

# Temozolomide Preferentially Depletes Cancer Stem Cells in Glioblastoma

Dagmar Beier,<sup>1</sup> Stefanie Röhl,<sup>1</sup> Deepu R. Pillai,<sup>1</sup> Stefanie Schwarz,<sup>1</sup> Leoni A. Kunz-Schughart,<sup>3</sup> Petra Leukel,<sup>1</sup> Martin Proescholdt,<sup>2</sup> Alexander Brawanski,<sup>2</sup> Ulrich Bogdahn,<sup>1</sup> Ariane Trampe-Kieslich,<sup>4</sup> Bernd Giebel,<sup>5</sup> Jörg Wischhusen,<sup>6</sup> Guido Reifenberger,<sup>4</sup> Peter Hau,<sup>1</sup> and Christoph P. Beier<sup>1</sup>

Departments of <sup>1</sup>Neurology and <sup>2</sup>Neurosurgery, University of Regensburg, Medical School, Regensburg, Germany; <sup>3</sup>ZIK OncoRay, Center for Radiation Research in Oncology, Dresden, Germany; <sup>4</sup>Department of Neuropathology, Heinrich-Heine-University, Medical School and <sup>5</sup>Institute of Transplantation Diagnostics and Cell Therapeutics, Heinrich-Heine-University, Düsseldorf, Germany; and <sup>6</sup>Interdisciplinary Center for Clinical Research, Junior Research Group "Tumor Progression and Immune Escape," University of Würzburg, Medical School, Clinics for Gynecology and Obstetrics, Würzburg, Germany

## Abstract

**The prognosis of patients suffering from glioblastoma (GBM) is dismal despite multimodal therapy. Although chemotherapy with temozolomide may contain tumor growth for some months, invariable tumor recurrence suggests that cancer stem cells (CSC) maintaining these tumors persist. We have therefore investigated the effect of temozolomide on CD133<sup>+</sup> and CD133<sup>-</sup> GBM CSC lines. Although differentiated tumor cells constituting the bulk of all tumor cells were resistant to the cytotoxic effects of the substance, temozolomide induced a dose- and time-dependent decline of the stem cell subpopulation. Incubation with sublethal concentrations of temozolomide for 2 days completely depleted clonogenic tumor cells *in vitro* and substantially reduced tumorigenicity *in vivo*. In *O*<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT)-expressing CSC lines, this effect occurred at 10-fold higher doses compared with MGMT-negative CSC lines. Thus, temozolomide concentrations that are reached in patients were only sufficient to completely eliminate CSC *in vitro* from MGMT-negative but not from MGMT-positive tumors. Accordingly, our data strongly suggest that optimized temozolomide-based chemotherapeutic protocols might substantially improve the elimination of GBM stem cells and consequently prolong the survival of patients.** [Cancer Res 2008;68(14):5706–15]

## Introduction

Human glioblastoma (GBM) is a highly aggressive brain tumor with a median survival of 14.6 months despite aggressive multimodal therapy (1). GBM are among the first solid cancers in which tumor cells with stem cell-like features, i.e., so-called cancer stem cells (CSC), were identified. They constitute a rare subpopulation of tumor cells that later differentiate into progenitor-like tumor cells or differentiated tumor cells (2). For several different brain tumors, including a subgroup of primary GBM, CD133<sup>+</sup> but not CD133<sup>-</sup> cells were found to be able to reconstitute the initial tumor *in vivo* when as little as 100 cells were injected

into nude mice (3–5). In a therapeutic setting, the proportion of CD133<sup>+</sup> cells (normally 5–30%) was enriched after irradiation *in vivo* and *in vitro*, resulting in an increased tumorigenicity of the remaining cells (4). However, CD133 does not seem to be essential for the stem cell-like properties of CSC (3, 6, 7): We have recently described a novel subgroup of primary GBM that contain no CD133<sup>+</sup> tumor cells. Instead, we have characterized CD133<sup>-</sup> CSC, which resemble CD133<sup>+</sup> CSC in terms of differentiation profile, tumorigenicity, and clonogenicity but show distinct molecular and cellular properties (3).

Temozolomide is the most commonly used chemotherapeutic agent in the therapy of GBM and is usually well-tolerated (1, 8). It achieves its cytotoxic effect mainly by methylating the *O*<sup>6</sup> position of guanine. This adduct can be removed by the DNA repair protein *O*<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT), which is expressed in a subgroup of GBM. Consequently, temozolomide displays its highest efficacy against tumors lacking MGMT expression due to a methylated MGMT promoter (9, 10). However, although temozolomide significantly increases the proportion of patients surviving for >2 years, long-term survivors are still rare (1). The biological properties of CSC provide a possible explanation for the failure of chemotherapy in the long term: CSC from several tumor entities overexpress multidrug resistance proteins (11, 12), which may protect them against cytotoxic drugs that kill progenitor and differentiated cells (2). CSC would then give rise to recurrent tumors. However, this model has not yet been confirmed experimentally for GBM.

We have now investigated the effect of temozolomide on CD133<sup>+</sup> and CD133<sup>-</sup> CSC lines using an established *in vitro* model of GBM stem cell lines (5, 13, 14). Surprisingly, we found that temozolomide preferentially eliminates CSC but spares more differentiated tumor cells.

## Materials and Methods

**GBM samples.** Native tumor tissue samples from GBM resections were provided by the local department of Neurosurgery. All tumors were histologically classified according to the WHO classification of central nervous system tumors (15). Experiments were approved by the local ethics committee (University of Regensburg, No. 05/105, June 14, 2005).

**Culture of primary GBM cells and spheres.** Samples were stored in sterile saline at 4°C and processed within 24 h after resection. They were washed and dissociated by mechanical and enzymatic means (1% trypsin, which does not alter CD133 cell surface expression). Erythrocytes were lysed using NH<sub>4</sub>Cl. Trypan blue staining confirmed >80% viability after the procedure. Tumor cells were then grown in stem cell-permissive DMEM-F12 medium supplemented with 20 ng/mL of each human recombinant

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

D. Beier and S. Röhl contribution equally to this work.

**Requests for reprints:** Christoph P. Beier, Department of Neurology, University of Regensburg, Universitätsstrasse 84, 93053 Regensburg, Germany. Phone: 49-941-941-3258; Fax: 49-941-941-3292; E-mail: Christoph.Beier@gmx.de.

©2008 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-07-6878

epidermal growth factor, human recombinant basic fibroblast growth factor (both from R and D Systems), and human leukemia inhibitory factor (Chemicon), and 2% B27 (Life Technologies). These culture conditions enable tumor cells to retain the molecular characteristics of the primary tumor with only minor changes in differentiation, expression pattern, and genetic mutation profile (3, 14). If not otherwise stated, 50% of the medium was substituted twice weekly. Temozolomide (Schering Plough; stock solution 150 mmol/L in DMSO) and 6-benzylguanine (6-BG; Sigma-Aldrich; stock solution 0.1 mmol/L in methanol) were diluted with stem cell-permissive medium. Thus, cells were treated with stem cell-permissive medium containing either temozolomide (5, 50, and 500  $\mu$ mol/L) or the DMSO control (0.3%) over the periods indicated. Metabolic activity was measured using 10% AlamarBlue (Biosource) that was added to the cell culture medium. The absorbance was determined after 16 h at 590 and 540 nm. The lactate dehydrogenase (LDH) assay (Promega; Cytotox96R) was performed according to the instruction manual.

**Flow cytometry.** For Carboxy-Fluorescein diacetate Succinimidyl Ester (CFSE) staining, cells were labeled for 10 min with 20  $\mu$ mol/L CFSE (Sigma Aldrich) and counterstained with CD133/2-PE (1:100; Miltenyi Biotec) or isotype control antibody. To label dead cells, 0.1  $\mu$ g/mL 7-Amino-Actinomycin D (7-AAD; Sigma Aldrich) was applied for 10 min. To analyze cell cycle and BrdUrd incorporation, cells were incubated with 10  $\mu$ mol/L BrdUrd for 12 h. Cells were fixed with 0.5% p-formaldehyde, incubated with 3 N HCl supplemented with 0.5% Tween, and double stained with anti-BrdUrd (1:25; Roche; secondary antibody: anti-mouse Cy5, 1:50) and 75  $\mu$ mol/L propidium iodide (Sigma Aldrich). Flow cytometric data were analyzed using WinMDI 2.8<sup>7</sup> and results were confirmed using ModFit LT 3.1 (Verity).

**Magnetic and fluorescence activated cell separation.** Cells were dissociated and resuspended in PBS containing 0.5% bovine serum albumin and 2 mmol/L EDTA. For magnetic labeling, CD133/1 Micro Beads were used (Miltenyi Biotec). Positive magnetic cell separation (MACS) was performed using several MACS columns in series.

For fluorescence-activated cell separation (FACS), cells were stained with CD133/2-PE (1:10; Miltenyi Biotec) or isotype control antibody (1:10, mIgG1 $\kappa$ -PE; Caltag Laboratories) and sorted on a BD FACSria (Becton Dickinson).

**Immunocytochemistry.** A sphere derived from one tumor cell was dissociated, treated with either temozolomide or DMSO (control) over the indicated period, and stained as previously described (3). Primary antibodies used were anti- $\beta$ -tubulin III (1:2,000; Promega), anti-glial fibrillary acidic protein (1:1,000; anti-GFAP; DAKO), anti-galactocerebroside C (1:500), and anti-nestin (1:500; both from Chemicon). Stainings were visualized using anti-mouse Alexa Fluor 488 (1:100; Molecular Probes) or anti-rabbit rhodamine antibody conjugates (1:100; Dianova). Nuclei were counterstained using 4',6-diamidino-2-phenylindole (1:10,000; Sigma-Aldrich).

**In vivo tumor model.** Tumor cells were suspended in 2  $\mu$ L PBS and intracranially injected into T-lymphocyte-deficient NMR1<sup>(nu/nu)</sup> mice as described previously (3, 16). All procedures were conducted in accordance with German laws governing animal care (Az. 621-2531.1-04/03). Tumor samples were snap frozen. Ten-micrometer sections were stained with H&E using standard protocols.

**Immunohistochemistry.** Fresh frozen sections (10  $\mu$ m) of mouse brain were fixed with acetone. Endogenous peroxidase activity was blocked by immersing the slide in 3% H<sub>2</sub>O<sub>2</sub> solution for 10 min at room temperature. Nonspecific binding of the primary antibody was blocked by incubating the slide with 0.2% fish skin buffer for 30 min at room temperature. Sections were stained using anti-ki67 antibody (1:50; Novocastra) or isotype control antibody for 1 h at room temperature. Staining was visualized using biotinylated secondary antibody and diaminobenzidine (ABC-kit Vectastain Standard and DAB kit, both from Vector Laboratories).

**Magnetic resonance imaging.** Three months after injection of tumor cells, mice were anesthetized as previously described (17) and tumor-like lesions were detected using a clinical magnetic resonance scanner at a field strength of 3.0 Tesla (Siemens Magnetom Allegra; Siemens Medical Solutions). The magnetic resonance imaging (MRI) of the mouse brain was carried out using a dedicated four-channel rat head phased-array coil assembly (Rapid Biomed). Initial scout images of the brain in three planes were acquired in 14 s using the localizer sequence with TR/TE values of 50/12.4 ms, respectively. The images were acquired using a TR/TE value of 4,000/73 ms using 4 averages and 1 concatenation with an acquisition time of 5 min and 8 s. The acquired images had an in-plane resolution of 200  $\mu$ m with a slice thickness of 0.7 mm. Tumor volume was determined using DatInf Measure 2.0.08.<sup>8</sup>

**MGMT promoter methylation-specific PCR.** The MGMT promoter methylation status was determined in CD133<sup>+</sup> and CD133<sup>-</sup> glioma CSC lines by using methylation-specific PCR as described elsewhere (18). A total of 200 ng of genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold kit (HIS Diagnostics). The primer sequences used to detect methylated MGMT promoter sequences were 5'-GTTTTAG-AACGTTTTGCGTTTCGAC-3' and 5'-CACCGTCCGAAAAAACTCCG-3'. This primer combination allows for the amplification of a 122-bp fragment from methylated DNA. The primer sequences used to detect unmethylated MGMT promoter sequences were 5'-TGTGTTTTAGATGTTTGTGTT-TTGAT-3' and 5'-CTACCACCATCCAAAAAACTCCA-3'. This primer combination allows for the amplification of a 129-bp fragment from unmethylated DNA. Each PCR product was separated on 2% agarose gels. As positive control sample, we used genomic DNA from the A172 glioma cell line (obtained from American Type Culture Collection), which carries a completely methylated MGMT promoter. Genomic DNA extracted from peripheral blood leukocytes served as unmethylated control sample. In addition, a control reaction without any template DNA was performed together with each PCR experiment.

**Quantitative real-time PCR.** RNA was extracted with the RNeasy kit (QIAGEN GmbH) and cDNA was synthesized using the Reverse Transcription System (Promega GmbH). Real-time PCR was performed with the QuantiTect SYBR Green PCR kit (QIAGEN) using the Stratagene Mx3000P Real-Time PCR system (Stratagene Europe). The following primer pairs used were as follows: MGMT, 5'-GCCGGCTTTCACCATCCCG-3' (forward) and 5'-GCTGCAGACCACTCTGTG GCACG-3' (reverse); 18s-RNA, 5'-CGGCTAC-CACATCCAAGGAA-3' (forward) and 5'-GCTGGAATTACCGCGCT-3' (reverse). PCR reactions were run as triplicates on 96-well plates. Temperature profile is as follows: 95°C (15 min) followed by 45 cycles with 95°C (20 s) to 58°C (30 s). The quality of the products was controlled by a melting curve. For quantification, standard curves were established by amplification of serial dilutions of cDNA for both, the target gene and the endogenous reference (18s-RNA).

**Western blot analysis.** For Western blot analysis of MGMT protein expression, 40  $\mu$ g of total cell lysate were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes by electroblotting. The membranes were blocked in 5% milk powder in 0.02% Tween PBS (TPBS) for 30 min and probed with antibodies against MGMT (1:1,000; a kind gift from Prof. B. Kaina, University of Mainz, Mainz, Germany) overnight at 4°C or  $\beta$ -actin (1:4,000; Abcam) for 1 h at room temperature. After 1 h of incubation with horseradish peroxidase-coupled secondary goat anti-rabbit or rabbit anti-mouse antibodies (Pierce) diluted 1:25,000 in TPBS, specific antibody binding was detected by using the enhanced chemiluminescence technique and the Fuji LAS 3000 imaging system. Recombinant human MGMT (Biomol) and total cell lysate from Jurkat cells were used as positive controls for the Western blot experiments.

**Statistics.** Data analysis was performed using JMP 5.1. The statistical tests used are indicated in the Figure Legends. All data are given as mean and SE. All experiments were performed with the CD133<sup>+</sup> cell lines R11, R18, R28, R44, and the CD133<sup>-</sup> cell line R8.

<sup>7</sup> <http://facs.scripps.edu/software.html>

<sup>8</sup> <http://www.datinf.de>

## Results

We investigated four CD133<sup>+</sup> (R11, R18, R28, and R44) and one CD133<sup>-</sup> (R8) CSC lines derived from primary astrocytic GBMs (3). In these cell lines, only a small subgroup of tumor cells (2–4%) fulfilled all stem cell criteria (3), whereas the proportion of CD133<sup>+</sup> cells ranged from 0% to 40%. The cells were cultured under medium conditions that recently proved to be effective to preserve molecular tumor characteristics compared with the primary tumor with only minor changes in differentiation, expression pattern, and genetic mutation profile even after long term culture (3, 14).

**Temozolomide does not induce cell death but efficiently inhibits proliferation in CD133<sup>+</sup> CSC lines.** Similar to “classic” FCS-cultured glioma cell lines (12, 19–21), all CD133<sup>+</sup> CSC lines tested were resistant to temozolomide-induced cell death at maximum concentrations reached in the spinal fluid (5 μmol/L temozolomide; ref. 22) and in the plasma (50 μmol/L temozolomide; refs. 22, 23). At higher concentrations, treatment with temozolomide for 7 days led to a reduction in the number of metabolically active, i.e., viable cells, compared with solvent-treated cells (Fig. 1A, left). To differentiate between an actual decline in cell numbers, i.e., cell death, and impaired proliferation, we compared the metabolic activity of control-treated cells after 2 days with temozolomide-treated cells after 7 days. This showed that 5 days of additional culture in the presence of 500 μmol/L temozolomide induced neither an increase nor a reduction in metabolic activity, which strongly suggests that the tumor cells had stopped proliferating (Fig. 1A, right).

To analyze cell death after temozolomide treatment, we used the LDH assay and CFSE. LDH is a cytoplasmatic enzyme that is released if a cell is dying. The LDH activity in the supernatant was only moderately increased after incubation with temozolomide in higher concentrations for 7 days (Fig. 1B, left). In addition, we labeled CD133<sup>+</sup> CSC lines with CFSE, a cytoplasmatic dye that is evenly distributed between daughter cells upon cell division but leaks if the cell is dying (24). The proportion of cells that had completely lost CFSE increased from 29% to 35% after 7 days incubation with maximal concentrations of temozolomide, suggesting that the substance only marginally induced cell death (Fig. 1B, right). Conversely, temozolomide significantly reduced the average calculated number of cell divisions per day from 0.65 to 0.16 (Fig. 1C, left) and the BrdUrd incorporation (Fig. 1C and D, right) indicating impaired proliferation of the cell lines. Depending on the cell line, temozolomide induced either G<sub>2</sub>-M arrest (Fig. 1D) or G<sub>2</sub> peak loss (data not shown). In all cell lines and in line with previous publications (25), the proportion of cells in the sub-G<sub>0</sub> peak indicating apoptotic cell death did not exceed 8% (Fig. 1D).

**Temozolomide depletes CD133<sup>+</sup> tumor cells in CD133<sup>+</sup> CSC lines.** In CD133<sup>+</sup> CSC lines, only a small subpopulation of CD133<sup>+</sup> CSC but not CD133<sup>-</sup> tumor cells maintains the growth of the cell lines (4, 5, 26, 27). Thus, we analyzed the proportion of CD133<sup>+</sup> tumor cells in temozolomide-treated CD133<sup>+</sup> CSC lines. Whereas the proportion of viable cells declined by ~8% after incubation with temozolomide, the proportion of CD133-expressing cells decreased in a time- and dose-dependent manner by up to 80% (Fig. 2A; data not shown). To monitor cellular proliferation and cell death of CD133<sup>+</sup> and CD133<sup>-</sup> cells, we labeled CD133<sup>+</sup> CSC lines with CFSE and costained against CD133. When treated with temozolomide, CD133<sup>-</sup> cells remained, whereas CD133<sup>+</sup> cells were largely depleted (Fig. 2B). Surprisingly, these data show that CD133<sup>+</sup> cells proliferate more

rapidly than CD133<sup>-</sup> tumor cells (on average 0.9 versus 0.36 cell divisions per day; Fig. 2B, inset).

**Temozolomide depletes CSC in CD133<sup>+</sup> CSC lines.** We have previously found that only 2% to 10% of CD133<sup>+</sup> GBM cells possess the potential for sphere formation (3, 28). Likewise, others have proposed that only a subset of CD133<sup>+</sup> tumor cells actually represent CSC (28, 29). Accordingly, a selective effect of temozolomide on CSC cannot be reliably assessed in the considerably larger CD133<sup>+</sup> population. We therefore investigated sphere formation, which is a measure for the presence of CSC (30, 31) and observed that temozolomide-treated tumor cells dose-dependently lost their clonogenic potential (Fig. 2C, left). This loss of stem-cell properties was observed at similar concentrations that were required to deplete CD133<sup>+</sup> cells (Fig. 2A). Treatment with temozolomide resulted in a reduced number of equally sized tumor spheres compared with control conditions (Fig. 2C, right). This suggests that temozolomide does not inhibit the growth of progenitor and differentiated cells derived from CSC that have resisted the challenge with temozolomide. Because both, reversible cell cycle arrest and depletion of CSC, would impair tumor sphere growth, we replaced the continuous exposure to temozolomide by pulse treatment for 2, 4, and 7 days. After 42 days, the number of spheres derived from CD133<sup>+</sup> CSC lines was determined. This revealed that incubation with 50 to 500 μmol/L temozolomide for 2 days was sufficient to completely abolish sphere formation (Fig. 2D).

**MGMT modulates temozolomide-mediated depletion of CSC in CD133<sup>+</sup> CSC lines.** Although the overall effects of temozolomide did not differ between the tested CSC lines, the temozolomide concentration required to deplete sphere-forming cells varied (Fig. 3A) and correlated with expression of MGMT mRNA (Fig. 3B). The MGMT expression in the CSC line R28 was associated with a significantly higher proportion of CD133<sup>+</sup> cells remaining after incubation with 50 μmol/L temozolomide for 7 days compared with the cell line R11 that was virtually devoid of MGMT mRNA (Fig. 3C). In pulse-treated cells, incubation with 50 μmol/L temozolomide for 2 days eliminated all CSC in the MGMT-negative CSC line R11. In contrast, in the MGMT-positive cell line R28, 10-fold higher concentrations (500 μmol/L) only incompletely eliminated CSC with mainly differentiated tumor cells remaining (Fig. 3D). As expected, the MGMT inhibitor 6-BG sensitized stem cell-like cells in this cell line to the deleterious effects of temozolomide, whereas the inhibitor had no effect on the MGMT-negative CSC line R11 (Fig. 4A; data not shown).

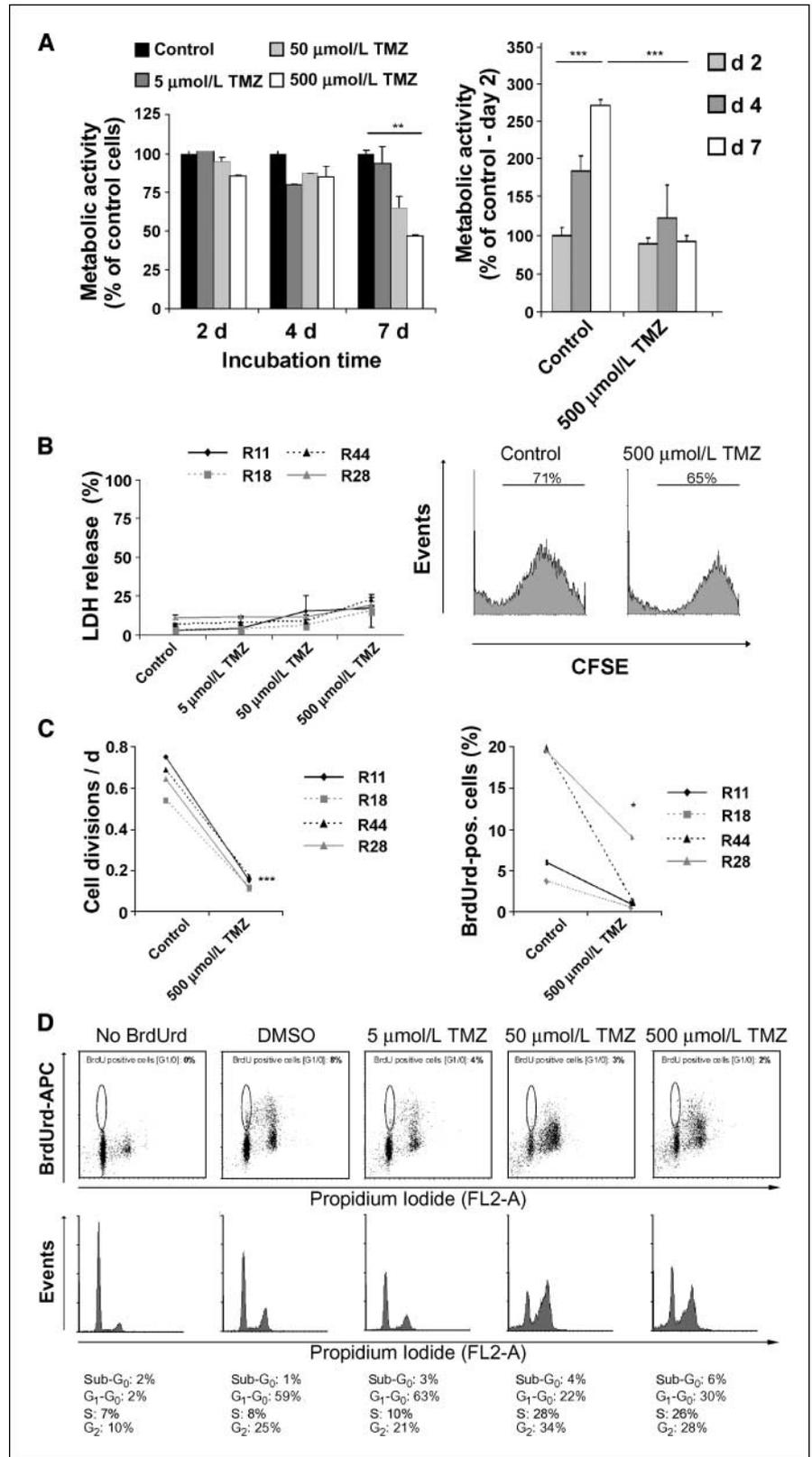
**MGMT promoter methylation and protein expression in CD133<sup>+</sup> CSC lines.** Because the MGMT mRNA status correlated with the susceptibility of glioma cells toward temozolomide, we hypothesized that differential MGMT promoter methylation and MGMT protein expression in CSC might be responsible for the selective depletion of stem cell-like cells from CSC lines. Whereas the MGMT promoter methylation pattern did not differ between CD133<sup>+</sup> and CD133<sup>-</sup> tumor cells (Fig. 4B), the MGMT protein expression was more pronounced in the CD133<sup>-</sup> subset of tumor cells (Fig. 4C). Accordingly, the increased susceptibility of CSC toward temozolomide is not due to a differential MGMT promoter methylation but is enhanced by differential MGMT protein expression in MGMT-positive cell lines.

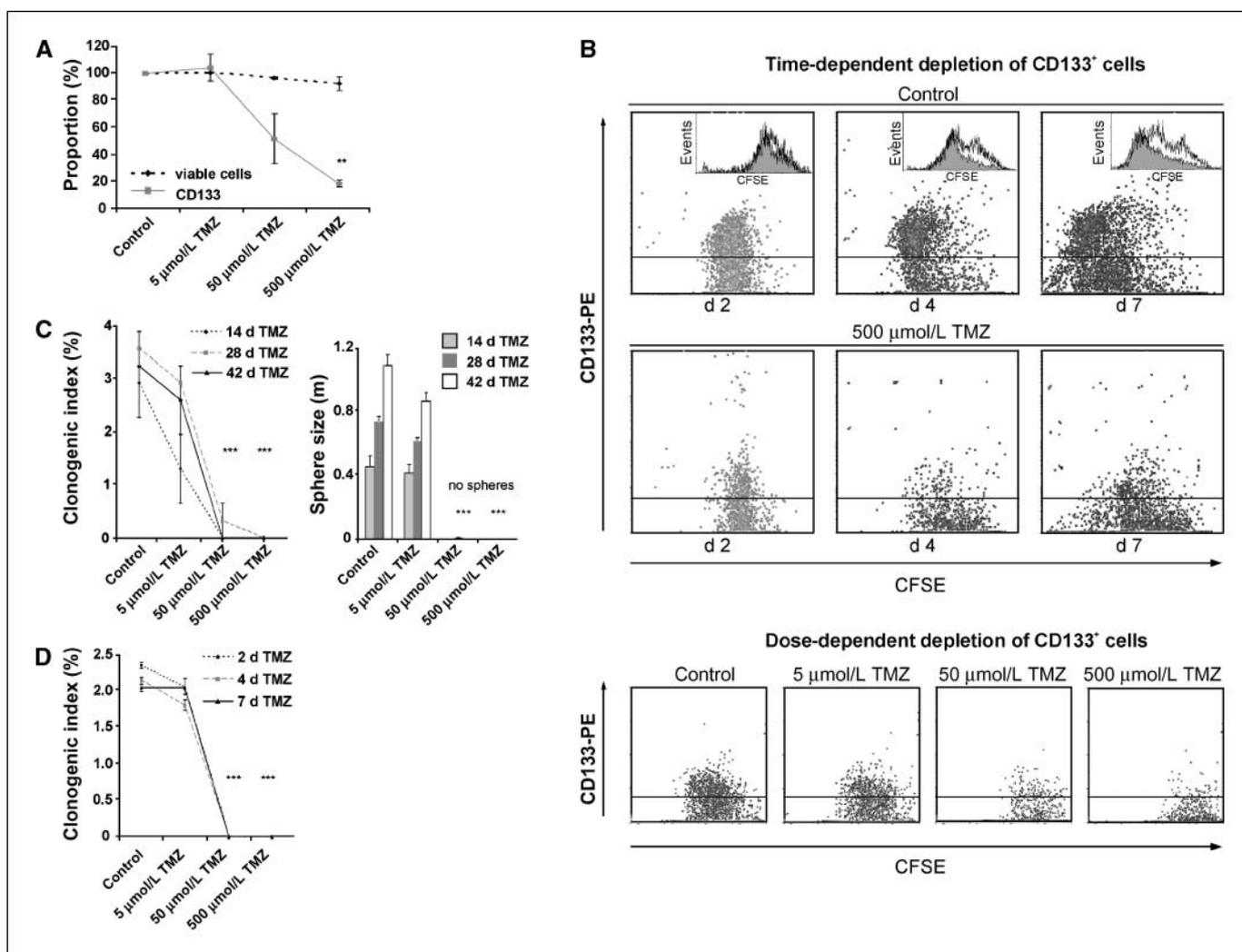
**Temozolomide treatment affects the differentiation potential of CD133<sup>+</sup> CSC.** We have previously shown that GBM-derived CSC can differentiate into cells expressing markers of all three neural lineages (3, 16). Therefore, we analyzed the

expression of both stem cell and differentiation markers in solvent-treated or temozolomide-treated CD133<sup>+</sup> CSC lines. As expected, the proportion of cells expressing the neural stem cell marker nestin declined upon treatment with temozolomide,

whereas the percentage of GFAP<sup>+</sup> cells (astrocytic lineage),  $\beta$ -tubulin-III<sup>+</sup> cells (neuronal lineage), and GalC<sup>+</sup> cells (oligodendroglial lineage) remained largely stable or even increased (Fig. 4D).

**Figure 1.** Temozolomide (TMZ) does not induce cell death but efficiently inhibits proliferation in CD133<sup>+</sup> CSC lines. **A**, CD133<sup>+</sup> CSC lines were treated for 2, 4, and 7 d with increasing doses of temozolomide. The metabolic activity corresponding to viable cells was determined by the AlamarBlue assay. The data are given as relative metabolic activity compared with the respective solvent-treated control cells at day 2, 4, and 7 (left), and compared with solvent-treated cells at day 2 (right; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ANOVA followed by Tukey-Kramer HSD test; results of CSC line R11 are shown). **B**, CSC lines were incubated with temozolomide for 7 d with the concentrations indicated. The LDH activity was determined in the supernatant and compared with untreated control cells [R11, R18, and R28 (all concentrations):  $P > 0.05$ ; R44 (control versus 500  $\mu$ mol/L):  $P < 0.05$ , left; ANOVA followed by Tukey-Kramer HSD test]. In addition, CD133<sup>+</sup> CSC lines labeled with CFSE were analyzed by flow cytometry after incubation for 7 d. CFSE-positive cells were defined (right; results of CSC line R11 are shown). **C**, the number of cell divisions (cd) was calculated by comparing the mean fluorescence of CFSE-labeled cells at d2 ( $F^{d2}$ ) and d7 ( $F^{d7}$ ) using the following equation:  $cd = [\log(F^{d2}) - \log(F^{d7}) / \log(2)]$  (left). CSC lines were incubated with temozolomide for 7 d as indicated and pulsed with BrdUrd for 12 h. Cells were then analyzed for DNA content and BrdUrd incorporation. The proportion of cells having completed at least one cell cycle [i.e., BrdUrd-positive (BrdUrd-pos) cells in the G<sub>0</sub>-G<sub>1</sub> phase of the cell cycle] in DMSO-treated cells (control) and cells treated with 500  $\mu$ mol/L temozolomide was determined (right; \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ; one-sided paired Student's *t* test). **D**, representative cytometries of anti-BrdUrd-Cy5 and propidium iodide double-stained cells of CSC line R11 are shown. As in C, cells were treated for 7 d with temozolomide and then pulsed with BrdUrd for 12 h.





**Figure 2.** Temozolomide depletes CD133<sup>+</sup> tumor cells in CD133<sup>+</sup> CSC lines. **A**, CD133<sup>+</sup> CSC lines were incubated with increasing doses of temozolomide for 7 d and the proportion of CD133<sup>+</sup> cells and of viable cells was determined using flow cytometry. Dead cells were detected using 7-AAD staining. The data are given as relative value compared with solvent-treated cells (\*\*,  $P < 0.01$ ; one-sided Student's  $t$  test); *points*, mean of three independent experiments with CSC line R11; *bars*, SE. **B**, CD133<sup>+</sup> CSC were enriched by FACS, labeled with CFSE, and incubated with temozolomide as indicated. Cells were counterstained with anti-CD133-PE, 10,000 cells were analyzed by flow cytometry, and displayed as a density plot. *Insets*, histograms of CD133<sup>+</sup> (*closed profile*) and CD133<sup>-</sup> (*open profile*) cells that were stained with CFSE and left to proliferate for 2, 4, or 7 d. **C**, 10 tumor cells per well were plated in a 96-well plate and incubated with temozolomide as indicated. Fresh medium supplemented with temozolomide was added twice weekly. Sphere number (*left*) and size (*right*) were determined after 14, 28, and 42 d. The calculated clonogenic index (spheres per 100 cells plated) and the average sphere size is given (results of CSC line R11 are shown). **D**, 1,000 tumor cells per well were plated in a 48-well plate and incubated with the indicated concentrations of temozolomide (diluted in "stem cell medium") for 2, 4, and 7 d. Subsequently, cells were cultured in temozolomide-free medium until day 42 when the number of spheres formed was determined (results of CSC line R11 are shown).

**Temozolomide also depletes CSC in CD133<sup>-</sup> CSC lines.** We have recently described a novel type of CD133<sup>-</sup> GBM CSC (3). They show molecular profiles that are distinct from those of CD133<sup>+</sup> GBM CSC and give rise to CD133<sup>-</sup> CSC lines that showed adherently but no neurosphere-like growing spheres. Similar to CD133<sup>+</sup> CSC lines, CD133<sup>-</sup> CSC lines were resistant to temozolomide-induced cell death, whereas tumor cell proliferation was significantly inhibited (Fig. 5A; data not shown). Because there are no specific markers for CSC in CD133<sup>-</sup> CSC lines, we determined the proportion of colony-forming cells as an indirect measure for the number of CSC. Upon treatment with 50 μmol/L temozolomide, the CD133<sup>-</sup> CSC largely lost their clonogenic potential (Fig. 5B). Incubation with 500 μmol/L temozolomide for 2 days caused no acute overall toxicity but completely eliminated clonogenic cells (Fig. 5A and C). This may be interpreted as a loss

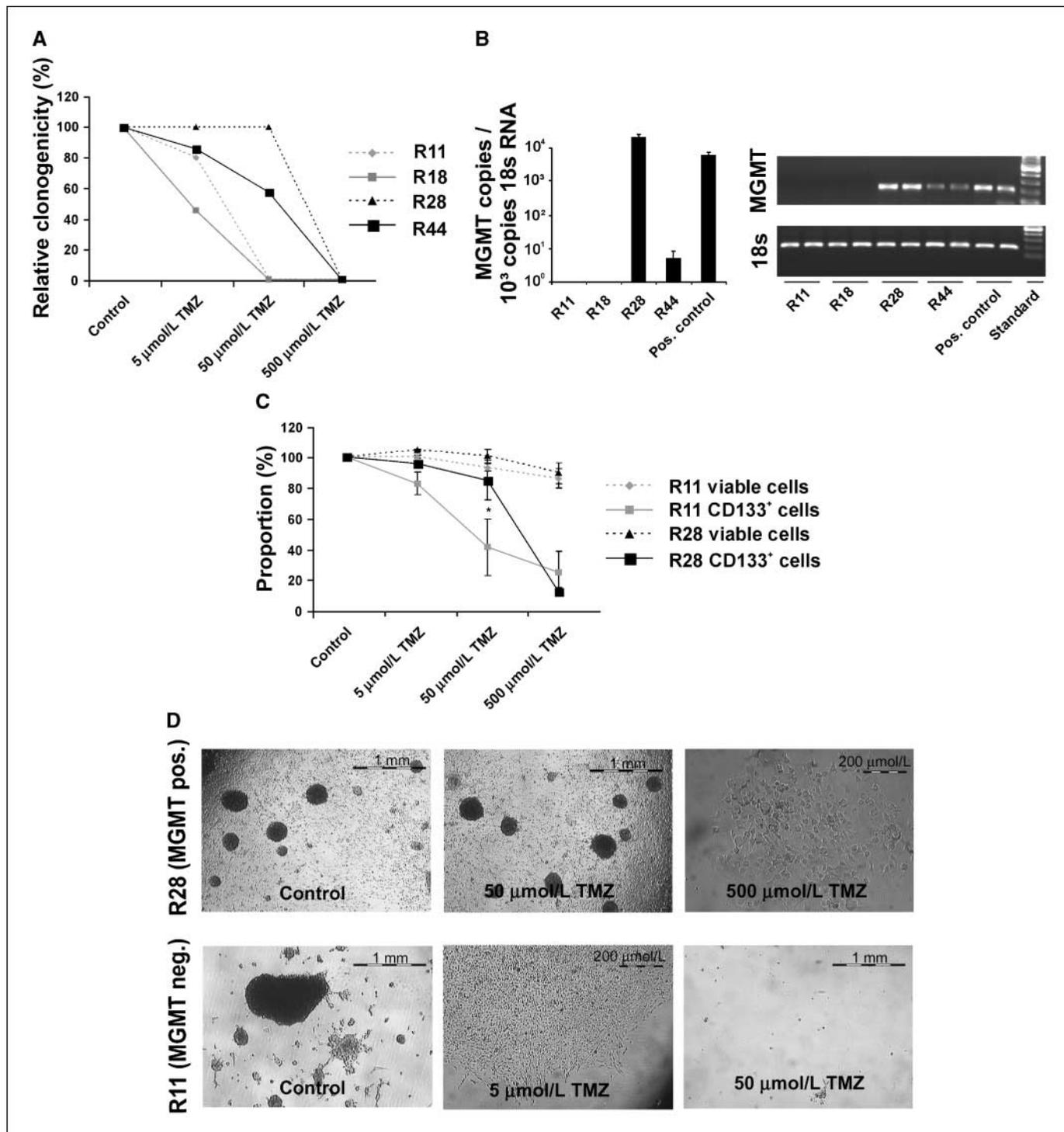
of cells with stem cell-like properties, which is corroborated by a massive decline in the nestin-positive population (Fig. 5D). In contrast, the fraction of the more differentiated subsets remained stable.

**Temozolomide abolishes the tumorigenicity of MGMT-negative CD133<sup>+</sup> CSC.** Finally, we investigated whether the loss of clonogenicity *in vitro* corresponded to a reduced tumorigenicity of CD133<sup>+</sup> CSC lines *in vivo*: CSC cultures (R11, MGMT negative; R28, MGMT positive) were incubated with increasing doses of temozolomide for 2 days, and 200,000 viable cells (assessed by trypan blue exclusion) were injected intracranially into nude mice. Again, temozolomide-treated cells were found to be completely viable (Supplementary Fig. S1). Twelve weeks after tumor inoculation, the tumor size was determined using 3T MRI. Pretreatment with temozolomide dose-dependently reduced the tumor size, i.e., the tumorigenicity of injected tumor cells (Fig. 6A).

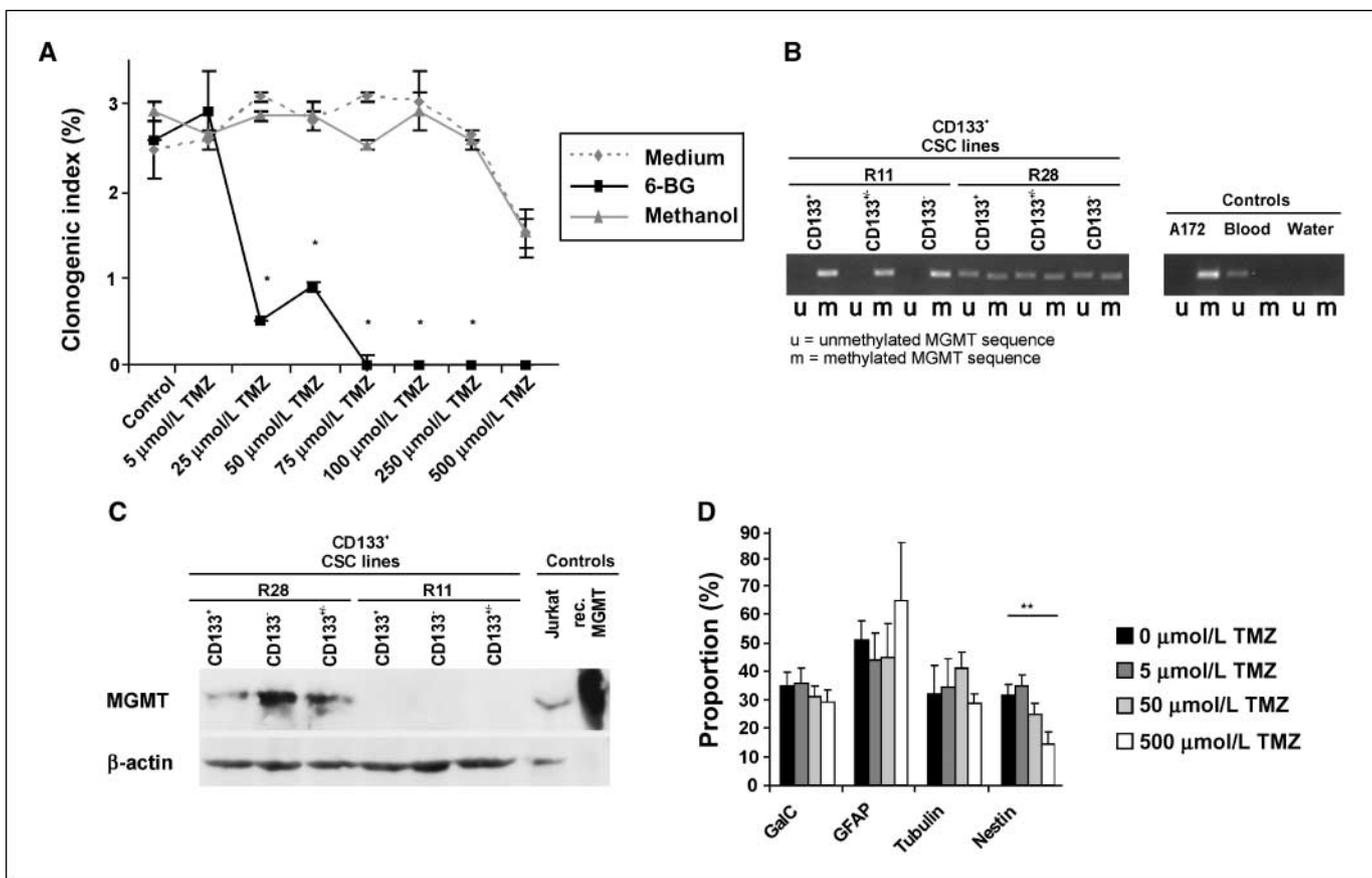
The results were reproduced with the CSC line R11 injected into additional 11 animals (Supplementary Table S1).

Animals were then sacrificed and brains were histologically analyzed. Again, we observed a dose-dependent massive decrease

in tumor size irrespective of the MGMT status of the CSC lines injected (Fig. 6B). Notably, lesions formed by the MGMT-positive CSC line (R28) and the MGMT-negative CSC line (R11) after pretreatment with 50 and 500  $\mu\text{mol/L}$  temozolomide showed a



**Figure 3.** MGMT modulates temozolomide-mediated depletion of CSC in CD133<sup>+</sup> CSC lines. **A**, 10 tumor cells per well were plated in a 96-well plate and incubated with temozolomide for 42 d. Medium supplemented with temozolomide was changed twice weekly. The relative clonogenicity compared with solvent-treated cells is given. **B**, the CD133<sup>+</sup> CSC lines were analyzed for MGMT-mRNA expression using quantitative PCR. The number of copies compared with 10<sup>3</sup> copies 18s RNA (left) and the corresponding agarose gel (right) are shown. **C**, the proportion of CD133<sup>+</sup> cells and of viable cells were determined by costaining with CFSE and CD133-PE. Cells were labeled with CFSE at day 0 and costained with CD133-PE after 7 d ( $n = 3$  experiments per cell line;  $P < 0.05$ , ANOVA followed by Tukey-Kramer HSD). **D**, 1,000 tumor cells were pulse treated for 2 d with the temozolomide concentrations indicated. Subsequently, cells were cultured in temozolomide-free medium until day 42. Representative pictures after 42 d are shown. *Pos. control*, positive control. *MGMT pos.*, MGMT positive. *MGMT neg.*, MGMT negative.



**Figure 4.** MGMT modulates temozolomide-mediated depletion of CSC in CD133<sup>+</sup> CSC lines. **A**, 6-BG established temozolomide-mediated CSC depletion in the MGMT-expressing CSC line R28. One thousand tumor cells were incubated for 2 d with temozolomide alone (*medium*) and combined with 50 µmol/L 6-BG or solvent control (*methanol*). Subsequently, cells were cultured in temozolomide-free medium until day 26 when the number of spheres formed was determined. The calculated clonogenic index (spheres per 100 cells plated) is given. (\*,  $P < 0.05$ ; two-sided Student's *t* test, compared with solvent control). **B**, CD133<sup>+</sup> and CD133<sup>-</sup> tumor cells within CD133<sup>+</sup> CSC lines (R28, R11, and two additional CD133<sup>+</sup> CSC lines; data not shown) were isolated using FACS. The MGMT promoter methylation status was analyzed in the respective fractions by methylation-specific PCR using primers specific for the methylated (*m*) and nonmethylated (*u*) promoter. Controls included A172 glioma cells where the MGMT promoter is methylated (21) and primary human lymphocytes. **C**, CD133<sup>+</sup> and CD133<sup>-</sup> cells of the tumor cell lines R11 and R28 were isolated by FACS and analyzed for MGMT expression by Western blot. The expression was quantified and compared with the MGMT expression in Jurkat cells. **D**, tumor cells from CD133<sup>+</sup> CSC lines were plated on coverslips and cultured for 48 h. Cells were then incubated with temozolomide for 7 d as indicated and stained for the indicated marker proteins (results of CSC line R11 are shown; \*\*,  $P < 0.01$ ; ANOVA followed by Tukey-Kramer HSD test).

highly different histologic phenotype. Although the MGMT-positive CSC line formed an infiltrating tumor with high cellularity, lesions formed by the pretreated MGMT-negative CSC line showed low cellularity, scar-like margins (Fig. 6C), and were virtually devoid of proliferating cells (Fig. 6D). This suggests that the injected pretreated MGMT-negative tumor cells survived *in vivo* but were not tumorigenic. The impaired tumorigenicity was seen at similar temozolomide concentrations that (a) eliminated CD133<sup>+</sup> cells and (b) reduced clonogenicity *in vitro* and, thus, corroborates the concept of temozolomide preferentially eliminating CSC in GBM.

## Discussion

Here, we describe that temozolomide preferentially depletes GBM cells with stem cell properties, whereas differentiated tumor cells are resistant to the agent. This finding clearly contradicts the predictions of the CSC hypothesis (2) and, therefore, warrants a rethinking of fundamental paradigms of glioma biology and therapy.

One part of the explanation may be that MGMT, the major mediator of temozolomide resistance in GBM, is overexpressed in

CD133<sup>-</sup> cells (Fig. 4C). However, the selectivity of the drug for CSC must have further reasons because temozolomide also induces the depletion of MGMT-positive CSC as well as the differential depletion of CSC in MGMT-negative CSC lines (Fig. 3C). Considering that temozolomide requires the formation of base mismatch and, therefore, cell cycle initiation to be effective, different rates of proliferation could also account for the differential susceptibility of stem cells and nonstem cells toward temozolomide. In this context, our finding that the CD133<sup>+</sup> compartment (which also comprises the actual CSCs) proliferates faster than the CD133<sup>-</sup> compartment (Fig. 2B) is certainly of interest. Moreover, this surprising observation raises the question whether the rapid deregulated growth of GBM might be due to rapidly dividing GBM stem cells. However, a definite answer to this question would require the identification of unambiguous markers for CSC in GBM. Because these are not available to date, we cannot exclude the possibility that CSC are—as previously suggested—a small slowly proliferating subset (32) that is hardly detectable within the considerably larger population of CD133<sup>+</sup> cells.

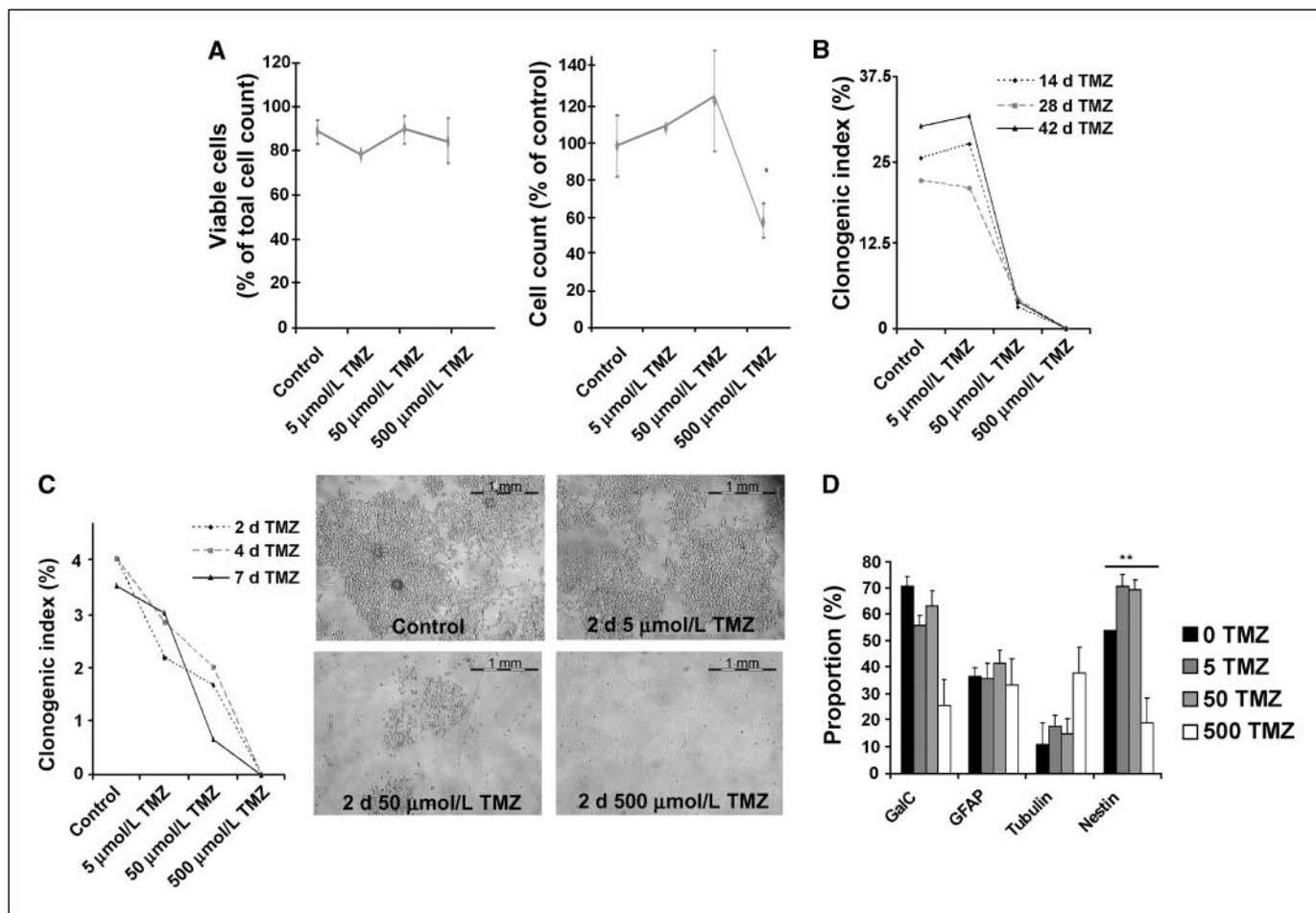
On a more functional level, we could show that temozolomide completely abolishes clonogenicity and tumorigenicity of

CD133-positive and CD133-negative CSC lines (Figs. 5B and C, and 6). It may therefore be hypothesized that temozolomide selectively kills CSC, which constitute such a small subset of the CD133<sup>+</sup> cells (<10%) that the elimination of these cells only becomes apparent after prolonged culture (Fig. 1). Alternatively, temozolomide might induce differentiation (33) or senescence (25) in stem cell-like tumor cells. Again, a conclusive answer to this question will only be possible once an unambiguous marker for CSC has been identified.

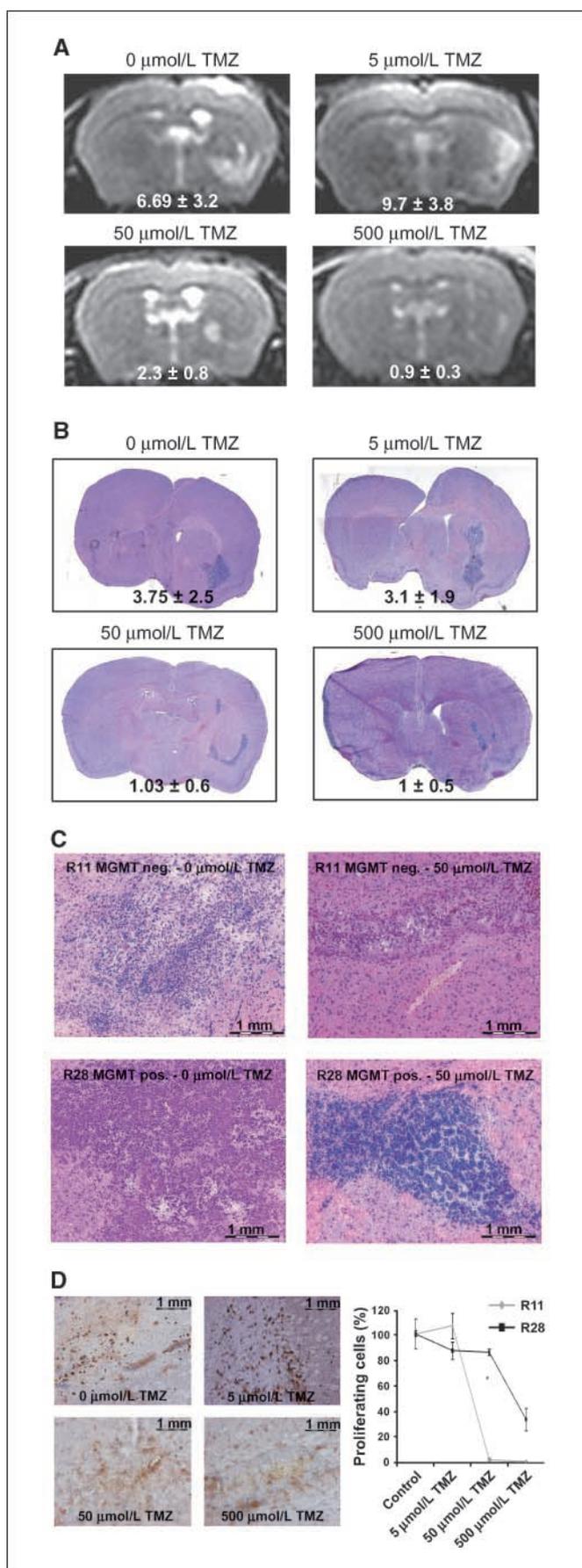
Our results clearly contradict recent reports that describe higher resistance of CD133<sup>+</sup> cells to chemotherapy compared with CD133<sup>-</sup> cells (19, 34). However, most of these studies use short-term metabolic assays to assess the viability of the tumor cells after treatment with extremely high (21, 25, 29, 35) temozolomide concentrations (500–2,000 μmol/L). Thus, the observed (still) low rates of metabolic inhibition (~20%) may be unspecific and not indicative for cell death. Moreover, these authors did not use culture conditions known to maintain the stemness of CSC (3–5, 14, 29, 30, 36–41) but rather cultured their cells in medium supplemented with FCS. These conditions may not be compatible

with the maintenance of the “stem cell state” because they are known to induce a multitude of genetic and transcriptional modifications and lead to the differentiation of CSC (14, 36, 39). The importance of suitable culture conditions for CSC-based assays is further confirmed by Clement and colleagues (29) who treated GBM CSC lines with 25 μmol/L temozolomide under defined serum-free culture conditions. In line with our results, these authors found that temozolomide induced a 50% to 80% decrease of proliferation in 3 of 4 tested GBM CSC lines with a marginal increase of caspase-3-positive tumor cells. In addition, they also describe slow rates of recovery 20 days after temozolomide treatment, which actually suggests an effect of temozolomide on CSC.

Although these data provide evidence for a selective depletion of CSC in GBM by temozolomide, we nevertheless found that all investigated CSC are completely resistant toward the cytotoxic effects of 5 μmol/L temozolomide, which corresponds to the peak concentration measured in the cerebrospinal fluid of patients (22). This resistance to temozolomide is in line with previous data



**Figure 5.** Temozolomide also depletes CSC in CD133<sup>-</sup> CSC lines. All experiments shown were carried out with the cell line R8 maintained by CD133<sup>-</sup> CSC (3). A, CD133<sup>-</sup> CSC were treated for 2 d with increasing doses of temozolomide. The proportion of viable cells (left) and the absolute cell number (compared with solvent-treated cells; right) were determined using the trypan blue exclusion assay (\*,  $P \leq 0.05$ ; one-sided Student's *t* test). B, 10 tumor cells per well were plated in a 96-well plate and incubated with temozolomide as indicated. Fresh medium supplemented with temozolomide was added twice weekly. The clonogenicity index (spheres per 100 cells plated) was determined after 14, 28, and 42 d. C, 1,000 tumor cells per well were plated in a 48-well plate and incubated with the indicated concentrations of temozolomide for 2, 4, and 7 d. Subsequently, cells were cultured in temozolomide-free medium until day 42. The clonogenic index and representative photographs are shown. D, tumor cells were plated on coverslips, adhered for 48 h, and then incubated for 7 d with the indicated concentrations of temozolomide. Expression of stem cell or neural lineage markers was assessed by immunocytochemistry (\*\*,  $P < 0.01$ ; ANOVA followed by Tukey-Kramer HSD).



(20) and may be due to the highly developed capacity of GBM cells to pump extraneous substances such as fluorochromes or chemotherapeutics out of the cell via ATP-binding cassette transporters (including MDR-1, MDP, and BCRP-1; refs. 11, 42). In addition, also MGMT-negative glioma cells may dispose of DNA repair mechanisms (25, 43) that counteract the cytotoxic effects when this alkylating agent is given at low doses. However, although concentrations required to eliminate MGMT-positive CSC ( $\sim 500 \mu\text{mol/L}$ ) cannot be reached *in vivo* (which easily explains the failure of temozolomide-based chemotherapy in the respective patients), CSC from MGMT-negative tumors could be eliminated by  $50 \mu\text{mol/L}$  temozolomide already. Although the exact temozolomide concentrations in the tumor cells are unknown, such concentrations have already been measured in the plasma of patients after standard treatment—and they might also be achieved in those regions of the tumor where the blood-brain barrier has been destroyed. Thus, an intriguing question arises: Why do patients with MGMT-negative tumors die despite the susceptibility of CSC to temozolomide? Considering the impaired blood-brain barrier that would enable temozolomide concentrations of up to  $50 \mu\text{mol/L}$  to be achieved in the tumor, we speculate that glioma cells may hide in otherwise healthy brain tissue in which temozolomide concentrations of  $5 \mu\text{mol/L}$  are likely. Accordingly, recurrent GBM in MGMT-negative tumors would derive from tumor stem cells that have invaded healthy brain tissues before therapy and were thus protected by the blood-brain barrier.

Importantly, our data may not only contribute to a better understanding of CSC in GBM, they also bear implications for the design and interpretation of clinical trials: In GBM, CSC might selectively be depleted (by cell cycle arrest, senescence, cell death, or differentiation), whereas the remaining tumor cells survive with reduced proliferative capacity. Thus, the tumor would not shrink under ongoing chemotherapy but remain stable for a prolonged period—which is exactly what is seen in many clinical responders. Moreover, the hypothesis that those CSC that drive a progressing tumor may not have been exposed to sufficiently high concentrations of temozolomide previously would justify further cycles of chemotherapy with the drug. Finally, our data support recent publications that suggest that dose-intensified temozolomide might be more effective than the conventional dosing scheme (10, 44–46). Thus, optimized temozolomide dosing schemes might even completely eradicate CSC from MGMT-negative tumors, thereby providing a cure (or permanent tumor containment) for affected patients by using a well-established chemotherapeutic agent.

**Figure 6.** Temozolomide abolished the tumorigenicity of MGMT-negative CD133<sup>+</sup> CSC. A to D,  $10^6$  tumor cells per condition of the CSC line R11 (MGMT negative) and R28 (MGMT positive) were incubated with the indicated concentrations of temozolomide for 48 h, and their viability was assessed using the trypan blue exclusion assay (data not shown). Viable cells ( $2 \times 10^5$ ) were then injected intracranially into NMRI<sup>(nu/nu)</sup> mice ( $n = 16$ ; 2 animals per group). A, 12 wk after implantation, tumor size was determined using 3T MRI (representative T2 weighted MRI are shown; tumor volume  $\pm$  SE is given). B, mice were sacrificed and tumor volume was analyzed using a caliper. The histologic tumor volume  $\pm$  SE is given. C, representative photographs depicting the morphology of tumor-like lesions formed by temozolomide-treated MGMT-expressing or MGMT-negative CSC lines are shown. D, tumor-like lesions formed by temozolomide-treated MGMT-positive or MGMT-negative CSC were stained for expression of the proliferation marker Ki-67 (pictures of lesions induced by R11 are shown). The proportion of Ki-67-positive cells in tumors formed by cells pretreated with temozolomide is given compared with tumors formed by control treated cells (\*,  $P < 0.05$ ; one-sided Student's *t* test).

## Disclosure of Potential Conflicts of Interest

P. Hau: Speakers honoraria. U. Bogdahn: Speakers honoraria. The other authors disclosed no potential conflicts of interest.

## Acknowledgments

Received 1/8/2008; revised 4/30/2008; accepted 5/2/2008.

**Grant support:** ReForM program of the Medical School, University of Regensburg, by the Bavarian Research Council, and an unrestricted educational

grant of Schering-Plough to the Department of Neurology, University of Regensburg.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Britta Friedensdorf for excellent technical assistance with the *MGMT* promoter methylation and Western blot analyses, Indra Tumurbat for her valuable help concerning Western blot optimization, Christiane Knobbe for the CD133 expression plasmid, and Prof. Bernd Kaina, University of Mainz, Germany, for kindly providing anti-*MGMT* antibodies.

## References

- Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;352:987–96.
- Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414:105–11.
- Beier D, Hau P, Proescholdt M, et al. CD133+ and CD133- glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res* 2007;67:4010–5.
- Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006;444:756–60.
- Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432:396–401.
- Wang J, Sakariassen PO, Tsinkalovsky O, et al. CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. *Int J Cancer* 2008;122:761–8.
- Günther HS, Schmidt NO, Phillips HS, et al. Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria. *Oncogene* 2007;27:2897–909. Epub 2007 Nov 26.
- Hau P, Koch D, Hundsberger T, et al. Safety and feasibility of long-term temozolomide treatment in patients with high-grade glioma. *Neurology* 2007;68:688–90.
- Hegi ME, Diserens AC, Gorlia T, et al. *MGMT* gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* 2005;352:997–1003.
- Chinot OL, Barrie M, Fuentes S, et al. Correlation between O6-methylguanine-DNA methyltransferase and survival in inoperable newly diagnosed glioblastoma patients treated with neoadjuvant temozolomide. *J Clin Oncol* 2007;25:1470–5.
- Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci U S A* 2004;101:781–6.
- Salmaggi A, Boiardi A, Gelati M, et al. Glioblastoma-derived tumorospheres identify a population of tumor stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. *Glia* 2006;54:850–60.
- Phillips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006;9:157–73.
- Lee J, Kotliarova S, Kotliarov Y, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell* 2006;9:391–403.
- Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol Berl* 2007;114:97–109.
- Beier D, Wischhusen J, Dietmaier W, et al. CD133 and Cancer stem cells predict prognosis in high grade oligodendroglial tumors. *Brain Pathol* 2007;18:370–7.
- Beier CP, Kolbl M, Beier D, Woertgen C, Bogdahn U, Brawanski A. CD95/Fas mediates cognitive improvement after traumatic brain injury. *Cell Res* 2007;17:732–4.
- Möller M, Wolter M, Felsberg J, Collins VP, Reifenberger G. Frequent promoter hypermethylation and low expression of the *MGMT* gene in oligodendroglial tumors. *Int J Cancer* 2005;113:379–85.
- Liu G, Yuan X, Zeng Z, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer* 2006;5:67.
- Eramo A, Ricci-Vitiani L, Zeuner A, et al. Chemotherapy resistance of glioblastoma stem cells. *Cell Death Differ* 2006;13:1238–41.
- Hermissin M, Klumpp A, Wick W, et al. O6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. *J Neurochem* 2005;96:766–76. Epub 2006 Jan 9.
- Ostermann S, Csajka C, Buclin T, et al. Plasma and cerebrospinal fluid population pharmacokinetics of temozolomide in malignant glioma patients. *Clin Cancer Res* 2004;10:3728–36.
- Brada M, Judson I, Beale P, et al. Phase I dose-escalation and pharmacokinetic study of temozolomide (SCH 52365) for refractory or relapsing malignancies. *Br J Cancer* 1999;81:1022–30.
- Lyons AB. Analysing cell division *in vivo* and *in vitro* using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods* 2000;243:147–54.
- Hirose Y, Berger MS, Pieper RO. p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells. *Cancer Res* 2001;61:1957–63.
- Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003;63:5821–8.
- Beier CP, Wischhusen J, Gleichmann M, et al. FasL (CD95L/APO-1L) resistance of neurons mediated by phosphatidylinositol 3-kinase-Akt/protein kinase B-dependent expression of lifeguard/neuronal membrane protein 35. *J Neurosci* 2005;25:6765–74.
- Dirks PB. Cancer: stem cells and brain tumours. *Nature* 2006;444:687–8.
- Clement V, Sanchez P, de Tribolet N, Radovanovic I, Ruiz i Altaba A. HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. *Curr Biol* 2007;17:165–72.
- Uchida N, Buck DW, He D, et al. Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci U S A* 2000;97:14720–5.
- Fomchenko EI, Holland EC. Stem cells and brain cancer. *Exp Cell Res* 2005;306:323–9.
- Ligon KL, Huillard E, Mehta S, et al. Olig2-regulated lineage-restricted pathway controls replication competence in neural stem cells and malignant glioma. *Neuron* 2007;53:503–17.
- Zucchetti M, Catapano CV, Filipposchi S, Erba E, D'Incalci M. Temozolomide induced differentiation of K562 leukemia cells is not mediated by gene hypomethylation. *Biochem Pharmacol* 1989;38:2069–75.
- Kang MK, Kang SK. Tumorigenesis of chemotherapeutic drug-resistant cancer stem-like cells in brain glioma. *Stem Cells Dev* 2007;16:837–47.
- Wick W, Wick A, Schulz JB, Dichgans J, Rodemann HP, Weller M. Prevention of irradiation-induced glioma cell invasion by temozolomide involves caspase 3 activity and cleavage of focal adhesion kinase. *Cancer Res* 2002;62:1915–9.
- Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* 2004;64:7011–21.
- Hemmati HD, Nakano I, Lazareff JA, et al. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci U S A* 2003;100:15178–83.
- Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*. *Glia* 2002;39:193–206.
- Li A, Walling J, Kotliarov Y, et al. Genomic changes and gene expression profiles reveal that established glioma cell lines are poorly representative of primary human gliomas. *Mol Cancer Res* 2008;6:21–30.
- Piccirillo SG, Reynolds BA, Zanetti N, et al. Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. *Nature* 2006;444:761–5.
- Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992;255:1707–10.
- Jackson EL, Garcia-Verdugo JM, Gil-Perotin S, et al. PDGFR  $\alpha$ -positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. *Neuron* 2006;51:187–99.
- Cahill DP, Levine KK, Betensky RA, et al. Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. *Clin Cancer Res* 2007;13:2038–45.
- Wick W, Steinbach JP, Küker WM, Dichgans J, Bamberg M, Weller M. One week on/one week off: a novel active regimen of temozolomide for recurrent glioblastoma. *Neurology* 2004;62:2113–5.
- Spence AM, Kiem HP, Partap S, Schuetze S, Silber JR, Peterson RA. Complications of a temozolomide overdose: a case report. *J Neurooncol* 2006;80:57–61.
- Brandes AA, Tosoni A, Cavallo G, et al. Temozolomide 3 weeks on and 1 week off as first-line therapy for recurrent glioblastoma: phase II study from gruppo italiano cooperativo di neuro-oncologia (GICNO). *Br J Cancer* 2006;95:1155–60.