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Oct-3/4 modulates the drug-resistant phenotype of glioblastoma cells through expression of ATP binding cassette transporter G2

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ABSTRACT

Background: Drug resistance is a major obstacle for the efficacy of chemotherapeutic treatment of tumors. Oct-3/4, a self-renewal regulator in stem cells, is expressed in various kinds of solid tumors including glioblastoma. Although Oct-3/4 expression has been implicated in the malignancy and prognosis of glioblastomas, little is known of its involvement in drug resistances of glioblastoma.
Methods: The involvement of Oct-3/4 in drug resistance of glioblastoma cells was assessed by lactate dehydrogenase assay, efflux assay of an anticancer drug, poly ADP-ribose polymerase cleavage, and in vivo xenograft experiments. Involvement of a drug efflux pump ATP binding cassette transporter G2 in Oct-3/4-induced drug resistance was evaluated by quantitative PCR analysis and knockdown by shRNA.
Results: Oct-3/4 decreased the susceptibility to chemotherapeutic drugs by enhancing excretion of drugs through a drug efflux pump gene, ATP binding cassette transporter G2. Moreover, the expression of Oct-3/4 was well correlated to ATP binding cassette transporter G2 expression in clinical GB tissues.
Conclusion: Oct-3/4 elevated the ATP binding cassette transporter G2 expression, leading to acquisition of a drug-resistant phenotype by glioblastoma cells.
General significance: If the drug-resistance of glioblastoma cells could be suppressed, it should be a highly ameliorative treatment for glioblastoma patients. Therefore, signaling pathways from Oct-3/4 to ATP binding cassette transporter G2 should be intensively elucidated to develop new therapeutic interventions for better efficacy of anti-cancer drugs.

1. Introduction

Glioblastoma (GB) is the most aggressive form of primary malignant brain tumor, and one of the most lethal neoplasms [1–3]. The World Health Organization defines grade IV glioma based on its microscopic appearance, and these high-grade gliomas exhibit a high mitotic index, infiltrative invasion into surrounding normal brain parenchyma, hyperproliferation of microvasculature and necrotic foci [4]. Despite significant advances in surgical and adjuvant therapies, the median survival period of GB patients is only 14.6 months, and its improvement in the last 20 years has been almost negligible [5]. In addition to the infiltrative growth of GB cells, their resistance to chemotherapy has been thought to hamper the development of efficacious treatments. O6-methylguanine-DNA methyltransferase repairs DNA damage caused by alkylating agents, a typical type of anti-cancer drug, which has been attributed to the chemotherapeutic resistance of GBs [6]. ATP-binding cassette (ABC) transporters are assumed to be another critical factor in such resistance by pumping out anticancer drugs from the cytoplasm [7,8]. The mechanisms underlying the resistance of GBs against chemotherapeutic drugs should be clearly elucidated to re-sensitize GB cells to chemotherapy.

Oct-3/4 is a member of the POU family of transcription factors, which is well known for its critical role in maintaining the pluripotency of stem cells [9]. Furthermore, a certain amount of Oct-3/4 is required to sustain

Abbreviations: GB, glioblastoma; ABC, ATP binding cassette transporter; PARP, poly ADP-ribose polymerase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EGFP, enhanced green fluorescence protein; Doxy, doxycycline; qPCR, quantitative PCR; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; HE, hematoxylin and eosin; MDR, multidrug resistance; HIF, hypoxia-inducible factor; PI3K, phosphatidylinositol 3′-kinase; AID, activation-induced cytidine deaminase

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the self-renewal of stem cells, and up-regulation or down-regulation of Oct-3/4 induces divergent cell fates. On the other hand, recent evidence has proposed a novel function of Oct-3/4 in tumorigenesis. Deregulated expression of Oct-3/4 has been detected in various human tumors and cancer cell lines, including brain tumors, breast cancer, oral squamous cell carcinoma, esophageal squamous cell carcinoma, and bladder cancer, and its expression is negatively correlated with the survival of patients [10–15]. It has also been reported that ectopic expression of Oct-3/4 causes dysplastic growth of epithelial tissue [16]. Moreover, enhanced expression of Oct-3/4 has been reported in cancer stem cells, a small population of cancer cells in tumors with stem-like properties [17,18]. Cancer stem cells possess a stronger tumorigenesis activity and resistance to radiotherapy and chemotherapy than those of non-cancer stem cells [19,20]. Therefore, these studies suggest that aberrant expression of Oct-3/4 contributes to the regulation of cancer stem cell genesis and neoplastic processes. Oct-3/4 expression levels have been positively correlated with glioma malignancy levels [15]. However, any correlation of chemotherapeutic drug resistance and the expression of Oct-3/4 in GB cells remain unclear.

In the present study, we investigated the relationship between Oct-3/4 and chemotherapeutic drug resistance and found that Oct-3/4 modulated the drug resistance of GB cells through expression of ABCG2.

2. Materials and methods

2.1. Cell culture

Human GB cell lines, U251, U87, and T98G, were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), 4.5 g/l glucose, and penicillin/streptomycin/amphotericin B mixture (Sigma, St Louis, MO) (DMEM-10% FBS).

2.2. Construction of expression plasmids and transfection

To generate an expression plasmid encoding human Oct-3/4, full-length cDNA of Oct-3/4 was amplified by PCR using cDNA derived from human GB tissue, sequenced, and then inserted into a pEGFP-C1 vector (Clontech, Palo Alto, WI), which was in frame with the 3′ end of enhanced green fluorescence protein (EGFP) coding sequences as previously described [21]. The amplified Oct-3/4 cDNA was also inserted into a pcDNA3 vector to express Oct-3/4 without EGFP for drug efflux assays.

The plasmids were transfected into U251 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) to establish cells that stably expressed EGFP, EGFP-Oct-3/4, or Oct-3/4. Cells were selected by culture in medium containing G418 (500 μg/ml; Invitrogen) as described previously [21].

2.3. Establishment of a Tet-off cell line

The EGFP-Oct-3/4 DNA fragment was excised from pEGFP-Oct-3/4 and inserted into a pTRE2hyg vector (Clontech) to control expression of EGFP-Oct-3/4 by the Tet-off system. To establish Tet-off cells, the Tet-off regulator plasmid, pTet-Off (Clontech), was transfected into U251 cells and then stable transformants expressing the Tet-sensitive transactivator, rTA, were selected by G418 as described above. These cells were further transfected with pTRE2hyg/EGFP-Oct-3/4, and the transfected cells were subjected to selection with G418 and hygromycin B (200 μg/ml; Invitrogen). To shut off EGFP-Oct-3/4 expression, the

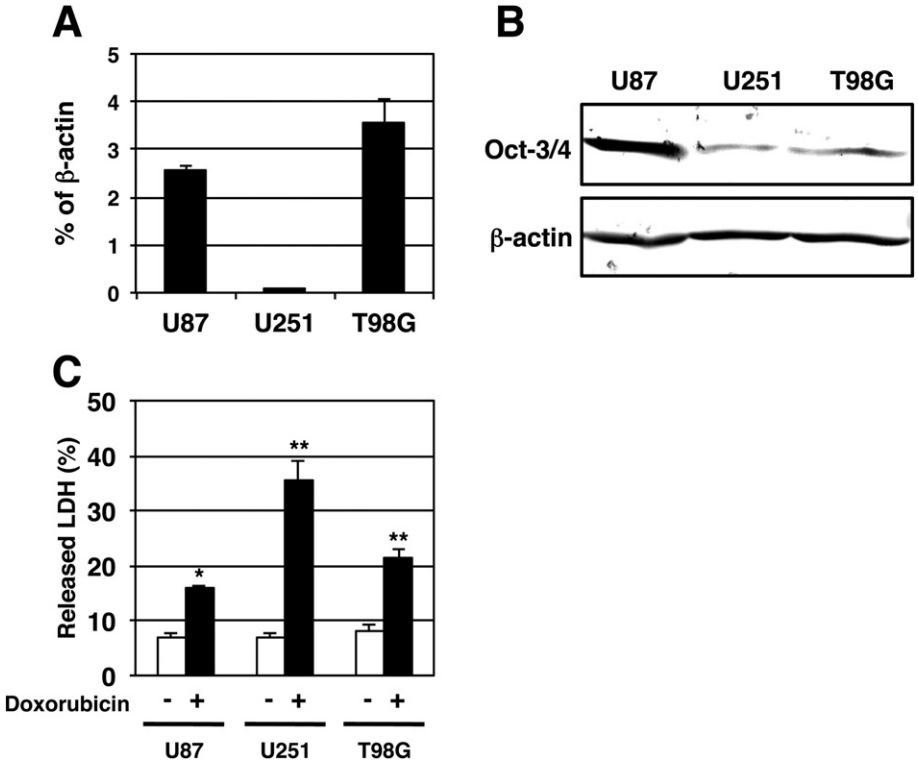

Fig. 1. (A) Expression of Oct-3/4 in human glioblastoma cell lines. qPCR analysis of Oct-3/4 mRNA expression in human glioblastoma cell lines. Results were normalized to β-actin mRNA expression levels. Values represent the mean ± SEM (n = 3). (B) Western blot analysis of Oct-3/4 protein expression (n = 3). Representative data are shown, and β-actin was used as the loading control. (C) Sensitivity of human glioma cell lines to doxorubicin. Each glioblastoma cell line was treated with doxorubicin (10 μM) for 24 h, and then cell damage was assessed by LDH assays. Results indicate the percentage (%) of LDH activity in the conditioned medium to the total LDH activity. Values represent the mean ± SEM (n = 5). *$p < 0.05$, **$p < 0.001$ vs untreated.

tetracycline analog doxycycline (Doxy) (1 μg/ml; Sigma) was added to the culture medium of U251/Tet-EGFP-Oct-3/4 cells.

2.4. RNA isolation and quantitative PCR analysis

Total RNA was collected using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA was prepared using ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO, Osaka, Japan). For quantitative PCR (qPCR) analysis, cDNA was diluted at 1:3, and 1 μl was used for triplicate qPCRs in an MJ mini instrument (BioRad, Hercules, CA) using Fast Start Universal SYBR Green (Roche Diagnostic Japan, Tokyo, Japan). All gene-specific mRNA expression values were normalized to β-actin expression levels. Primer sequences used in this study are as follows: Oct-3/4; forward 5′-AGCGAACCAGTATCGAGAAC-3′ and reverse 5′-GCCTCAAAATCCTCTCGTTG-3′, ABCG2; forward 5′-ACCCTTATGATGGTGGCTTA-3′ and reverse 5′-ATGCAATGGTTGTGAGAT TG-3′, ABCB1; forward 5′-TTCCTTCAGGGTTTCACATT-3′ and reverse 5′-AATTACAGCAAGCCTGGAAC-3′, ABCC1; forward 5′-TGTTGGTCTCTGTG TTCCTG-3′ and reverse 5′-CGTTTTCGCCTAAAAGAAAG-3′, β-actin; forward 5′-AGAAGAGCTATGAGCTGCCTGACG-3′ and reverse 5′-TACTTG CGCTCAGGAGGAGCAATG-3′.

2.5. Western blot analysis

Cells were lysed with Laemmli's sample solution. The lysates were electrophoresed, transferred onto nitrocellulose membranes, and immunoblotted with antibodies against β-actin (mouse monoclonal, clone AC-15; Sigma), Oct-3/4 (rabbit polyclonal; Rockland, Gilbertsville, PA), GFP (rabbit polyclonal; Delta Biolabs, Muraoka Drive Gilroy, CA), or poly ADP-ribose polymerase (PARP) (rabbit monoclonal, Cell Signaling Technology, Danvers, MA). After incubation with an alkaline phosphatase-conjugated secondary antibody (Promega KK, Tokyo, Japan), immunoreactions were developed using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.6. Lactate dehydrogenase assay

To assess chemotherapeutic drug-induced cytotoxicity, cells were seeded at 2×10^3 cells/well in a 96-well plate, allowed to attach overnight, and then exposed to chemotherapeutic drugs at various concentrations in DMEM-10% FBS for 24 h. Carboplatin and VP16 were purchased from Sigma, and doxorubicin was purchased from Kyowa Kirin (Tokyo, Japan). After chemotherapeutic drug treatments, relative cell viability was determined with a Cytotoxicity Detection Kit (Roche Diagnostic Japan) according to the manufacturer's instructions by analyzing the activity of lactate dehydrogenase (LDH) released from damaged cells into the culture medium as described previously [22]. LDH activity was analyzed by measuring absorbance of the water soluble-formazan product at 490 nm (reference at 690 nm) with a scanning multiwell spectrophotometer Flex Station 3 (Molecular Devices Japan, Tokyo, Japan). Data were expressed as the percentage of released LDH activity to the total LDH activity that was determined by lysing the cells with a solution of 1% Triton X-100.

2.7. In vivo xenograft experiments and immunohistochemistry

U251/Tet-EGFP-Oct-3/4 cells (2×10^6 cells in 200 μl 1:1 phosphate-buffered saline (PBS)/Matrigel (Becton Dickinson, Bedford, MA)) were subcutaneously implanted into the right thighs of 6-week-old male BALB/c nude mice (Japan Charles River, Yokohama, Japan). Tumor size

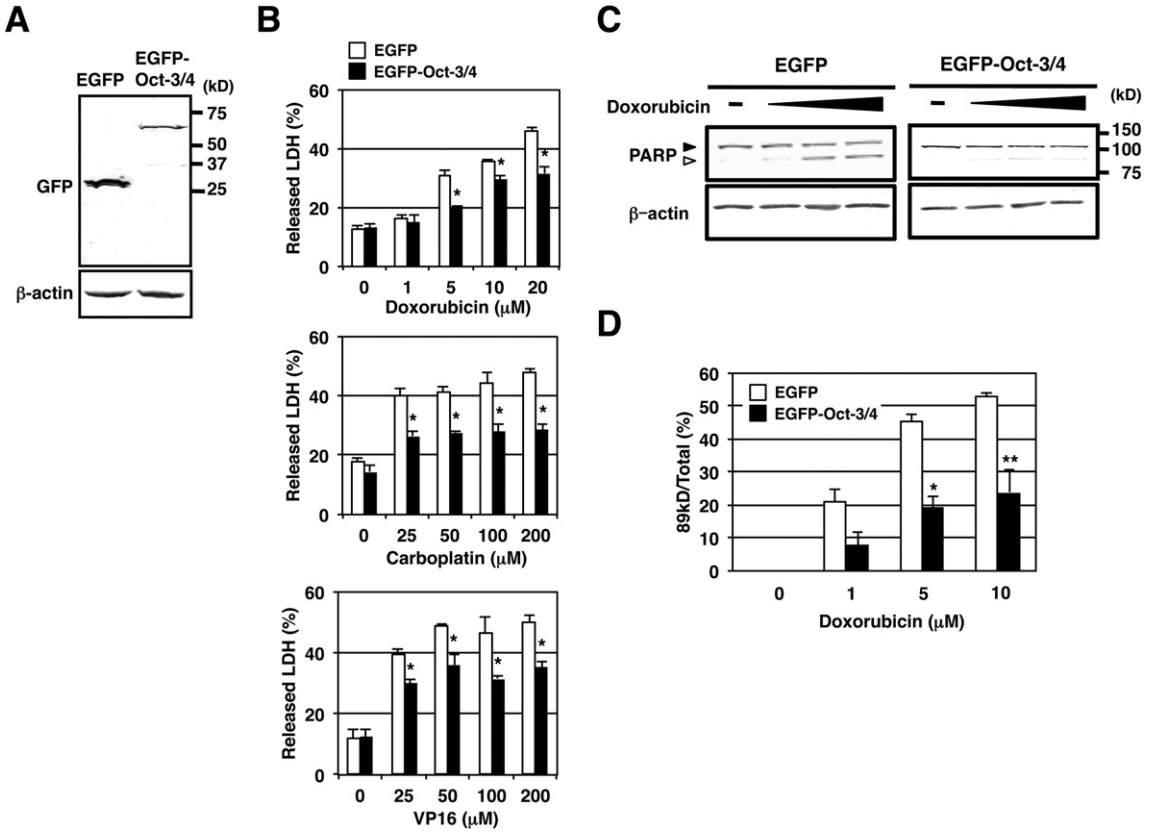

Fig. 2. Enhanced drug resistance of Oct-3/4 expressing cells to anticancer drugs. (A) U251 cells with transfected pEGFP-C1 or pEGFP-Oct-3/4 were confirmed the gene expression by western blot analysis using an anti-GFP antibody. (B) Cells were treated with various concentrations of doxorubicin, carboplatin, and VP16 for 24 h, and then LDH activities in conditioned media were determined. Values represent the mean ± SEM (n = 5). *p < 0.001 vs U251/EGFP cells. (C) Cells were exposed to increasing concentrations of doxorubicin (0, 1, 5, and 10 μM) for 24 h. PARP cleavage, indicative of apoptotic cell death, was detected by western blot analysis. (D) Relative protein levels of cleaved PARP in doxorubicin-treated cells. Results indicate the percentage (%) of cleaved PARP to total PARP. Values represent the mean ± SEM (n = 3). *p < 0.01, **p < 0.001 vs U251/EGFP cells.

was measured before and after doxorubicin treatment, and tumor volume (V) was evaluated by the equation $V = L \times W^2 \times 0.5$, where L is the length and W is the width of the tumor. Tumors were allowed to develop in all mice (1097.84 ± 86.68 mm^3, $n = 20$) for 6 weeks after implantation, and randomly divided into four groups. Either saline or doxorubicin (0.2 mg/kg/day) was intraperitoneally administered to the mice for 2 weeks. To suppress EGFP-Oct-3/4 expression, drinking water containing 0.25 mg/ml doxycycline (1–1.5 mg/day) was given to the mice during the doxorubicin treatment. The effect of the doxycycline treatment was evaluated by the disappearance of EGFP fluorescence under excitation light for GFP (LEDGFP-3W, Optocode, Tokyo, Japan) (Supplemental Fig. 1).

Subcutaneous tumors were dissected, fixed with 4% paraformaldehyde in PBS, paraffin embedded, and sliced into 5-μm-thick sections. Sections were then subjected to hematoxylin and eosin (HE) staining. For immunohistochemistry, sections were incubated with an anti-GFP antibody (rabbit polyclonal; Delta Biolabs) and anti-Ki67 antibody (mouse monoclonal, clone MIB-1; Dako, Tokyo, Japan). Immunoreaction was visualized with diaminobenzidine (DAB) as chromogen using ImmPACT DAB EqV peroxidase substrate kit (Vector Laboratories Inc., Burlington, CA) according to the manufacturer's instructions. Tumor sections were counterstained with hematoxylin staining. All animal experiments were performed in accordance with the guidelines of Ehime University Committee for Ethics of Animal Experimentation.

2.8. Drug efflux assay

Cells were seeded at 5×10^5 in 6-cm culture dishes, and incubated with doxorubicin (10 μM) for 3 h at 37 °C (uptake period). Labeled cells were washed twice with PBS and then incubated in fresh DMEM-10% FBS for an additional 3 h at 37 °C (efflux period) with or without verapamil (50 μM; Sigma). Autofluorescence of doxorubicin at 488 nm, which is indicative of the cellular doxorubicin content, was assessed on a FACS Calibur flow cytometer and analyzed by CellQuest software (Becton Dickinson, Tokyo, Japan).

2.9. Human GB tissues

Human GB samples for isolation of total RNA were resected tumor specimens obtained from GB patients at Ehime University Hospital. The resected tissues were reevaluated as GB by the pathological diagnosis. The study protocol was approved by the local ethics committee at Ehime University Hospital, and informed consent was obtained from all patients.

2.10. Statistical analysis

Values were expressed as the mean ± standard error of the mean (SEM). Data were subjected to two-tailed Student's t-tests (unpaired) or analysis of variance with Tukey's post-hoc test. Significance was set at $p < 0.05$. Correlations between Oct-3/4 and ABCG2 mRNA expression in GB patients were determined with bivariate analysis using Pearson's correlation coefficient.

3. Results

3.1. Oct-3/4 enhances chemotherapeutic drug resistance in GB cells

qPCR and immunoblot analyses showed various levels of Oct-3/4 expression in the three human GB cell lines (Figs. 1A and B). We first

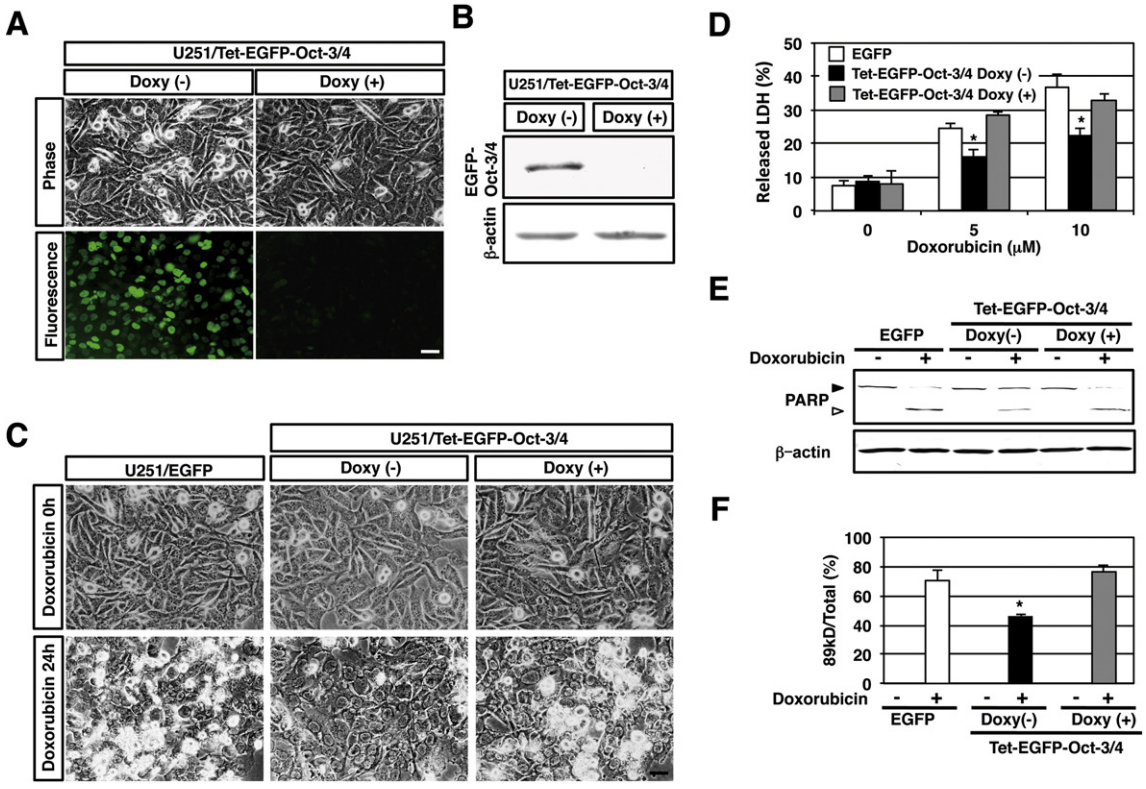

Fig. 3. Suppression of Oct-3/4 expression leads to increased sensitivity to doxorubicin in vitro. Cells were transduced with tet-EGFP-Oct-3/4 system, and then cultured with or without doxycycline (1 μg/ml) for 48 h. EGFP-Oct-3/4 expression was confirmed by EGFP fluorescence (A) and western blot analysis (B). (C) Prior to doxorubicin treatment, U251/tet-EGFP-Oct-3/4 cells were cultured in the presence of doxycycline for 48 h to suppress EGFP-Oct-3/4 expression. The cells were then re-seeded at 2×10^4 cells/well in 4-well plates, followed by treatment with doxorubicin (10 μM) for 24 h. Phase contrast micrographs of the cells are shown. Scale bar = 50 μm. (D) Cells were treated with the indicated concentrations of doxorubicin for 24 h and then released LDH in conditioned media was determined. Values represent the mean ± SEM ($n = 5$). *$p < 0.001$ vs U251/EGFP and U251/Tet-EGFP-Oct-3/4 Doxy (+) cells. (E) Cells were incubated with doxorubicin (10 μM) for 24 h. PARP cleavage was then detected by western blot analysis. (F) Relative protein levels of cleaved PARP in doxorubicin-treated cells. Data were expressed as the mean ± SEM ($n = 3$). *$p < 0.05$ vs U251/EGFP cells.

assessed the effect of Oct-3/4 levels on resistance to the chemotherapeutic drug doxorubicin, which is classified as an anthracycline antitumor antibiotic, in GB cell lines (Fig. 1C). All cell lines were exposed to doxorubicin for 24 h, and then cell damage was evaluated by LDH assays. U251 cells, which expressed Oct-3/4 at a lower level than that in the other two cell lines, displayed higher sensitivity to treatment with doxorubicin as revealed by higher LDH release. This result suggested the involvement of Oct-3/4 expression in the chemotherapeutic drug resistance of GB cells.

To examine whether enhanced expression of Oct-3/4 was sufficient to confer drug resistance to GB cells, we established U251 cells with stable expression of EGFP or EGFP-Oct-3/4 (Fig. 2A). The established cells were incubated with various concentrations of the doxorubicin, carboplatin (an alkylating agent), and VP16 (a topoisomerase inhibitor) for 24 h, and then cell viability was determined by LDH assays (Fig. 2B). U251 cells with Oct-3/4 overexpression displayed stronger resistance to doxorubicin than that of cells expressing EGFP alone. The forced expression of Oct-3/4 also decreased susceptibility to carboplatin and VP16 treatment. To further confirm the decreased susceptibility to chemotherapeutic drugs in U251/EGFP-Oct-3/4 cells, we analyzed cleavage of poly ADP-ribose polymerase (PARP), which is indicative of apoptotic cell death. PARP is one of the primary targets of activated caspase-3, and the 116 kD uncleaved PARP protein can be proteolytically cleaved to produce an 89 kD protein by caspase-3 [23]. As shown in Fig. 2C, both cell lines showed PARP cleavage induced by doxorubicin treatment, but the level of PARP cleavage was decreased in Oct-3/4-overexpressing cells (Fig. 2D). Next, we introduced the Tet-off system into U251 (U251/Tet-EGFP-Oct-3/4) cells to shut off Oct-3/4 expression by addition of a tetracycline derivative, doxycycline (Doxy). The expression of EGFP-Oct-3/4 had almost ceased in the presence of Doxy for 24 h (Figs. 3A and B). To assess the effect of Oct-3/4 expression on doxorubicin cytotoxicity, we first examined changes in cell

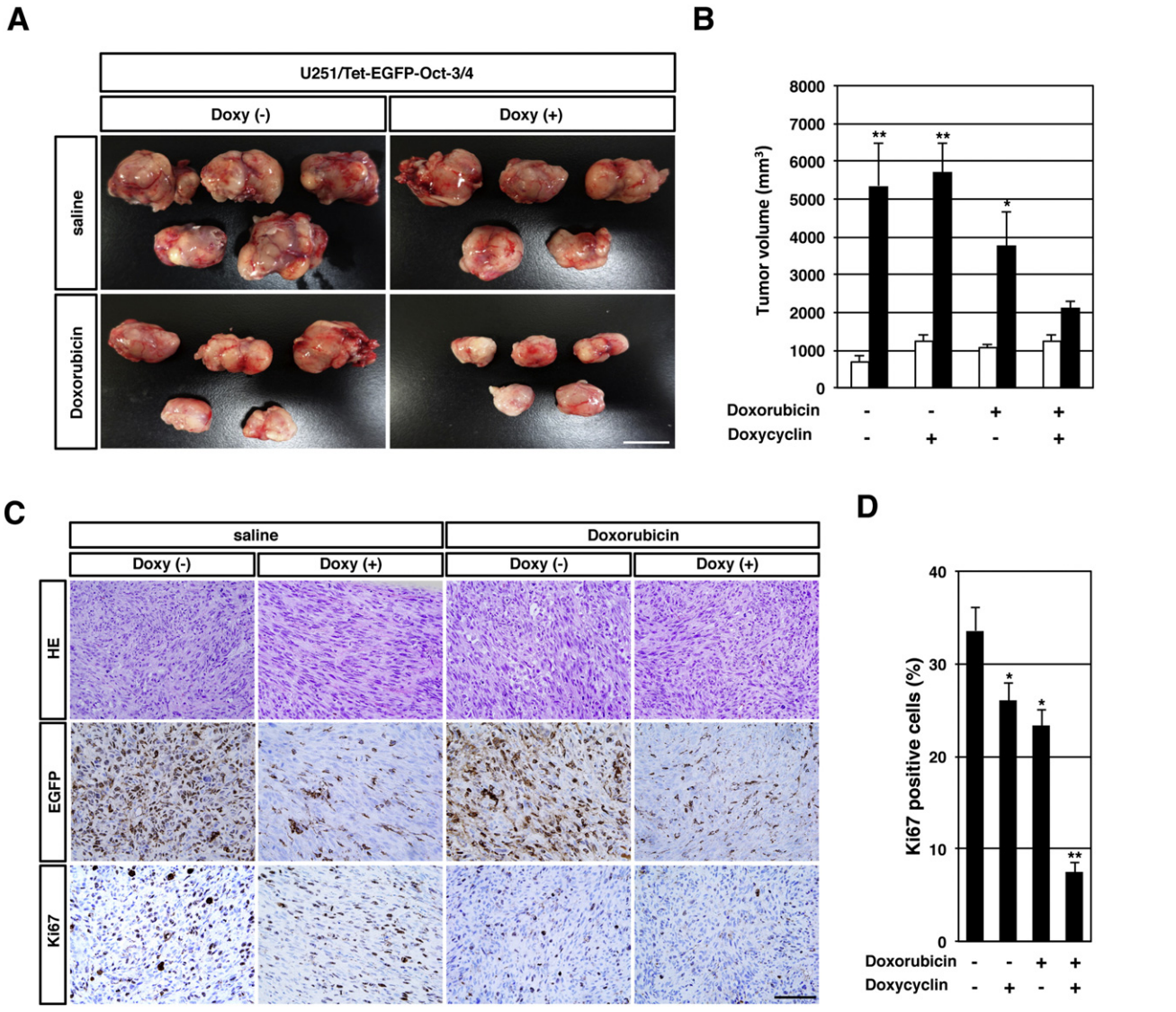

Fig. 4. Suppressed Oct-3/4 expression increased the sensitivity to doxorubicin in vivo. (A) U251/Tet-EGFP-Oct-3/4 (2×10^6) cells were subcutaneously implanted into the right thighs of male nude mice. Six weeks after implantation, saline or doxorubicin (0.2 mg/kg/day) was intraperitoneally given for 2 weeks with or without doxycycline. Gross appearance of dissected tumors from each group of mice was shown ($n = 5$). Scale bar = 2 cm. (B) Volumes of the tumors at the beginning (white bar) and the end (black bar) of doxorubicin/doxycycline treatment are shown. Values are represented as mean ± SEM ($n = 5$). $^*p < 0.05$ and $^{**}p < 0.001$ compared to the tumors at the beginning of each treatment. (C) Tumors dissected after doxorubicin treatment were sectioned and stained with HE and antibodies against EGFP or Ki67. Scale bar = 100 μm. (D) Ki67 positive cells in four optic fields observed with a ×40 objective lens were counted. Values indicate the percentage of Ki67 positive cells against total cells, and represent the mean ± SEM ($n = 3$). $^*p < 0.05$ and $^{**}p < 0.01$ compared to doxorubicin (−)/doxycycline (−) group.

morphology. Similar to U251/EGFP cells, Doxy (+) cells exhibited damaged morphology characterized by rounding up, hypertrophic nuclei, and membrane blebbing, suggesting increased vulnerability to doxorubicin compared with that of Doxy (−) cells (Fig. 3C). Consistent with these morphological changes, the suppressed LDH release and PARP-cleavage in Doxy (−) cells were canceled by Doxy treatment, indicating that Oct-3/4 is critically involved in resistance against chemotherapeutic drugs (Figs. 3D, E and F). To further evaluate the involvement of Oct-3/4 in drug resistance, we treated mice bearing xenografts of U251/Tet-EGFP-Oct-3/4 cells with doxorubicin. Six weeks after implantation, all mice developed subcutaneous tumors and they were administered with saline or doxorubicin (0.2 mg/kg/day) intraperitoneally for 2 weeks. EGFP-Oct-3/4 expression was abolished by drinking water containing Doxy (Supplemental Fig. 1 and Fig. 4C). Doxy-treated xenografts showed that the enhanced sensitivity to doxorubicin, and tumor growth was suppressed (Figs. 4A and B). In the doxorubicin (+)/doxycycline (+) group, Ki67 positive tumor cells were much decreased without affecting the microvessel densities (Figs. 4C, D and Supplemental Fig. 2).

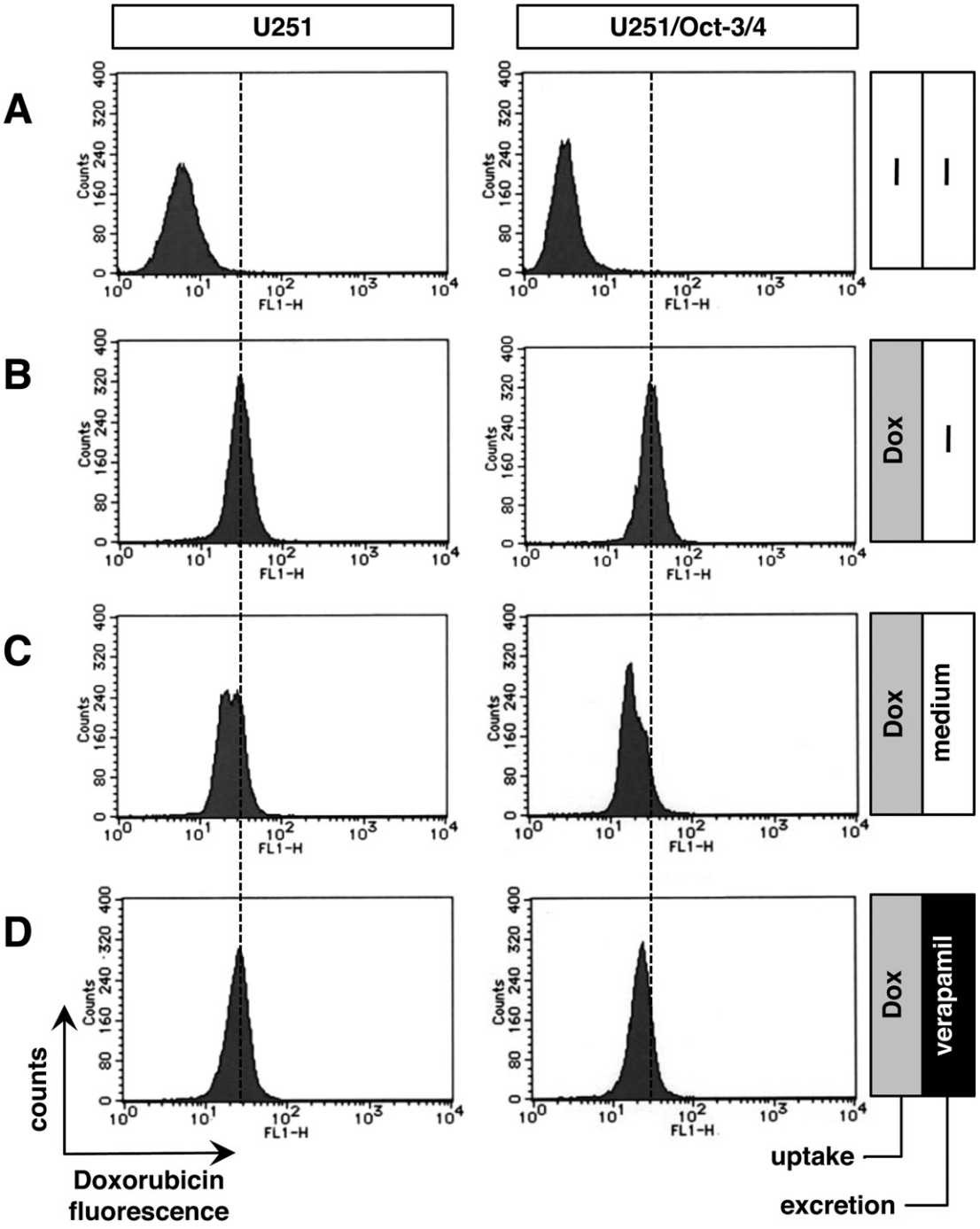

Fig. 5. Oct-3/4 contributes to doxorubicin efflux. The levels of internalized doxorubicin based on autofluorescence of the drug were evaluated by flow cytometry. From top to bottom, histograms present unlabeled cells (A), cells treated with doxorubicin (B), cells treated with doxorubicin after an efflux period of 3 h in fresh growth medium (C), and cells treated with doxorubicin after an efflux period of 3 h in fresh growth medium containing verapamil (50 μM) (D). Dotted lines represent the peak of doxorubicin fluorescence in cells. Representative data are shown (n = 3).

3.2. Oct-3/4 enhances drug excretion ability

The capacity for drug excretion was evaluated with flow cytometry by detecting the autofluorescence of intracellular doxorubicin. In this analysis, drug excretion was reflected by a left shift in the histogram of intracellular doxorubicin autofluorescence in comparison with cells before the excretion period (Figs. 5B and C). Although U251 cells took up doxorubicin, Oct-3/4-overexpressing cells highly excreted doxorubicin during the excretion period (Fig. 5C). Thus, Oct-3/4 may enhance excretion of chemotherapeutic drugs, which contribute to drug resistance. Moreover, the doxorubicin excretion was abolished by verapamil, a broad spectrum inhibitor of ABC transporters (Fig. 5D), suggesting that ABC transporters are involved in the excretion of the drug.

3.3. Oct-3/4 enhances mRNA expression of ABC transporters

The ABC transporter family is known to contribute to the acquisition of multidrug resistance (MDR) because of their drug excretion activity. Therefore, the mRNA expression of ABC transporters in U251/EGFP-Oct-3/4 cells was investigated by qPCR. Fig. 6A shows that the mRNA expression of ABCG2 was significantly induced in U251/EGFP-Oct-3/4 cells compared with that in control cells. However, the mRNA expression of ABCB1 and ABCC1 was similar to control and Oct-3/4-overexpressing cells. Enhanced expression levels of ABCG2 mRNA (Fig. 6Ba) and protein (Fig. 6Bb) in U251/Tet-EGFP-Oct-3/4 cells were reduced to the levels in control cells by Doxy treatment. Furthermore, expression levels of ABCG2 mRNA (Fig. 6Ca) and protein (Fig. 6Cb) in GB cell lines were correlated with Oct-3/4 expression (Figs. 1A and 6C). To further confirm whether enhanced drug resistance in U251/EGFP-Oct-3/4 cells was mainly dependent on ABCG2, we investigated chemotherapeutic drug resistance in ABCG2 knockdown U251/EGFP-Oct-3/4 cells (Fig. 6D). As shown in Fig. 6E, knockdown of ABCG2 in U251/EGFP-Oct-3/4 cells resulted in increased LDH release in response to doxorubicin treatment, suggesting the involvement of ABCG2 in the enhanced chemotherapeutic drug resistance of U251/EGFP-Oct-3/4 cells. Furthermore, mRNA expression of Oct-3/4 and ABCG2 was measured in GB tissues from 20 patients by qPCR. As shown in Fig. 7, there was a positive correlation between Oct-3/4 and ABCG2 expressions in GB patients.

4. Discussion

In this study, we established U251 cells overexpressing Oct-3/4 to investigate the functional involvement of Oct-3/4 in drug resistance. We found that up-regulated expression of ABCG2 in Oct-3/4-expressing cells resulted in higher resistance to chemotherapeutic drugs than that of the control cells. Furthermore, the clinical data showed that Oct-3/4 mRNA expression in human GB tissues was positively correlated with ABCG2 mRNA expression. These results indicate that up-regulated expression of Oct-3/4 may contribute to the development of the MDR phenotype through expression of ABCG2 in GB patients.

Several mechanisms have been proposed for the promoting effects of Oct-3/4 on ABCG2 expression. Krishnamurthy et al. reported that hypoxia-inducible factor 1 (HIF1) directly binds to the hypoxia response element in the ABCG2 promoter and activates transcriptional activity of the ABCG2 gene [24]. Consistent with this study, we found that a HIF1α inhibitor suppressed Oct-3/4-induced ABCG2 expression in U251/EGFP-

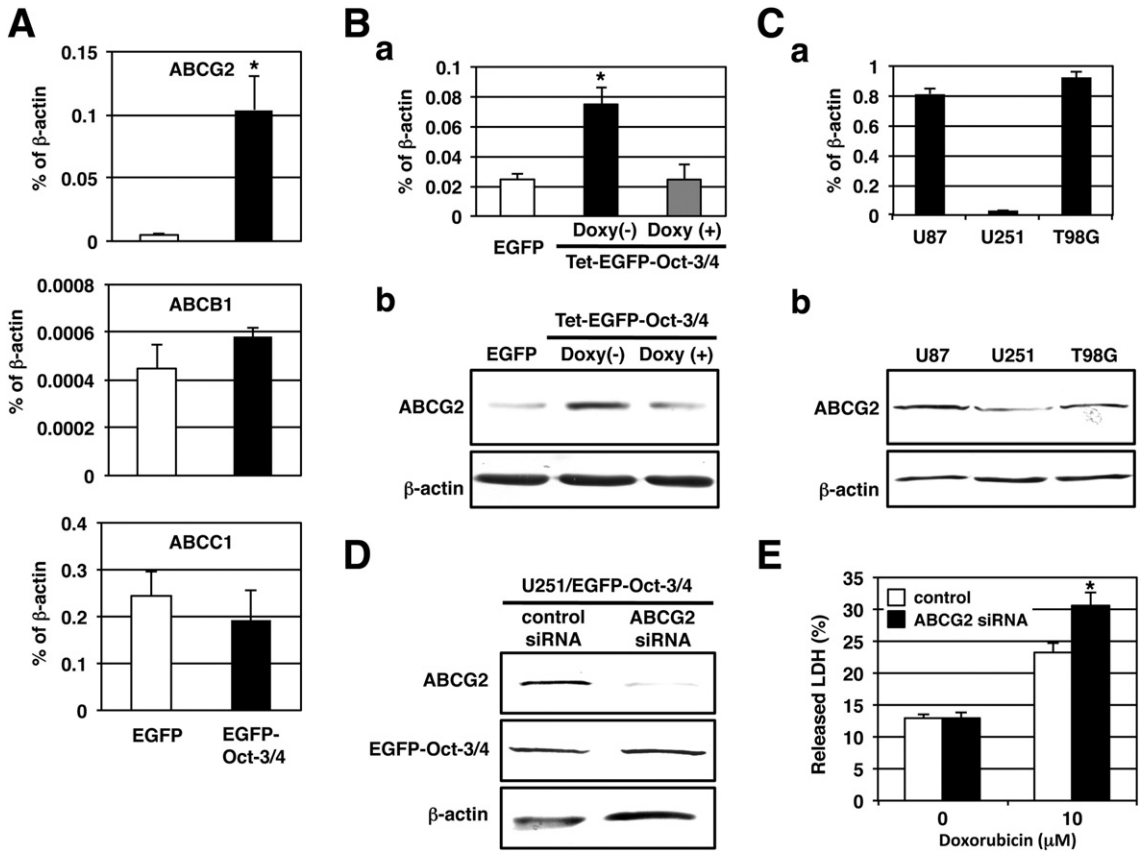

Fig. 6. Oct-3/4-induced up-regulation of ABCG2 mRNA and protein. (A) qPCR analysis of ABC transporter mRNA expression in Oct-3/4-expressing cells. mRNA expression levels were normalized to β-actin mRNA levels. Values represent the mean ± SEM (n = 5). *p < 0.05 vs U251/EGFP cells. (B) U251/Tet-EGFP-Oct-3/4 cells were cultured in the presence of doxycycline for 48 h. ABCG2 mRNA and protein expression was then determined by qPCR and western blot analyses, respectively. qPCR data (a) represent the mean ± SEM (n = 3). *p < 0.001 vs U251/EGFP cells. Representative data of western blot analysis (b) is shown (n = 3). (C) qPCR (a) and western blot (b) analyses of ABCG2 mRNA and protein expression in human glioblastoma cell lines. (D) Effect of ABCG2 suppression by siRNA on Oct-3/4-enhanced drug resistance. ABCG2 protein levels in cell lysates of untargeted control or ABCG2 (Sigma; NM_004827) knockdown U251/EGFP-Oct-3/4 cells were evaluated by western blot analysis. Representative data are shown (n = 3). EGFP-Oct-3/4 and β-actin were used as loading controls. (E) Cells were treated with doxorubicin (10 μM) for 24 h, and then the released LDH in the culture medium was analyzed. Values represent the mean ± SEM (n = 5). *p < 0.001 vs control siRNA.

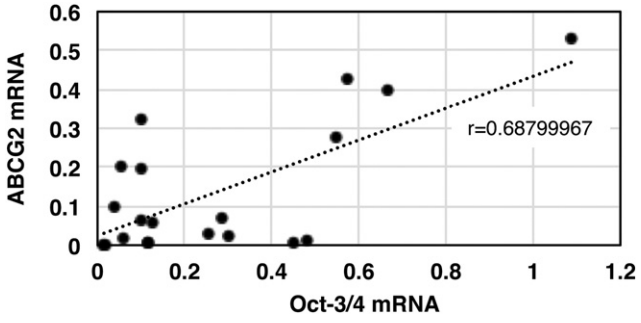

Fig. 7. Analysis of the correlation between Oct-3/4 and ABCG2 expression in surgically resected GB tissues. mRNA expression of Oct-3/4 and ABC transporters in 20 GB patients was analyzed by qPCR. The correlation was then assessed as described in the Materials and Methods section, and the score of correlation was given in the graph.

Oct-3/4 cells under normoxic condition (Supplemental Fig. 3). Although HIF1α is well known to be stabilized under hypoxic condition, AKT, a downstream target of phosphatidylinositol 3′-kinase (PI3K), has also been known as a regulator of HIF1α under normoxic condition [25–27]. We recently found that Oct-3/4 induces activation of AKT, resulting in the accumulation of HIF1α protein in GB cells [28]. It has been reported that PI3K/AKT regulates ABCG2 expression in glioma progenitor cells [29]. Taken together, these studies suggest that enhanced ABCG2 expression by Oct-3/4 is regulated at least in part via the AKT–HIF1 pathway under normoxic condition. On the other hand, Marques et al. identified an Oct-3/4-binding sequence in the promoter region of ABC transporters, although ABC transporters have not been confirmed as a direct target of Oct-3/4-dependent transcription [30]. Further studies are required to elucidate the precise mechanism underlying the transcriptional regulation of ABC transporters.

The mechanism by which GB cells acquire the MDR phenotype is quite complex and not fully understood, but an enhanced expression of Oct-3/4 has been found in several cancer cell lines with the MDR phenotype as compared to parental cells [30–33]. Interestingly, reduced demethylation of CpG sites in the promoter region of Oct-3/4 has been found in liver cancer MDR cells, suggesting that Oct-3/4 expression is controlled by epigenetic regulation during acquisition of the drug-resistant phenotype [31]. Epigenetic dysregulation and gene mutations in somatic stem cells may lead to the development of cancer stem cells [34]. During reprogramming of somatic cells to induced pluripotent stem cells, the Oct-3/4 promoter is demethylated by activation-induced cytidine deaminase (AID, also known as AICDA) [35]. Therefore, AID may also be a possible key regulator in the acquisition of the MDR phenotype by upregulating expression of Oct-3/4. In addition to the drug-resistant phenotype, MDR cells have various features such as increased self-renewal, increased cell motility, and an enhanced tumorigenic potential [7]. We recently demonstrated that Oct-3/4 may be involved in the increased migration and invasion of GB cells via integrin signaling and matrix metalloproteinase-13 activity [21,36]. Therefore, in addition to drug resistance, Oct-3/4 may contribute to many features of MDR cells.

Resistance against chemotherapeutic drugs is the major criterion that characterizes cancer stem cells [37,38]. Furthermore, it has been thought that the existence of cancer stem cells explains why conventional anticancer therapies often cannot completely eradicate tumors, resulting in eventual recurrence. In addition to being a key regulator in the self-renewal process, Oct-3/4 protects embryonic stem cells from apoptosis induced by anticancer drugs or cytotoxic stresses [39]. ABCG2 is highly expressed in CD133-positive cancer stem cells, and has been subsequently established as a novel cancer stem cell marker in human cells [40,41]. Moreover, ABCG2 has been also utilized to identify unlabeled cells with cancer stem cell properties, such as side population cells, by their ability to pump out the fluorescent dye Hoechst 33342 [42]. These studies, together with the present findings, suggest that the capacity for chemotherapeutic drug efflux in cancer stem cells and MDR cells may be regulated by Oct-3/4 via activating its downstream target genes such as ABCG2.

The mortality rate of GB remains high because patients often suffer relapses when GBs become resistant to chemotherapeutic drugs. Therefore, treatments that suppress GB cells with highly invasive and drug-resistant phenotypes would be highly ameliorative for GB patients. From this viewpoint, the present study may provide evidence that a signaling pathway from Oct-3/4 to ABCG2 is a promising target to prevent GB cells turning into cells with MDR.

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Transparency document

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