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Inhibition of ABCG2 enhances chemo-sensitivity of murine glioma stem cell-like cells to temozolomide and reduces spheroid-forming capability

Hiromasa Yoshioka[1], Takeshi Okuda[1], Mitsugu Fujita[2], Takao Inoue[3], Takayuki Tasaki[1], Shuichi Izumoto[1], Amami Kato[1]

[1]*Department of Neurosurgery, Kinki University Faculty of Medicine, Osakasayama, Osaka 589-8511, Japan*
[2]*Department of Microbiology, Kinki University Faculty of Medicine, Osakasayama, Osaka 589-8511, Japan*
[3]*Department of Pathology, Kinki University Faculty of Medicine, Osakasayama, Osaka 589-8511, Japan*

Abstract

Current evidence indicates that glioma stem cell-like cells (GSCs) in humans play critical roles in the pathogenesis of carcinogenesis of glioblastoma (GBM). The GSCs are known to overexpress members of the adenosine triphosphate-binding cassette (ABC) family transporters to exhibit multidrug resistance. Eradication of the GSC compartment is therefore essential to achieve a stable and long-lasting remission of GBM. To elucidate the characteristics of GSCs in detail, we generated murine GSC lines from *Sleeping Beauty* transposon-mediated spontaneous GBM. Using these several cell lines, we evaluated the significance of ABC transporters in the GSC kinetics by cell morphology assays, flow cytometry, and quantitative RT-PCR for mRNA expressions. As a consequence, we show that siRNA-mediated ABCG2 inhibition enhances the sensitivity of GSCs to temozolomide (TMZ) and in turn reduces their spheroid-forming capability. Furthermore, we show that GSCs treated with *Abcg2*-specific siRNA become sensitive to TMZ and reduce their spheroid-forming capability. In conclusion, our data suggest that targeting of drug transporters in GSCs is a promising strategy to enhance their chemo-sensitivity for achieving a long-lasting remission of GBM.

Key words: glioblastoma, cancer stem cell, drug resistance, ABC transporter

Background

Glioblastoma multiforme (GBM) is the most common primary brain tumor and one of the most lethal cancers with an average life expectancy of less than 1 year.[1] Treatment of GBM patients is extremely challenging because complete surgical resection is rarely achievable, and GBM is refractory to current chemotherapy regimens based on temozolomide (TMZ).[2] It is therefore urgent to develop novel therapeutic strategies for GBM.

In recent years, numerous studies have reported the presence of stem cell-like cells in solid tumors.[3] Likewise, GBM is known to possess glioma stem cell-like cells (GSCs).[4] Based on a line of previous studies,[5] GSCs are defined as built-in pluripotent cells that express a stem cell marker CD133 and nestin, an intermediate filament protein found in undifferentiated central nervous system cells, at high levels. In addition, they are known to exhibit resistance to chemotherapy and play a critical role in repopulating tumor masses. That is, although chemotherapy

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can kill the majority of glioma cells and induce a temporary regression, it results in disease relapse by the enrichment of chemo-resistant GSCs in the end.[6] To achieve a long-lasting remission of GBM, therefore, we need to develop new therapeutic approaches that target both the tumor bulk and the GSC compartment.

Animal models that mimic the complexity of human GBM would be useful for understanding GBM biology and predicting therapeutic responses. In this regard, we and others have recently developed a novel *Sleeping Beauty (SB)* transposon-mediated spontaneous murine GBM model.[7–10] This system allows us to trace tumor initiation and progression by in vivo bioluminescent imaging. Of more importance, these murine tumors share many features with the human diseases including glial marker expression, pseudopalisading necrosis, and brain invasion.[7] Thus, this model appears suitable for investigating gliomagenesis including GSC biology. In addition, we have already established new GBM cell lines using this system.[7,8] It is therefore intriguing whether human-equivalent GSCs can be established from these spontaneous murine tumors.

Among various putative mechanisms that explain the chemo-resistance of GSCs, the ATP-binding cassette (ABC) transporters are thought to be the most responsible for this phenomenon.[11] The ABC transporters belong to a superfamily of membrane pumps that catalyze the ATP-dependent transport of various endogenous compounds and xenobiotics (such as chemotherapeutic agents) out of the cell.[12] A line of functional studies of ABC transporters revealed that both $CD34^+$ hematopoietic stem cells 13 and $CD133^+$ cancer stem cell-like cells 14 express several ABC transporters at high levels. Based on these studies, ABC transporters are considered a prominent functional marker for stem cell-like cells including GSCs.[14]

In the field of stem cells, the cells that efflux dyes such as Hoechst332342 or Vybrant Violet at high levels are so-called 'side population (SP)' cells.[15,16] As most cells accumulate the violet dye, SP can be isolated by dual-wavelength flow cytometry based of their capability to efflux the dye where the action of the ABC transporters is critically required.[17] This phenotype was originally described in murine bone marrow preparation, where this fraction was found to be enriched for long-term hematopoietic stem cells.[18] In turn, the SP cells have been found in cancer cells, including glioma cells.[6] That is, GSCs are distinguishable from normal glioma cells based on the same dye-efflux capability.[6]

Based on these findings, we hypothesized that GSCs would express ABC transporters at high levels and to promote their chemo-resistance to TMZ. To elucidate this question, we generated a number of murine GSC lines from *SB*-mediated spontaneous GBM in this study. As a consequence, murine GSCs exhibited various types of ABC transporters at high levels and chemo-resistance to TMZ. In addition, siRNA-mediated ABCG2 inhibition enhanced their chemo-sensitivity with a reduction of the spheroid-forming capability. In summary, our data suggest that murine GSC appears useful to investigate GSC biology in detail and that targeting of multidrug transporters in GSCs is a promising strategy to enhance their chemo-sensitivity and therefore to achieve a long-lasting remission of GBM.

Methods

Chemicals and antibodies

Chemicals were obtained from Sigma-Aldrich unless indicated otherwise. TMZ was dissolved in 10% DMSO/phosphate-buffered solution (PBS) to make a 100 mM stock solution. Fluorescently-labeled antibodies were obtained as follows: anti-CD133 (eBioSciences), anti-nestin (Abcam), anti-ABCG2 (Abcam), and isotype-matched controls (BD BioSciences).

Mice

Balb/c wild-type mice were obtained from CLEA and maintained under specific pathogen-free conditions at the Animal Research Center of Kinki University Faculty of Medicine. All procedures were approved by the Institutional Animal Care and Use Committee.

Tumor models

The procedure to induce spontaneous murine GBM has been described previously.[7–10] DNA transfection reagent (*in vivo*-jetPEI) was obtained from Polyplus Transfection. The following DNA plasmids were used for glioma induction: pT2/C-Luc//PGK-SB13, pT/CAGGS-NRASV12, pT2/shP53, and PT3.5/CMV-EGFRvIII (0.125 μg for each). To trace tumor growth, we conducted bioluminescence imaging using an IVIS200 (Caliper Life Sciences).

The procedure to inoculate tumor cells into

the brain of adult mice has been described previously.[19] Briefly, using a Hamilton syringe (Hamilton), cells were suspended in 2 ul PBS and stereotactically injected through an entry site at the bregma, 3 mm to the right of the sagittal suture and 4 mm below the surface of the skull of anesthetized mice using a stereotactic frame (Stolting).

Induction of spheroid-forming cells

The procedure to establish adherent cell lines and spheroid-forming cells from SB transposon-mediated spontaneous murine GBM has been described previously.[8] Briefly, neurospheroid cultures were established from acute cell dissociation of the murine GBM tissues, and the cells were maintained in DMEM/F12 medium supplemented with B27, EGF, and bFGF (20 ng/ml each).[4] In all experiments, the cells were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere. In some experiments, the cells derived from the line GB03 were resuspended as described above and then treated with TMZ for 48 hours in a dose-escalation manner; the concentration used was as follows: 2,000, 1000, 500, 250, 125, 62.5, 31.25 uM. After the 48-hour incubation, cell viability assay using WST-1 (Roche) was performed on the indicated days according to the manufacturer's instructions.

Flow cytometry

The procedure has been described previously with minor modifications.[19] Briefly, cells were detached from culture dishes with Accutase (Millipore), and viable cells were stained with the fluorescently-labeled antibodies described above. For SP analysis, the cells were stained with Vybrant DyeCycle Violet (Life Technologies) according to the manufacturer's instructions.[20] Flow data were obtained using an Attune Acoustic Focusing Cytometer (Life Technologies) and analyzed using WinList software (Verity Software House).

Immunofluorescence studies

The procedure has been described previously with minor modifications.[21] Briefly, $3\text{-}10\times10^3$ cells were plated on glass coverslips. On the following day after plating, the cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and blocked in 10% FBS. The coverslips were then incubated with the fluorescently-labeled antibodies described above. Nuclei were counterstained with DAPI. Fluorescent images were obtained using a BioZero Fluorescence Microscope (Keyence).

Quantitative real-time polymerase chain reaction (RT-PCR)

The procedure has been described previously.[9] The following primer/probe sets were obtained from Life Technologies: *Abcb1* (Mm00440736_m1), *Abcc1* (Mm00456156_m1), *Abcg2* (Mm00496364_m1). Amplification data were obtained using a StepOnePlus Real Time PCR System (Life Technologies). Each sample was tested in triplicate, and relative gene expression data were analyzed by the 2-ΔCT method.

Small interfering RNA in murine GSCs and spheroid size scoring

Murine GSCs were transfected with siRNA against *Abcg2* or control siRNA (Santa Cruz Biotechnology) using Lipofectamine RNAi MAX (Life Technologies) according to the manufacturer's instructions with minor modifications.

To evaluate the spheroid-forming capability of the murine GSCs, 5 representative areas were selected in culture. Then, the number of spheroid formations was counted in the selected areas along with the following grading criteria: grade 1, small spheroid (countable cell aggregation); grade 2, moderate spheroid (relatively large but countable); grade 3, large spheroid (uncountable cell aggregation). Scoring was performed by more than two researchers.

Data analysis

The procedure has been described previously.[22] Briefly, statistical significance of differences between two groups was determined by Student's t-test; one-way analysis of variance with Holm's post-hoc test was used for multiple group comparison. All data were analyzed by R Environment (R Project). $P<0.05$ was considered to be statistically significant.

Results

Glioma stem-like cells are inducible from murine spontaneous glioma

We have previously shown that spontaneous GBM is inducible in the mouse brain by intracerebroventricular transfection of *Nras*, small hairpin RNA against *p53*, and/or *EgfrvIII* using the *SB* transposon system.[7–10] The tumors induced in this system resemble human GBM based on appearance (Fig. 1A) and pathological characteristics (Fig. 1B).[7] To establish spheroid-forming stem cell-like cells from these tumors, we used culture conditions that have been estab-

lished for isolation of neural stem cells.[4] Within 24-48 hours of primary culture, murine brain tumors yielded a minority fraction of cells that formed neurosphere-like clusters (tumor spheroids; Fig. 1C) whereas the remaining populations of tumor cells were adherent (data not shown). We established 7 cell lines that tend to form tumor spheroids (Table 1).

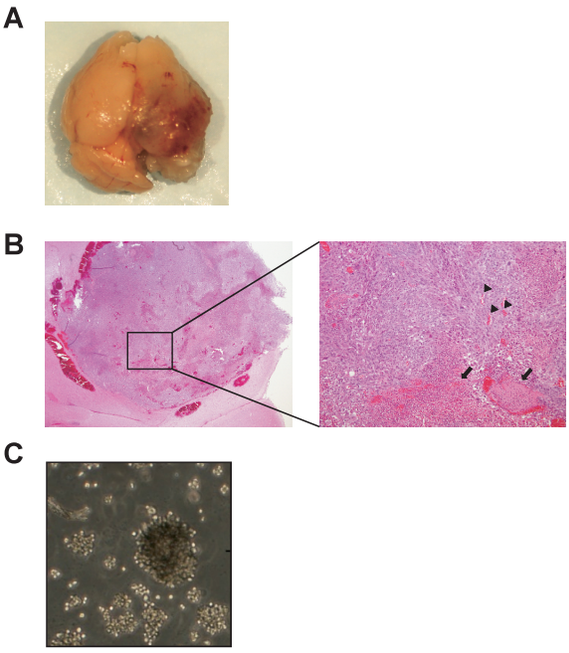

Fig. 1 lioma stem cell-like cells (GSCs) are inducible from murine spontaneous glioma
A, Balb/c wild-type mice receiving *Sleeping Beauty* (*SB*) transposon-compatible oncogene transfection in the right ventricles developed brain tumors that mimic human glioblastoma (GBM). B, a representative histopathological view of the *SB*-induced tumor tissues. Arrows: pseudopallisading necrosis. Arrow heads: vascular formation. Original magnification: 100x. C, neurosphere culture induced spheroid-forming cells from the *SB* glioma tissues. A representative view was obtained from GS02. Original magnification: 40x.

Table 1 Characteristics of established murine GSC lines

Line ID	Median of Survival	MFI of CD133	MFI of Nestin	IC_{50} of TMZ
GS01	38	92	533	N/A
GS02	56	103	432	683
GS03	41	83	245	773
GS04	58	75	723	724
GS05	43	125	263	612
GS06	42	132	326	N/A
GS07	62	80	420	N/A

MFI: mean fluorescent intensity. IC_{50}: half maximal inhibitory concentration. N/A: not applicable.

Murine glioma stem-like cells form glioma-like brain tumors *in vivo*

To confirm the carcinogenicity of the spheroid-forming cells *in vivo*, the established cells (GS02, GS03, and GS06, respectively) were inoculated into the striatum of syngeneic Balb/c wild-type mice at 1,000 cells/brain. All mice receiving intracerebral transplants of the spheroid-forming cells died due to brain tumor growth within 1-2 months after inoculation (Fig. 2A). Histopathological studies revealed that the developed tumors invaded extensively from the transplantation sites, which mimics human GBM (Fig. 2B). The tumors were infiltrating and featured nuclear atypia, necrotic areas, increased vascularization, and endothelial proliferation.

Murine glioma stem-like cells express their specific markers at high levels.

We next sought to evaluate whether the spheroid-forming cells would express GSC markers.[5] To this end, we performed immunofluorescent staining for GSCs (Fig. 3). All the spheroid-forming cells showed immunoreactivity for CD133 (stem cell marker; Fig. 3A) and nestin (neuron marker) at high levels (Fig. 3B). In contrast, the spheroid-forming cells did not express markers for differentiated neurons, astrocytes, or oligodendrocytes (data not shown). These findings suggest that the induced spheroid-forming cells share a very similar phenotype with human GSCs.[4] That is, spheroid-forming murine GBM lines that resemble human GSCs were successfully induced from SB transposon-mediated spontaneous GBM. Based on these data, we refer to the spheroid-forming GBM cells as murine GSCs from this point on.

Murine GSCs exhibit ABC transporters at high levels

Based on a line of previous finding that increased expression of ABC transporters is a characteristics of stem cell-like cells including GSCs,[14] we sought to evaluate the expression levels of relevant ABC transporters. To this end, we extracted mRNA from both the adherent cells and spheroid-forming GSCs to perform quantitative RT-PCR for several relevant ABC transporters (Fig. 4). As a result, we observed a significant increase in *Abcb1* (Fig. 4A) and *Abcg2* (Fig. 4C) in the spheroid-forming GSCs compared with the adherent cells; *Abcc1* showed no

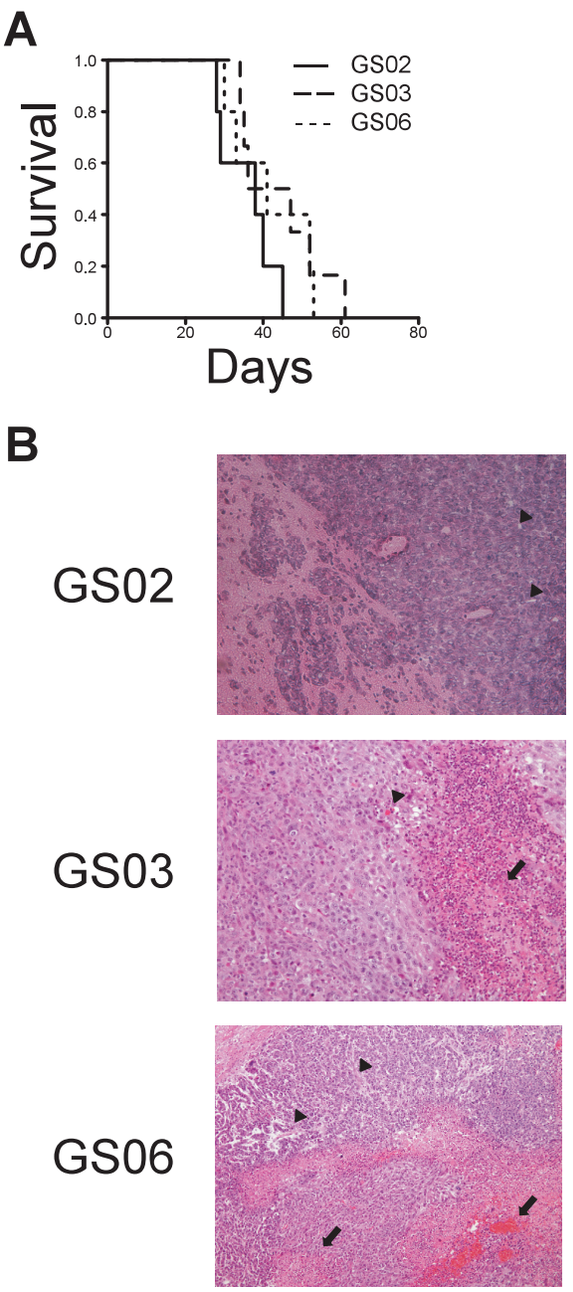

Fig. 2 Murine glioma stem-like cells form glioma-like brain tumors *in vivo*
The spheroid-forming murine GBM cells (1,000 cells/brain) formed a tumor mass in the brain of syngeneic mice on days 40-45 after inoculation. A, symptom-free survivals of the mice inoculated with the indicated cell lines in the brain. B, histopathological views of the tumor tissues induced by the indicated cell lines. Arrows : pseudopallisading necrosis. Arrow heads : vascular formation. Original magnification : 100x.

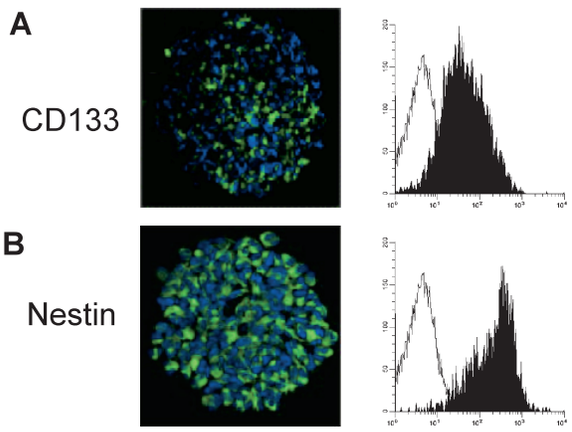

Fig. 3 Murine glioma stem-like cells express their specific markers at high levels
Representative data of CD133 (A) and nestin (B) expressions on the spheroid-forming cells. Left, fluorescent microscopic view. Nuclei were stained with DAPI. The representative data were obtained from GS02, and data from other cell lines are summarized in Table 1. Original magnification : 100x. Right, flow cytometry. Shaded histograms, spheroid-forming cells ; open histograms, corresponding adherent cells.

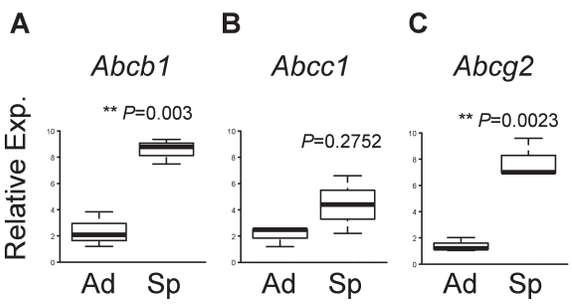

Fig. 4 Murine GSCs exhibit the adenosine triphosphate-binding cassette (ABC) transporters at high levels
Total RNA was extracted from the spheroid-forming murine GBM cells (GS01, GS03, and GS06) and their counterpart adherent cells to perform quantitative RT-PCR for the expression levels of *Abcb1* (A), *Abcc1* (B), and *Abcg2* (C). Values are relative to *Gapdh* expression levels. Lines within boxes denote means ; box upper and lower bounds indicate SD ; whiskers indicate minimum and maximum values. Data were obtained from multiple lines, and experiments were repeated at least three times. *P*-values are based on Student's t-test.

significant difference (Fig. 4B). These data suggest that the increased expressions of ABC transporters in GSCs contribute to their SP-skewed phenotype.

Murine GSCs exhibit the drug-efflux ability at high levels

Recent studies suggest that the increased expression of various ABC transporters leads to chemo-resistance of human GSCs to TMZ.[17,23] To confirm whether the murine GSCs exhibit a

similar phenotype due to the increased expression of ABC transporters, we first treated several murine GSC lines as well as the counterpart adherent cell lines with TMZ in a dose-escalation manner *in vitro* (Fig. 5A and Table 1). TMZ treatment effectively killed the GS03-derived adherent cells with 251 uM of half maximal inhibitory concentration in average (IC_{50}, top). In contrast, consistent with human GSCs, the GS03-derived, spheroid-forming GSCs were found resistant to TMZ with 773 uM of IC_{50} (bottom).

Then, given that drug resistance of GSCs requires the action of ABC transporters,[18] we therefore evaluated the drug-efflux ability of these cells by performing a dye exclusion-based SP assay on the murine GSCs (Fig. 5B) using a newly-developed Vybrant DyeCycle Violet dye.[20] Under normal culture conditions, we observed a small fraction of SP among adherent cells (top left) and a relatively larger fraction among spheroid-forming GSCs (bottom left). When the cells were treated with 250 uM TMZ, we observed a different pattern of dye exclusion. TMZ treatment increased SP cells in both the adherent cells (top right) and the spheroid-forming GSCs (bottom right). These data suggest that, although TMZ can kill chemo-sensitive normal populations, it selectively enriches the SP cells that efflux chemotherapeutic agents, and therefore is chemo-resistant.

ABCG2 inhibition in murine GSC enhances their chemo-sensitivity to TMZ.

Recent studies have demonstrated the superiority of ABCG2 in drug efflux compared with other ABC transporters in cancer stem cell-like cells.[24] Therefore, we directed our focus to the impact of ABCG2 on the kinetics/phenotype of the murine GSCs. To this end, we used small interfering RNA (siRNA) against *Abcg2* for the murine GSC (Fig. 6A and 6B). Under normal culture conditions or when transfected with control siRNA, the GSCs consistently expressed ABCG2 at high levels (Fig. 6A, left). In contrast, when the cells were transfected with the *Abcg2*-specific siRNA, the cells exhibited a reduction in ABCG2 expressions (Fig. 6A, right). RT-PCR demonstrated the consistent results (Fig. 6B). These data suggest that ABCG2 expressions in the murine GSCs are controllable using specific siRNA.

Next, to confirm whether ABCG2-impaired GSCs could gain sensitivity to TMZ, we further treated the siRNA-transfected cells with TMZ (Figs. 6C and 6D). The siRNA transfection

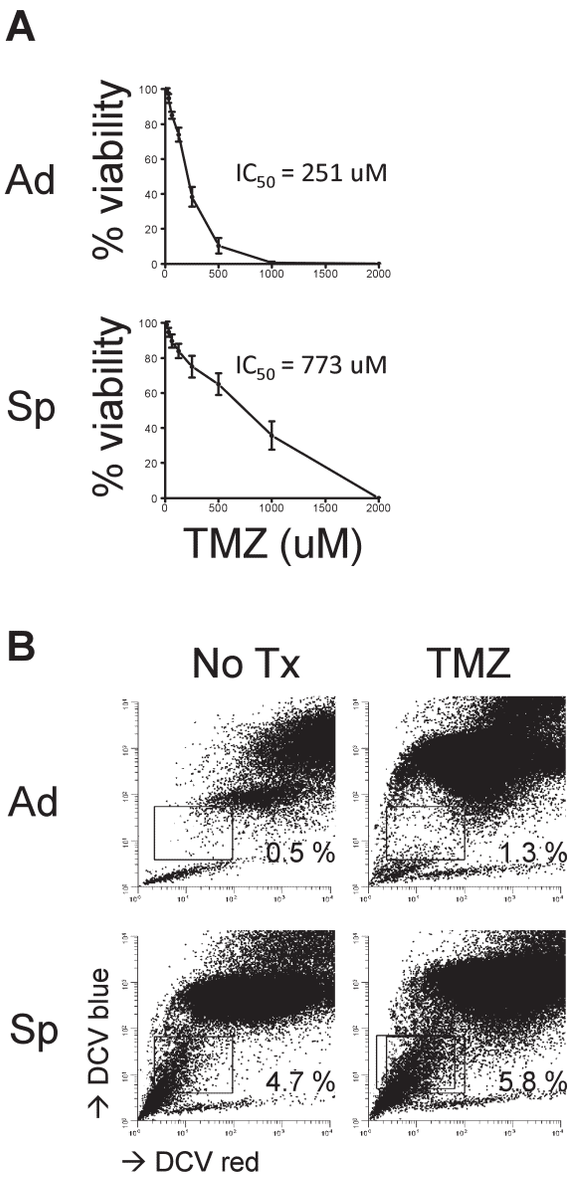

Fig. 5 Murine GSCs exhibit drug-efflux ability at high levels.
A, adherent cell (Ad) and spheroid-forming cells (Sp) induced from the SB tumors were treated with temozolomide (TMZ) for 48 hours at the indicated concentrations *in vitro*. Cell viability was evaluated by WST-1 assay. Representative graphs were obtained from the line GS03 and its counterpart. Data from other cell lines are summarized in Table 1. IC_{50} : half maximal inhibitory concentration. B, the adherent cell (Ad) and spheroid-forming (Sp) cells were treated with 250 uM TMZ *in vitro* for 48 hours. The cell were stained with Vybrant DyeCycle Violet and analyzed by flow cytometry. Representative data were obtained from the line GS03. Numbers indicate the frequency of side populations in culture.

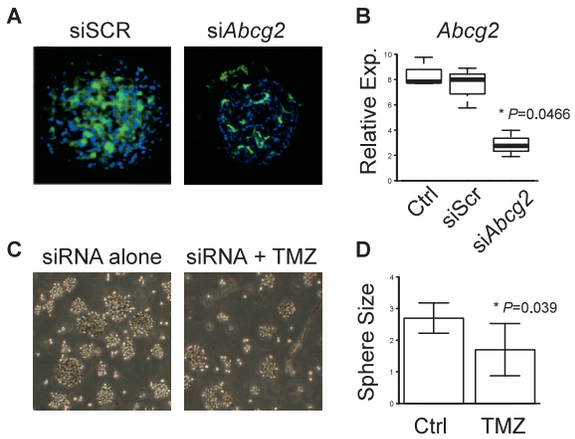

Fig. 6 ABCG2 inhibition in murine GSC enhances their chemo-sensitivity to TMZ
A, the spheroid-forming GSCs were transfected with siRNA against murine *Abcg2*, and the ABCG2 expressions were evaluated by fluorescent imaging. Original magnification : 100x. B, total RNA was extracted from the siRNA-transfected GSCs (GS01, GS03, and GS06) to perform quantitative RT-PCR for the expression levels of *Abcg2*. Data were obtained from multiple lines, and experiments were repeated at least three times. *P*-values are based on Holm's post-hoc test. C, the GSCs were transfected with *Abcg2*-specific siRNA and subsequently treated with 250 uM TMZ for 48 hours. Original magnification : 40x. D, enumerated score of the spheroids in culture. Data were obtained from multiple lines, and experiments were repeated at least three times.

alone did not change the morphology of tumor spheroids (Fig. 6C, left). However, when the siRNA-transfected cells were subsequently treated with TMZ, the cells exhibited a significant reduction in tumor spheroid formation (Fig. 3C, right). To perform statistical analysis for this phenomenon, we scored the degree of the tumor spheroids in culture (Fig. 6D). The cells treated with both specific siRNA and TMZ significantly reduced their spheroid-forming capability compared with the controls. These data suggest that TMZ-sensitive GSCs are unable to form tumor spheroids, probably by losing the cell-to-cell-contact and invasive ability.

Discussion

Discovery of a stem cell-like subpopulation inside GBM has highlighted the need for new therapeutic approaches that target both the tumor bulk and the GSC compartment, in order to achieve a stable and long-lasting remission. In this regard, this is the first report documenting the usefulness of GSCs induced from *SB* transposon-mediated spontaneous GBM (Figs. 1-3) as a novel tool to elucidate GSC biology. In addition, we demonstrated that the GSCs express various ABC transporters at high levels (Figs. 4 and 5) and that siRNA-mediated ABCG2 inhibition in the GSCs enhances their chemo-sensitivity to TMZ and reduces their spheroid-forming capability (Fig. 6). The data obtained in this study collectively suggest that targeting of drug transporters in GSCs to enhance their chemo-sensitivity is a promising strategy to achieve a long-lasting remission of GBM.

We established GSCs from *SB* transposon-mediated spontaneous murine GBM (Figs. 1-3 and Table 1). These murine tumors share many features with the human GBM including glial marker expression, pseudopalisading necrosis, and brain invasion.[7] Since this novel system well-mimics the complexity of human GBM, it is useful for understanding glioma biology and predicting therapeutic responses.[8−10] Moreover, this system possesses a striking technical advantage in that it allows us to use any type of mouse strain for the GBM induction. Therefore, although we used the *Abcg2*-specific siRNA for the GSCs in this study (Fig. 6), we can theoretically induce *Abcg2*-impaired GBM by using commercially-available *Abcg2*-deficient mice (Jackson Laboratory) as a host. Indeed, we are in the process of developing *Abcg2*-impaired GSCs by modifying this mouse system.

We observed the increased expressions of various ABC transporters in the GSCs (Fig. 4). In this regard, multidrug resistance is the principal mechanism by which many cancers develop resistance to chemotherapeutic agents and is a major factor in the failure of chemotherapy. Aberrant expression of ABC transporters is one of the main mechanisms responsible for multidrug resistance in cancer cells.[25] Overexpression of several ABC transporters (including ABCB1, ABCC1, and ABCG2) results in an ATP-driven efflux of drugs from cancer cells, thereby leading to decreased intracellular drug concentrations and lower toxicity. Consistent with this, we observed the increased expressions of several ABC transporters in the spheroid-forming GSCs compared with the counterpart adherent cells. Moreover, we observed that inhibition of ABCG2 in the GSCs enhances their chemo-sensitivity to TMZ (Fig. 6) ; this finding supports the relevance of ABC transporters in multidrug resis-

tance. Although there is no definitive evidence of TMZ as a substrate for the ABCG2 transporter thus far, some indirect evidence suggests that TMZ is also a substrate for this transporter.[26] We are in the process of performing an *in silico* structural analysis to elucidate the interaction between TMZ and ABCG2.

In summary, using a novel murine GSC system that were derived from *SB* transposon-mediated spontaneous GBM, we show that GSCs exhibit various types of ABC transporters at high levels to induce resistance to TMZ. In addition, siRNA-mediated ABCG2 inhibition enhances their chemo-sensitivity to TMZ, and the TMZ-sensitive GSCs reduce their spheroid-forming capability under the presence of TMZ. Taken together, these data suggest that targeting of multidrug transporters in GSCs to enhance their chemo-sensitivity is a promising strategy to achieve a long-lasting remission of GBM.

Abbreviations

GBM : glioblastoma multiforme, GSC : glioma stem cell-like cell, TMZ : temozolomide, *SB* : *Sleeping Beauty*, ABC : ATP-binding cassette, SP : side population, PBS : phosphate-buffered solution, DAPI : 4',6-diamidino-2-phenylindole, IC_{50} : half maximal inhibitory concentration

Competing Interests

The authors have no competing interests to disclose.

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References

1. Stupp R, *et al*. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 352 : 987–996
2. van den Bent MJ, Hegi ME, Stupp R. (2006). Recent developments in the use of chemotherapy in brain tumours. *Eur J Cancer*. 42 : 582–588
3. Galli R, *et al*. (2004). Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res*. 64 : 7011–7021
4. Singh SK, *et al*. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Res*. 63 : 5821–5828
5. Medema JP. (2013). Cancer stem cells : the challenges ahead. *Nat Cell Biol*. 15 : 338–344
6. Charles N, Holland EC. (2009). Brain tumor treatment increases the number of cancer stem-like cells. *Expert Rev Neurother*. 9 : 1447–1449
7. Wiesner SM, *et al*. (2009). De novo induction of genetically engineered brain tumors in mice using plasmid DNA. *Cancer Res*. 69 : 431–439
8. Fujita M, *et al*. (2010). Role of type 1 IFNs in antiglioma immunosurveillance-using mouse studies to guide examination of novel prognostic markers in humans. *Clin Cancer Res*. 16 : 3409–3419
9. Fujita M, *et al*. (2011). COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. *Cancer Res*. 71 : 2664–2674
10. Kohanbash G, *et al*. (2013). GM-CSF promotes the immunosuppressive activity of glioma-infiltrating myeloid cells through interleukin-4 receptor-alpha. *Cancer Res*. 73 : 6413–6423
11. Moitra K, Lou H, Dean M. (2011). Multidrug efflux pumps and cancer stem cells : insights into multidrug resistance and therapeutic development. *Clin Pharmacol Ther*. 89 : 491–502
12. Rees DC, Johnson E, Lewinson O. (2009). ABC transporters : the power to change. *Nat Rev Mol Cell Biol*. 10 : 218–227
13. Bunting KD. (2002). ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells*. 20 : 11–20
14. Dean M, Fojo T, Bates S. (2005). Tumour stem cells and drug resistance. *Nat Rev Cancer*. 5 : 275–284
15. Challen GA, Little MH. (2006). A side order of stem cells : the SP phenotype. *Stem Cells*. 24 : 3–12
16. Golebiewska A, Brons NH, Bjerkvig R, Niclou SP. (2011). Critical appraisal of the side population assay in stem cell and cancer stem cell research. *Cell Stem Cell*. 8 : 136–147
17. Bleau AM, *et al*. (2009). PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. *Cell Stem Cell*. 4 : 226–235
18. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med*. 183 : 1797–1806
19. Fujita M, *et al*. (2008). Inhibition of STAT3 promotes the efficacy of adoptive transfer therapy using type-1 CTLs by modulation of the immunological microenvironment in a murine intracranial glioma. *J Immunol*. 180 : 2089–2098
20. Boesch M, *et al*. (2012). DyeCycle Violet used for side population detection is a substrate of P-glycoprotein. *Cytometry A*. 81 : 517–522
21. Fujita M, *et al*. (2004). Aurora-B dysfunction of

multinucleated giant cells in glioma detected by site-specific phosphorylated antibodies. *J Neurosurg*. 101 : 1012–1017
22. Nishio N, *et al*. (2012). Zoledronate sensitizes neuroblastoma-derived tumor-initiating cells to cytolysis mediated by human gammadelta T cells. *J Immunother*. 35 : 598–606
23. Martin V, *et al*. (2013). Melatonin-induced methylation of the ABCG2/BCRP promoter as a novel mechanism to overcome multidrug resistance in brain tumour stem cells. *Br J Cancer*. 108 : 2005–2012
24. Tang DG. (2012). Understanding cancer stem cell heterogeneity and plasticity. *Cell Res*. 22 : 457–472
25. Gottesman MM, Fojo T, Bates SE. (2002). Multidrug resistance in cancer : role of ATP-dependent transporters. *Nat Rev Cancer*. 2 : 48–58
26. Persson AI, Weiss WA. (2009). The side story of stem-like glioma cells. *Cell Stem Cell*. 4 : 191–192