody (Chemicon). Tumor section CD133 staining was performed with non-conjugated antibody (Miltenyi Biotec) as described (7) and for *in vitro* cells after 50 mM CINH₄ incubation for autofluorescence treatment. Preparations were examined using Leica software and a Leica SP2 acoustical optical beam splitter microscope with two passes with a Kalman filter and a 1024X1024 collection box.

Statistical Analysis—The results shown represent the means ± S.E. of the number of experiments indicated in every case. Statistical analysis was performed by analysis of variance, and post hoc analysis was performing using Student's *t* test.

RESULTS

Glioma-derived Stem Cells Express CB Receptors—To investigate the potential effects of cannabinoids on GSCs, we first analyzed whether these cells express CB receptors. GSCs derived from GBM biopsies and human glioma U87MG and U373MG cell lines were cultured and generated neurosphere structures equivalent to those formed by normal neural stem cells (Fig. 1A). Clonal GSC cultures were subjected to subsequent neurosphere passages and showed unlimited self-renewal ability (supplemental Fig. 1A). Thus, we characterized in detail their stem-like cell characteristics. Immunostaining evidenced a high expression of the neural stem cell markers Musashi-1 and nestin (Fig. 1A), with many cells coexpressing both proteins (supplemental Fig. 1B). In addition, flow cytometry analysis showed a CD133-positive cell population (supplemental Fig. 1C), the size of which depended on the tumor of origin. The CB₁ and CB₂ receptors were shown by double immunofluorescence to colocalize in nestin-positive cells both *in vitro* (Fig. 1A) and in glioma xenografts (Fig. 1B); the CB₁ and CB₂ receptors were present in 49 ± 5 and 31 ± 7% of the nestin-positive cells, respectively. Western blotting of GSC cultures was also used to analyze the presence of CB receptors (Fig. 1C). Reverse transcription PCR analysis confirmed the expression of stemness markers, including CD133, nestin, Musashi-1, SOX2, and NOTCH1, and the pluripotency embryonic stem cell marker OCT4 in GSCs (Fig. 1D). These findings correlated with enhanced CB receptor expression of GBM- and U87MG-GSC

⁵ See www.superarray.com for a detailed list of the genes analyzed.

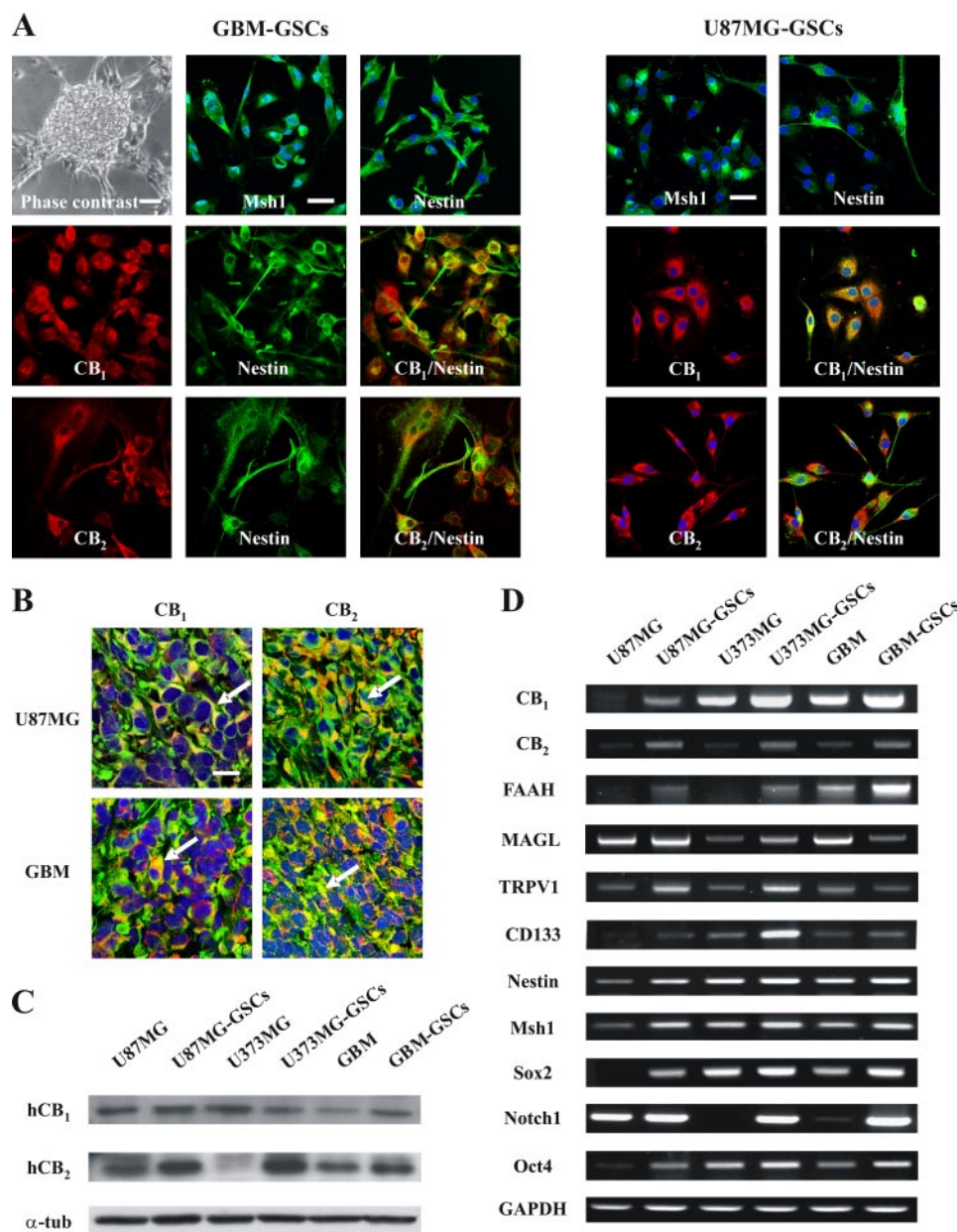


FIGURE 1. Glioma stem-like cells express CB receptors. *A*, immunofluorescence of GBM- and U87MG-GSCs reveals the expression of Musashi-1 (*Msh1*) and nestin (green) together with the presence of CB₁ and CB₂ receptors (red). Scale bars = 40 μ m. *B*, shown is CB receptor and nestin expression in glioma xenografts. Scale bar = 20 μ m. *C*, GBM-, U87MG-, and U373MG-GSCs express human (*h*) CB₁ and CB₂ receptors as shown by Western blotting. α -tub, α -tubulin. *D*, reverse transcription PCR analysis determined the expression of transcripts for the following components of the eCB system: the CB₁ and CB₂ receptors, TRPV1, fatty acid amide hydrolase (*FAAH*), and monoacylglycerol lipase (*MAGL*). The indicated stem cell-related gene transcripts were analyzed in GSCs and their differentiated counterparts. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

populations compared with their respective differentiated counterparts. In particular, GSCs were significantly enriched in CB₂ receptors at both the transcript and protein levels, in line with the correlation between astrocytoma malignancy and CB₂ receptor expression (16). Other elements of the endocannabinoid (eCB) signaling system were also present in GSCs, including TRPV1 and the hydrolases monoacylglycerol lipase and fatty acid amide hydrolase, enzymes responsible for eCB degradation (Fig. 1D). Finally, GSCs were evaluated for their ability to produce endogenous cannabinoid ligands upon incubation

with the calcium ionophore A23187 (5 μ M, 2 min) and subsequent identification by LCQ mass spectrometry (supplemental Table 1). Several major species of 2-monoacylglycerols were detected, specifically palmitoylglycerol and stearoylglycerol. In addition, 2-arachidonoylglycerol and *N*-arachidonoylglycine, a metabolite of anandamide, were present in the samples at trace levels. Gas chromatography-mass spectrometry analysis after derivatization with *N,O*-bis(trimethylsilyl)trifluoroacetamide confirmed the data obtained by LCQ mass spectrometry (supplemental Table 1).

CB Receptor Activation Contributes to the Regulation of Glioma Stem Cell Gene Expression—To identify the potential action of cannabinoids on GSCs, we investigated the changes in gene expression induced by the synthetic cannabinoid agonist HU-210 (30 nM) in GBM-GSCs. HU-210 significantly altered the expression of 11 genes (Fig. 2A) of the 266 genes analyzed. Among them, seven genes involved in regulation of the cell cycle and cell proliferation (*CDK4* (cyclin-dependent kinase 4), *CDKN1B* (cyclin-dependent kinase inhibitor 1B), *FGFR1* (EGF receptor 1), *FGFR3* (EGF receptor-3), EGF receptor, EGF, and integrin α 4) were down-regulated by cannabinoid stimulation. In addition, the transcript levels of neuronal *MAP2* and the tumor suppressor *RBL1* (retinoblastoma-like 1) were increased. These results were confirmed by real-time quantitative PCR analysis of three transcripts (Fig. 2B) and suggest that CB receptor activation regulates essential GSC functions such as cell proliferation and differentiation. However, GSC proliferation

and self-renewal were not affected by cannabinoid stimulation during several neurosphere passages (data not shown). The impact of CB receptor activation on differentiation-related genes was therefore determined by quantitative PCR analysis. HU-210 treatment increased the mRNA levels of the glia-specific markers GFAP and S-100 β in a CB₁ receptor-dependent manner as evidenced by SR141716 antagonism (Fig. 3, A and B, respectively). In addition, CB₁ receptor activation resulted in increased expression of the early neuronal marker β -tubulin III (Fig. 3C).

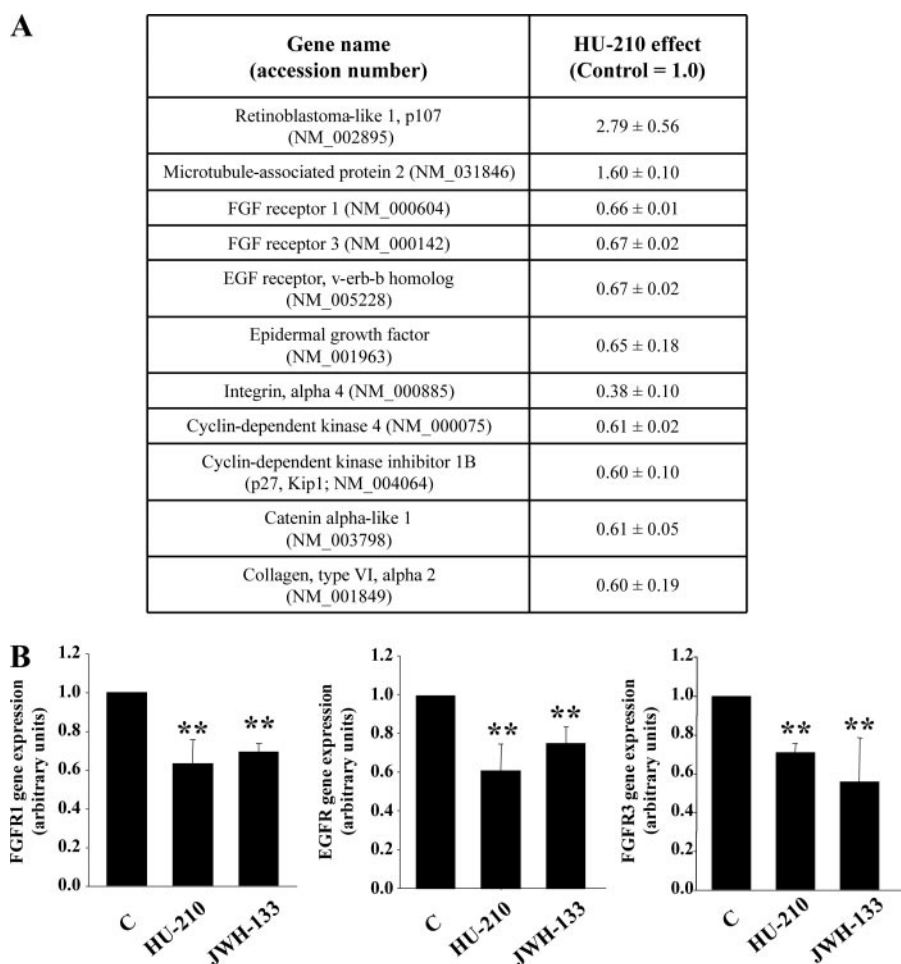


FIGURE 2. Gene expression changes in cannabinoid-treated glioma stem-like cells. A, HU-210-induced changes in GBM-GSCs were analyzed by stem gene array. Selected changes in gene expression were significantly different ($p < 0.01$ versus control cells). B, cannabinoid regulation of the EGF receptor (*EGFR*), *FGFR1*, and *FGFR3* transcripts was confirmed by quantitative PCR. Results correspond to three independent experiments. **, $p < 0.01$. C, control.

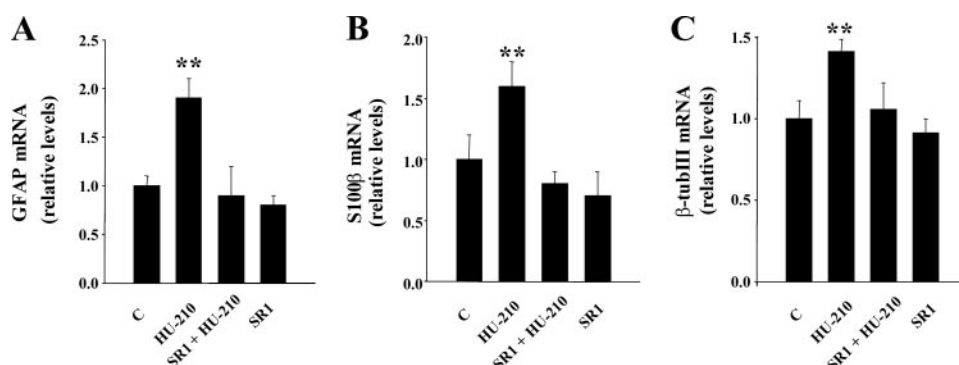


FIGURE 3. Cannabinoid regulation of glioma stem-like cell gene expression *in vitro*. A–C, real-time PCR analysis of GFAP, S-100 β , and β -tubulin III (*β -tubIII*) expression, respectively, in GSCs treated with 30 nM HU-210, 2 μ M SR141716 (SR1), or both and normalized to 18 S rRNA transcript levels. Results correspond to three independent experiments. **, $p < 0.01$ versus the control (C).

Cannabinoids Promote Neural Differentiation of Glioma Stem Cells—On the basis of the results of cannabinoid regulation of differentiation-related genes, we next analyzed the regulation of GBM-GSC differentiation by the CB₁ and CB₂ receptor agonists HU-210 and JWH-133 alone or in combination with the receptor antagonists SR141716, SR144528, and capsaizepine. Activation of the CB₁ and CB₂ receptors, as demon-

strated by the use of their respective antagonists, decreased the nestin-positive cell population (Fig. 4A) and increased the more differentiated cell population expressing the glial markers GFAP and S-100 β (Fig. 4, B and C, respectively) or the neuronal marker β -tubulin III (Fig. 4D). Capsazepine did not modify the cannabinoid-induced decrease in nestin-positive cells and the induction of GFAP- and β -tubulin III-positive cells, although it counteracted the S-100 β -positive cell increase (supplemental Fig. 2). Histone methylation status has been shown to correlate with changes in neural progenitor cell differentiation (20), and thus, methylation of histone H3 at Lys⁹ was monitored. HU-210 and JWH-133 increased the number of trimethyl-histone H3 (Lys⁹)-labeled cells in a CB receptor-dependent manner as evidenced by SR141716 and SR144528 antagonism (Table 1). In summary, these experiments confirmed the neural progenitor ability of GSC cultures that, under differentiation conditions, attach and recapitulate their endogenous differentiation program. Aberrant GSC differentiation was observed, as previously reported (5, 6, 21). Similarly, cannabinoids induce both neuronal (β -tubulin III) and glial (GFAP) gene expression (Fig. 3). Many cells were observed to coexpress both glial and neuronal markers (supplemental Fig. 1D), which, in the case of normal neural stem-derived cells, segregate in different cell compartments.

Cannabinoids Inhibit Gliomagenesis Initiated by Glioma Stem Cells—Stem-like cells are considered to be the initiating cell population of tumorigenesis. Thus, when injected in nude mice, U87MG- and GBM-GSCs induced tumor formation at cell numbers that were 30- and 10-fold lower, respectively, than their differentiated counterparts (0.25×10^6 U87MG-GSCs and 1×10^6 GBM-GSCs were injected to initiate tumor formation). As cannabinoid receptor activation regulates GSCs, we sought to determine their impact on the ability of GSCs to initiate glioma generation *in vivo*. U87MG-GSCs previously cultured in the presence of 30 nM HU-210 or JWH-133 were

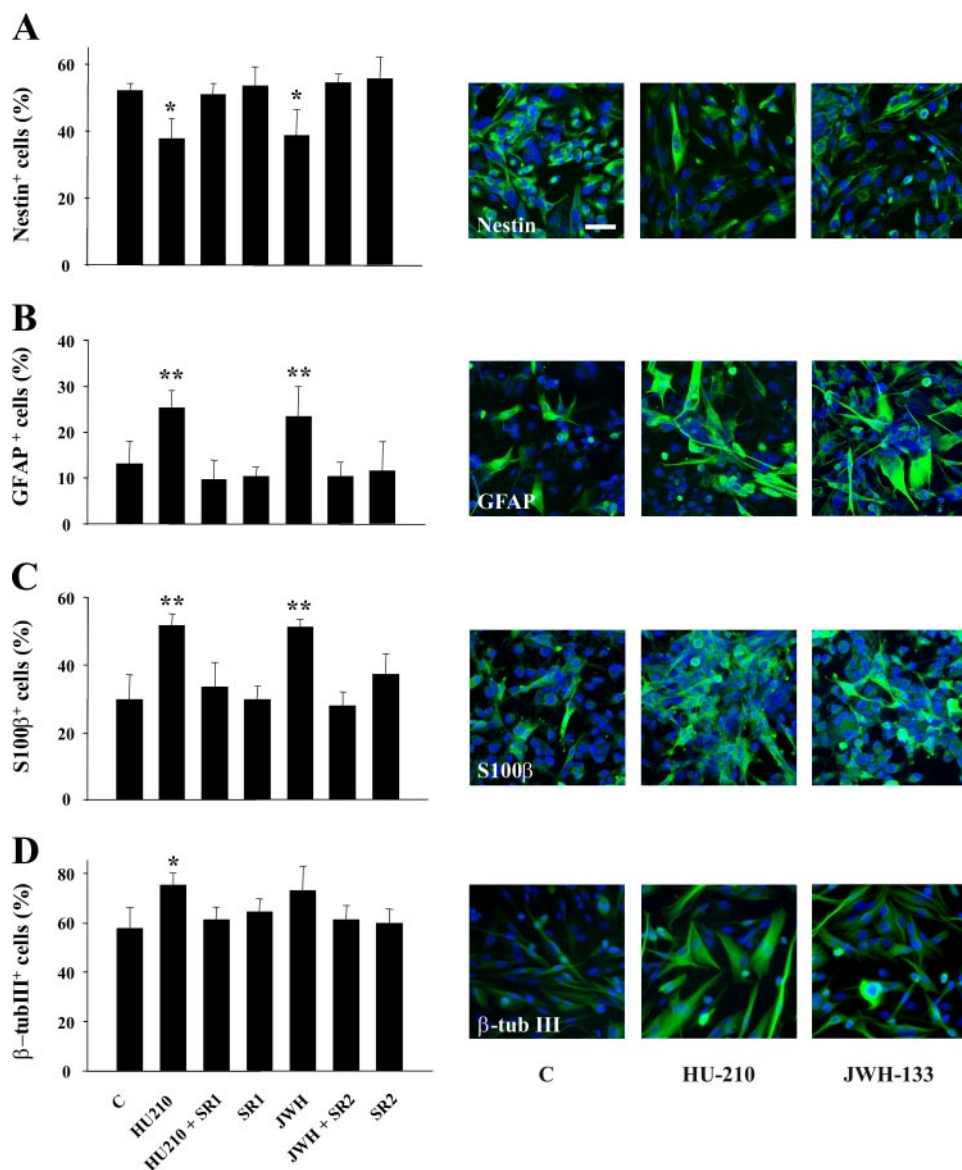


FIGURE 4. Cannabinoid regulation of glioma stem-like cell differentiation *in vitro*. Left panels, quantification of nestin-positive (A), GFAP-positive (B), S-100 β -positive (C), and β -tubulin III (β -tubIII)-positive (D) cells (green) versus total cells. Cannabinoid-induced differentiation was analyzed after a 4-day differentiation period of GBM-GSCs in the presence of vehicle (control (C)), 30 nM HU-210 alone or with 2 μ M SR141716 (SR1), and 30 nM JWH-133 alone or with 2 μ M SR144528 (SR2). Each antagonist was also tested alone. Results correspond to three independent experiments. *, $p < 0.05$ versus the control; **, $p < 0.01$. Right panels, representative immunofluorescence images of vehicle- and cannabinoid-treated cells. Cell nuclei are shown in blue. Scale bar = 55 μ m.

TABLE 1

Cannabinoid regulation of histone H3 methylation

Methylation of histone H3 at Lys⁹ (meK9-H3) was determined in GSCs after differentiation in the presence of vehicle (Control), 30 nM HU-210 alone or with 2 μ M SR141716, 2 μ M SR141716 alone, 30 nM JWH-133 alone or with 2 μ M SR144528, or 2 μ M SR144528 alone.

	meK9-H3 ⁺ cells
	%
Control	100 \pm 11
HU-210	188 \pm 32 ^a
HU-210 + SR141716	96 \pm 15
SR141716	98 \pm 12
JWH-133	209 \pm 35 ^a
JWH-133 + SR144528	95 \pm 16
SR144528	92 \pm 15

^a $p < 0.01$ versus the control.

less efficient as tumor-initiating cells (Table 2). Moreover, cannabinoid-treated GSCs generated tumors with a lower growth rate, resulting in smaller tumor size compared with vehicle-treated cells (Fig. 5A and Table 2). Similarly, HU-210- and JWH-133-treated GBM-GSCs were less efficient in initiating gliomagenesis (Fig. 5B and Table 2). In particular, HU-210 notably reduced tumor growth, whereas in the case of JWH-133, tumors were visible only 2 months after the rest of the animals had been killed (Fig. 5B and data not shown). Samples of GSC-derived gliomas were obtained, and their ability to form primary spheres was determined. Tumors generated by cannabinoid-treated GSCs showed decreased neurosphere-forming activity (Fig. 6A) and reduced cell proliferation (Ki-67-positive cells) (Fig. 6, B and C). These observations confirm that cannabinoids inhibit stem-like cell-initiated gliomagenesis.

To analyze cannabinoid regulation of the differentiation status of GSC-derived tumors, progenitor markers were analyzed by immunofluorescence. Tumors derived from cannabinoid-treated cells showed decreased nestin immunoreactivity (Fig. 7A), a finding that was also observed in gliomas treated *in vivo* with JWH-133 (54 \pm 7% relative immunoreactivity versus 100 \pm 8% in vehicle-treated tumors). In addition, the expression of vimentin, a progenitor marker that has been correlated with glioma malignancy (22, 23), was also decreased (48 \pm 5% relative immunoreactivity in JWH-133-treated tumors versus 100 \pm 9% in vehicle-treated tumors). Next, we analyzed the expression of differentiated markers in cannabinoid-treated derived gliomas. CB receptor activation increased the expression of the neuronal markers MAP2 and β -tubulin III (Fig. 7, B and C) and increased S-100 β glial immunoreactivity (Fig. 7D). In agreement with a previous report (6), GFAP immunoreactivity could not be detected, but an increase in its transcript levels was observed (250 \pm 55% upon HU-210 treatment and 160 \pm 20% upon JWH-133 treatment versus 100 \pm 10% upon vehicle treatment).

DISCUSSION

The recent discovery of brain cancer stem cells has important implications both for the development of new therapeutic

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TABLE 2

Cannabinoid regulation of glioma stem-like cell-initiated gliomagenesis

HU-210- and JWH-133-treated cells were injected subcutaneously into mice, and the efficiency of tumorigenesis (number of mice that generated tumors relative to total injected mice and the corresponding percentages), relative tumor growth, and tumor weight at the end of the experiment were evaluated.

	U87MG-GSCs			GBM-GSCs		
	Gliomagenesis efficiency	Tumor cell growth	Tumor weight	Gliomagenesis efficiency	Tumor cell growth	Tumor weight
Vehicle	6/6(100)	100 ± 14	1.13 ± 0.16	5/5(100)	100 ± 17	0.43 ± 0.08
HU-210	4/6(67)	53 ± 4 ^a	0.60 ± 0.07 ^a	3/5(60)	12 ± 3 ^a	0.05 ± 0.02 ^a
JWH-133	4/6(67)	39 ± 6 ^a	0.44 ± 0.10 ^a	3/5(60)	0 ^a	0 ^a

^a *p* < 0.01 versus the control.

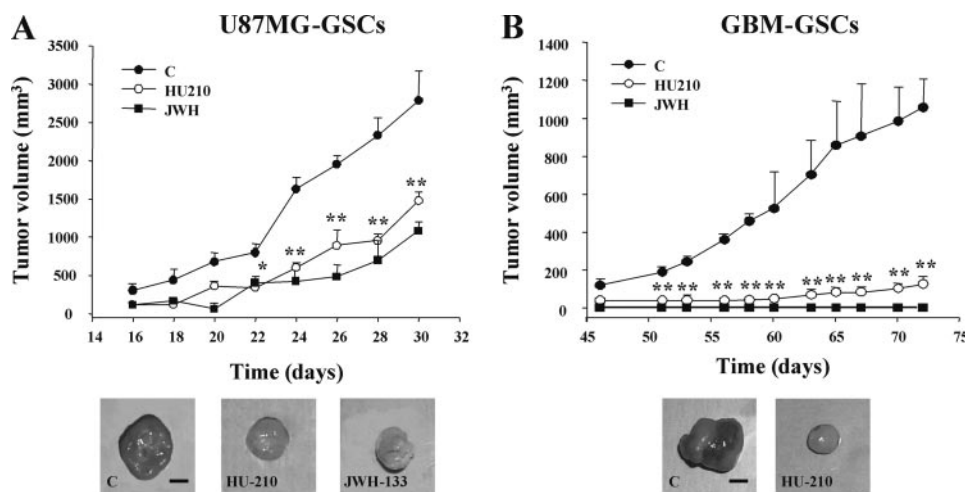


FIGURE 5. Cannabinoids inhibit glioma stem cell-initiated gliomagenesis. A and B, tumor growth of U87MG- and GBM-GSCs, respectively, treated with vehicle (control (C); ●), 30 nM HU-210 (○), or JWH-133 (■) and injected subcutaneously into immunodeficient mice. Representative images of dissected tumors are shown below. Scale bars = 0.5 (A) and 0.3 (B) cm. *, *p* < 0.05 versus the control; **, *p* < 0.01.

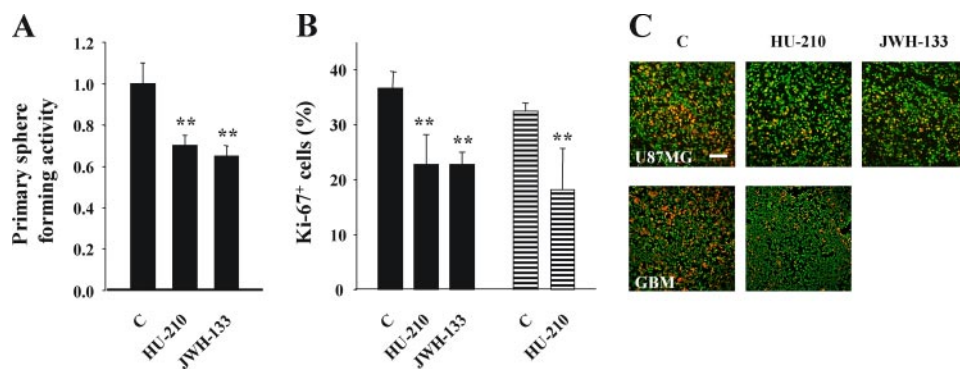


FIGURE 6. Cannabinoid inhibition of tumor-derived neurosphere formation and *in vivo* cell proliferation. A, shown is the primary sphere formation from the indicated tumor biopsies. B, cell proliferation was quantified by Ki-67 immunoreactivity in U87MG-derived (black bars) and GBM-derived (striped bars) tumors obtained from cannabinoid- and vehicle (control (C)) treated GSCs. **, *p* < 0.01 versus the control. C, representative pictures are shown for each condition. Ki-67 is shown in red, and cell nuclei are shown in green. Scale bar = 90 μm. Results correspond to three independent experiments.

strategies for glioma management and for the evaluation of potential pitfalls and benefits of currently available treatments (1–3). Here, we show that GSCs express different elements of the eCB system, including G protein-coupled receptors (CB₁ and CB₂), the ionotropic receptor TRPV1, and eCB-degrading enzymes (fatty acid amide hydrolase and monoacylglycerol lipase), and that cannabinoid agonists target the stem-like cell compartment of brain tumors, promote GSC differentiation in a receptor-dependent manner, and reduce gliomagenesis *in vivo*. Although no overt differences between CB₁ and CB₂

receptor-mediated actions in GSCs were evident in this study, potential variations in their molecular mechanisms of action may occur. For instance, it is known that the CB₁ receptor (but not the CB₂ receptor) is coupled to the modulation of various Ca²⁺ and K⁺ channels (13) and that CB₁ receptor activation is selectively regulated by cholesterol-enriched membrane microdomains (24). In addition, the different molecular species of eCBs differ in their affinity for the two CB receptor types. Thus, 2-arachidonoylglycerol rather than anandamide has been proposed as the preferential ligand for CB₂ receptors (25). Furthermore, CB₂ receptor-selective enrichment in GSCs might help to explain the observed correlation between CB₂ receptor expression and glioma cell malignancy (16). On the other hand, capsaizepine was able to prevent some of the cannabinoid actions on GSC differentiation, suggesting that these ligands may also affect TRPV1 function by as yet unknown mechanisms. Altogether, these observations support that the coexpression of different CB receptors in GSCs would allow these cells not only to be pharmacologically targeted by CB₂ receptor-selective non-psychotropic ligands (14), but also to respond differentially depending on the molecular com-

position of the eCB tone present in the tumor niche. Those eCB molecules may be produced by GSCs and surrounding cells of neuronal and glial origin (13). In this respect, altered levels of eCBs have been reported in human GBM biopsies compared with normal brain tissue, suggesting that this family of extracellular lipid cues may be involved in the endogenous antitumoral response (26, 27).

The malignancy of human brain tumors inversely correlates with their degree of differentiation as shown by increased nestin, Musashi-1, and doublecortin expression (28–30), whereas

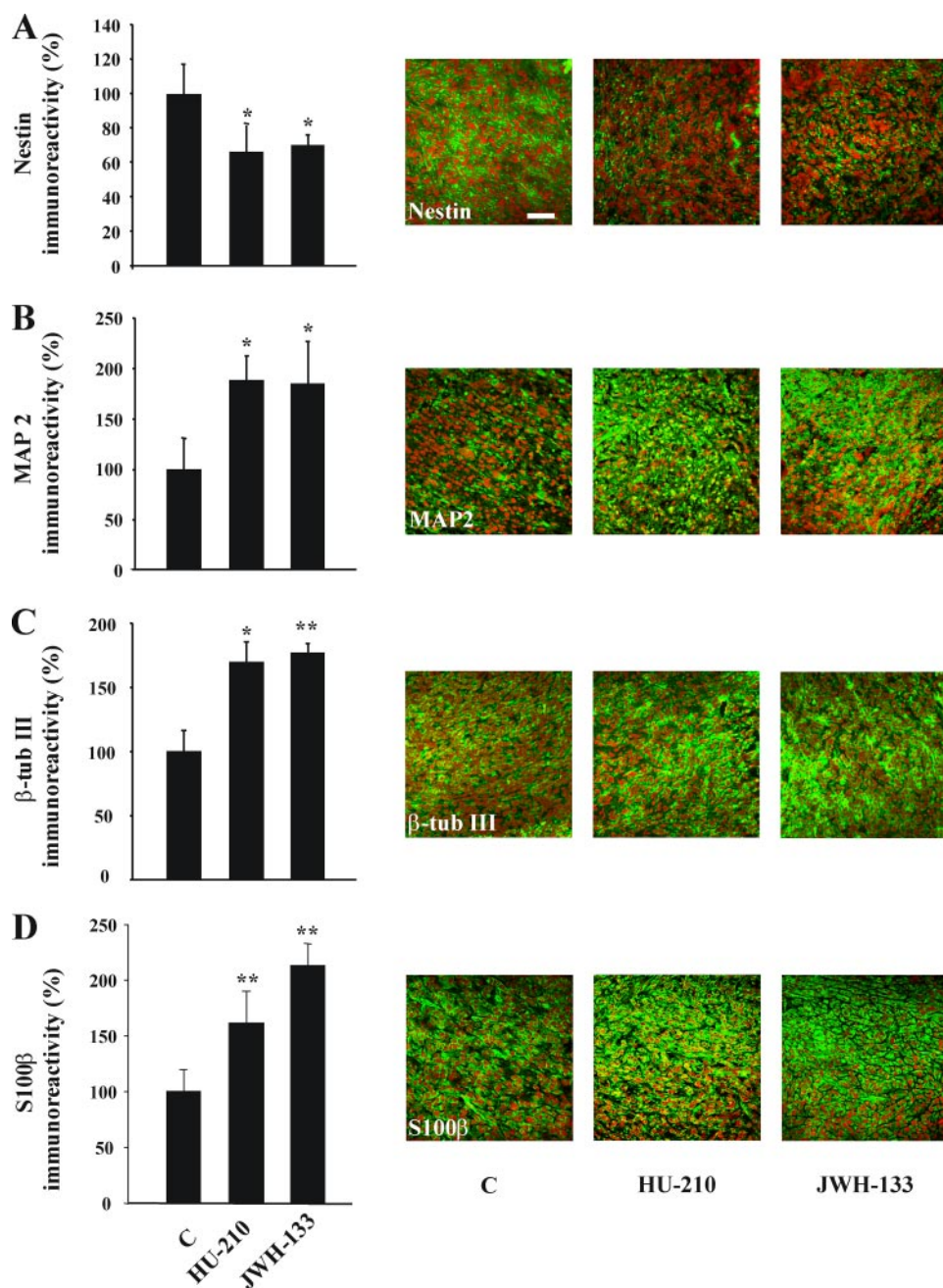


FIGURE 7. **Cannabinoid regulation of glioma stem-like cell differentiation *in vivo*.** Left panels, quantification of nestin (A), MAP2 (B), β -tubulin III (β -tub III; C), and S-100 β immunoreactivity (D) in HU-210- and JWH-133-treated U87MG-GSC-derived tumors. *, $p < 0.05$ versus the control (C); **, $p < 0.01$. Right panels, neural markers are shown in green, and cell nuclei are shown in red. Representative immunofluorescence images of cannabinoid-treated tumors are shown. Results correspond to three independent experiments. Scale bar = 80 μ m.

their mitotic activity is inversely correlated with their increased expression of mature glial and neuronal markers (22, 23, 31). Thus, compared with GBM, low-grade astrocytomas, oligodendrogliomas, and neuroblastomas have a better prognosis and much more efficient therapeutic management (1, 2). Genetic modeling of glioma origin has shown that, in addition to differentiated astrocytes (32), neural stem cells constitute a potential niche for malignant transformation that may be more permissive for malignization (11, 33). These findings were followed by the identification of brain tumor-initiating cells through their

selective expression of CD133 (7, 8) or their ability to exclude Hoechst 33342 (9, 10). The existence of a brain tumor-initiating cell phenotype with stem cell features may lead in the future to potential therapeutic strategies based on enforced stem cell differentiation aimed at decreasing brain tumor-initiating ability (8, 34). In this context, a strong correlation between poor glioma prognosis and the expression of a signature of neurogenesis-related genes has been reported recently (23). Similarly, radial glial progenitor cells constitute the putative origin for ependymoma (35), and neural stem cells may cause cerebellar tumors (34). Finally, brain stem-like cells have been shown to reproduce a brain tumor phenotype in a more reliable manner compared with differentiated transformed cells (36). However, it should be kept in mind that, in addition to much evidence supporting a role for stem-like cells in gliomagenesis and tumor biology (3), new studies are required to provide definitive proof of the concept (37). In particular, a better understanding of the molecular mechanisms responsible for the transformation of normal neural stem cells (38, 39) and dedifferentiation of neural cells (32, 33) and of the alternative origins of GSCs (40) is required.

Cannabinoids are known to exert an antitumoral action against gliomas (15–17), an effect that has been extended to a variety of tumors of different origins (12). Our new data support that, in addition to inducing apoptosis of differentiated transformed cells (12), cannabinoids promote differentiation of GSCs and inhibit tumor initiation. These observations are in line with recent

findings demonstrating that cannabinoid stimulation promotes differentiation of non-transformed adult neural progenitors (18, 41). Moreover, the expression of CB receptors by brain tumor-initiating cells may reflect their normal developmentally regulated expression pattern by non-transformed neural stem cells such as subventricular zone progenitors (42) and cortical radial progenitors and hippocampal nestin type I cells (19, 41). These findings are thus related to the link between the normal neural stem cell compartment and tumor development (2, 3). In conclusion, our results demonstrate the action of cannabinoids

on glioma stem-like cells and thus may open new avenues for cannabinoid-based antitumoral strategies.

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