Cancer Research

# ID4 Imparts Chemoresistance and Cancer Stemness to Glioma Cells by Derepressing miR-9*–Mediated Suppression of SOX2

Hye-Min Jeon[1], Young-Woo Sohn[1], Se-Young Oh[1], Sung-Hak Kim[1], Samuel Beck[1], Soonhag Kim[2], and Hyunggee Kim[1]

## Abstract

Glioma stem cells (GSC) possess tumor-initiating potential and are relatively resistant to conventional chemotherapy and irradiation. Thus, they are considered to be major drivers for glioma initiation, progression, and recurrence. However, the precise mechanism governing acquisition of their drug resistance remains to be elucidated. Our previous study has shown that inhibitor of differentiation 4 (ID4) dedifferentiates *Ink4a/Arf*$^{-/-}$ mouse astrocytes and human glioma cells to glioma stem-like cells (induced GSCs or iGSCs). In this article, we report that ID4-driven iGSCs exhibit chemoresistant behavior to anticancer drugs through activation of ATP-binding cassette (ABC) transporters. We found that ID4 enhanced SOX2 protein expression by suppressing microRNA-9* (miR-9*), which can repress SOX2 by targeting its 3′-untranslated region. Consequently, ID4-mediated SOX2 induction enhanced ABCC3 and ABCC6 expression through direct transcriptional regulation, indicating that ID4 regulates the chemoresistance of iGSCs by promoting SOX2-mediated induction of ABC transporters. Furthermore, we found that short hairpin RNA-mediated knockdown of SOX2 in ID4-driven iGSCs resulted in loss of cancer stemness. Moreover, ectopic expression of SOX2 could dedifferentiate *Ink4a/Arf*$^{-/-}$ astrocytes and glioma cells to iGSCs, indicating a crucial role of SOX2 in genesis and maintenance of GSCs. Finally, we found that the significance of the ID4-miR-9*-SOX2-ABCC3/ABCC6 regulatory pathway is recapitulated in GSCs derived from patients with glioma. Together, our results reveal a novel regulatory mechanism by which ID4-driven suppression of miR-9* induces SOX2, which imparts stemness potential and chemoresistance to glioma cells and GSCs. *Cancer Res; 71(9); 3410–21. ©2011 AACR.*

## Introduction

Glioblastoma multiforme (GBM), the most common and aggressive form of brain malignancies, remains mostly incurable and has about 1-year median survival postdiagnosis, despite considerable advances in diagnosis, therapies, and understanding of its molecular pathways (1–5). Recent studies have revealed that a cancer-cell subpopulation in brain tumors—glioma stem cells (GSC)—exhibits tumor-initiating ability, self-renewal, and aberrant differentiation and is responsible for glioma initiation and progression (6, 7). Interestingly, GSCs are remarkably resistant to chemotherapy and irradiation, which are first-line treatment options for patients with malignant gliomas (8, 9). Therefore, GSCs are considered major culprits for glioma recurrence after therapy (10).

**Authors' Affiliations:** [1]School of Life Sciences and Biotechnology, Korea University; and [2]Department of Applied Bioscience, CHA Stem Cell Institute, CHA University, Seoul, Republic of Korea

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

**Corresponding Author:** Hyunggee Kim, School of Life Sciences and Biotechnology, Korea University, Seoul 136-713, Republic of Korea. Phone: 82-2-3290-3059; Fax: 82-2-953-0737; E-mail: hg-kim@korea.ac.kr

**doi:** 10.1158/0008-5472.CAN-10-3340



Although resistance to temozolomide is associated with the expression of O6-methylguanine-DNA-methyltransferase, which is abundantly detected in CD133-positive GBMs (11), a major cause of chemoresistance in GSCs is the activation of multiple drug resistance genes, such as those encoding ATP-binding cassette (ABC) transporters (8). ABC transporters can cause efflux of molecules across the cell membrane by using ATP and are highly expressed in normal and cancer stem cells compared with their differentiated counterpart cells (12, 13). A recent study has shown that PI3K/Akt signaling, which is activated by PTEN loss, confers chemoresistance to GSCs by enhancing ABCG2 activity (14). However, the mechanism underlying the expression of various ABC transporters involved in the chemoresistance of GSCs remains poorly understood.

MicroRNAs (miRNA or miR) are an evolutionarily conserved group of small noncoding RNAs that play pivotal roles in regulating most biological processes of normal development and various diseases, including cancers, by suppressing mRNA stability and/or translation (15). Recently, the biological consequences of miRNAs have been revealed in many studies showing that miRNAs specifically regulate maintenance and differentiation of embryonic stem cells (ESC) and induce pluripotent stem cells (iPSC; ref. 16). For example, miR-145, which is highly upregulated during differentiation, can

repress pluripotency in human ESCs by suppressing many reprogramming factors, including OCT4, SOX2, and KLF4 (17). Conversely, miR-291-3p, miR-294, and miR-295, which are specifically expressed in ESCs, can increase the efficiency of iPSC genesis by inducing OCT4, SOX2, and KLF4 (18). These reprogramming factors are upregulated in various human malignancies, and some are encoded by classical oncogenes (19–21). In addition, SOX2 is highly expressed in patients with glioma and plays a crucial role in GSC maintenance (22). However, the precise roles, such as tumor initiation, self-renewal, aberrant differentiation, and chemo/radioresistance, of these reprogramming factors in GSC nature remain to be elucidated.

Previously, we have shown that inhibitor of differentiation 4 (ID4) induces dedifferentiation of *Ink4a/Arf*$^{-/-}$ mouse astrocytes, generating GSC-like cells (iGSCs) through activation of cyclin E and Notch signaling (23). However, the molecular mechanism governing the chemoresistance of ID4-driven iGSCs has not been elucidated. In this article, our data show that ID4 confers chemoresistance to GSCs by inducing the expression of 2 SOX2-mediated ABC transporters—ABCC3 and ABCC6—through direct suppression of miRNA-9* (miR-9*), a SOX2-targeting miRNA.

## Materials and Methods

### Cell culture and conditions

Human glioma cell lines (A172, A1207, LN18, and LN229) were purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium/high glucose supplemented with 10% FBS (Hyclone), 1% penicillin and streptomycin (Life Technologies), and 2 mmol/L L-glutamine (Life Technologies). Human glioma stem cells (hGSC-1, hGSC-2, and hGSC-3; ref. 24) and glioma cells were grown in suspension culture with neurobasal medium (NBE; Invitrogen) supplemented with modified N2, B27, epidermal growth factor (EGF; 20 ng/mL; R&D Systems), and basic fibroblast growth factor (bFGF; 20 ng/mL; R&D Systems). For neurosphere-formation assays, cells were seeded at a density of 2 cells/mm$^2$ in 12-well plates and then grown in suspension with NBE supplemented with N2, B27, EGF, and bFGF (all 20 ng/mL), as described previously (23). EGF and bFGF were replaced every 3 days, and the neurosphere numbers were determined after 14 days.

Luciferase reporter gene assay, RNA analysis, plasmid and gene transduction, Western blot analysis, immunofluorescence assay, bromodeoxyuridine (BrdU) incorporation assay, fluorescence activated cell-sorting (FACS) assay, *in vivo* tumorigenicity assay, apoptosis analysis, side population cell analysis, and statistical analysis were done. Details of experimental procedures are provided in the Supplementary Information.

## Results

### ID4 primes anticancer drug resistance through induction of ABC transporters

To investigate whether ID4 confers chemoresistance to cells, we established ID4-overexpressing glioma cells (A172-ID4 and A1207-ID4) by transduction of retroviral vector-encoding *ID4* genes, as well as ID4-depleted cells (LN229-shID4) using short hairpin RNA interference (shRNAi). Compared with their controls, cell death evaluated on flow cytometric analysis revealed that A172-ID4 and A1207-ID4 were significantly resistant whereas LN229-shID4 was sensitive to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-induced apoptosis (Supplementary Fig. S1). Our previous study has shown that ID4 allows *Ink4a/Arf*$^{-/-}$ astrocytes and glioma cells to acquire GSC properties through activation of cyclin E and Jagged1-Notch1 signaling (23). Thus, we examined whether these pathways were required for ID4-mediated antiapoptosis by treating cells with BCNU, in combination with purvalanol A (a cyclin E inhibitor; ref. 25) and/or DAPT (a Notch inhibitor), and found that cyclin E and Notch signaling were not involved in ID4-mediated chemoresistance of A172, A1207, and LN229 (Supplementary Fig. S1). A recent study had shown that GSCs acquire anticancer drug resistance through PI3K/Akt-mediated ABCG2 activation (14) and, therefore, we investigated whether ID4-driven chemoresistance was associated with ABC transporter activity. First, we cotreated cells with BCNU and the pan-ABC transporter inhibitor verapamil (14) and found that verapamil suppressed BCNU-induced apoptosis in A172-ID4 (Fig. 1A) but not in LN229-shID4 (Fig. 1A), indicating that ID4 causes cells to retain chemoresistance, at least in part, through ABC transporter activity.

Next, we carried out semiquantitative real-time PCR (RT-PCR) analysis to examine which ABC transporters are differentially regulated by ID4 and found that the levels of ABCC3, ABCC6, and ABCA2 were significantly elevated in A172-ID4 (Supplementary Fig. S2A) and the levels of ABCC3, ABCC4, ABCC6, and ABCG2 were increased in *Ink4a/Arf*$^{-/-}$ astrocyte-ID4 (Supplementary Fig. S2B). On the contrary, the levels of ABCC2, ABCC3, ABCC6, and ABCG2 were markedly reduced in LN229-shID4 (Supplementary Fig. S2C). In addition, we examined the expression of ABC transporters in 2 human GSCs (hGSC) derived from GBM patients (24) and found that the levels of ABCC3 and ABCC6 were diminished in ID4-depleted hGSC1 (Supplementary Fig. S2D) and the levels of ABCC3, ABCC6, and ABCG2 were decreased in ID4-depleted hGSC2 (Supplementary Fig. S2E). Thus, to further investigate a possible role of ABC transporters in ID4-driven chemoresistance, we focused on ABCC3 and ABCC6. We depleted ABCC3 and ABCC6 in ID4-overexpressing cells by using shRNAi and confirmed their depletion in A172-ID4 and A1207-ID4 by quantitative RT-PCR (qRT-PCR) and Western blot assays (Fig. 1B; Supplementary Fig. S3A and B). The depletion of ABCC3 and ABCC6 in ID4-overexpressing cells resulted in a marked increase in apoptosis (Fig. 1C), suggesting that ID4-mediated chemoresistance to BCNU is partially attributed to increased ABCC3 and ABCC6 expression.

### ID4 upregulates SOX2 by suppressing miR-9* expression

The preceding data raise the question pertaining to how ID4 upregulates ABCC3 and ABCC6 mRNA expression, because ID4 is a helix–loop–helix (HLH) protein that lacks a DNA-binding domain, and it can suppress transcription by acting as

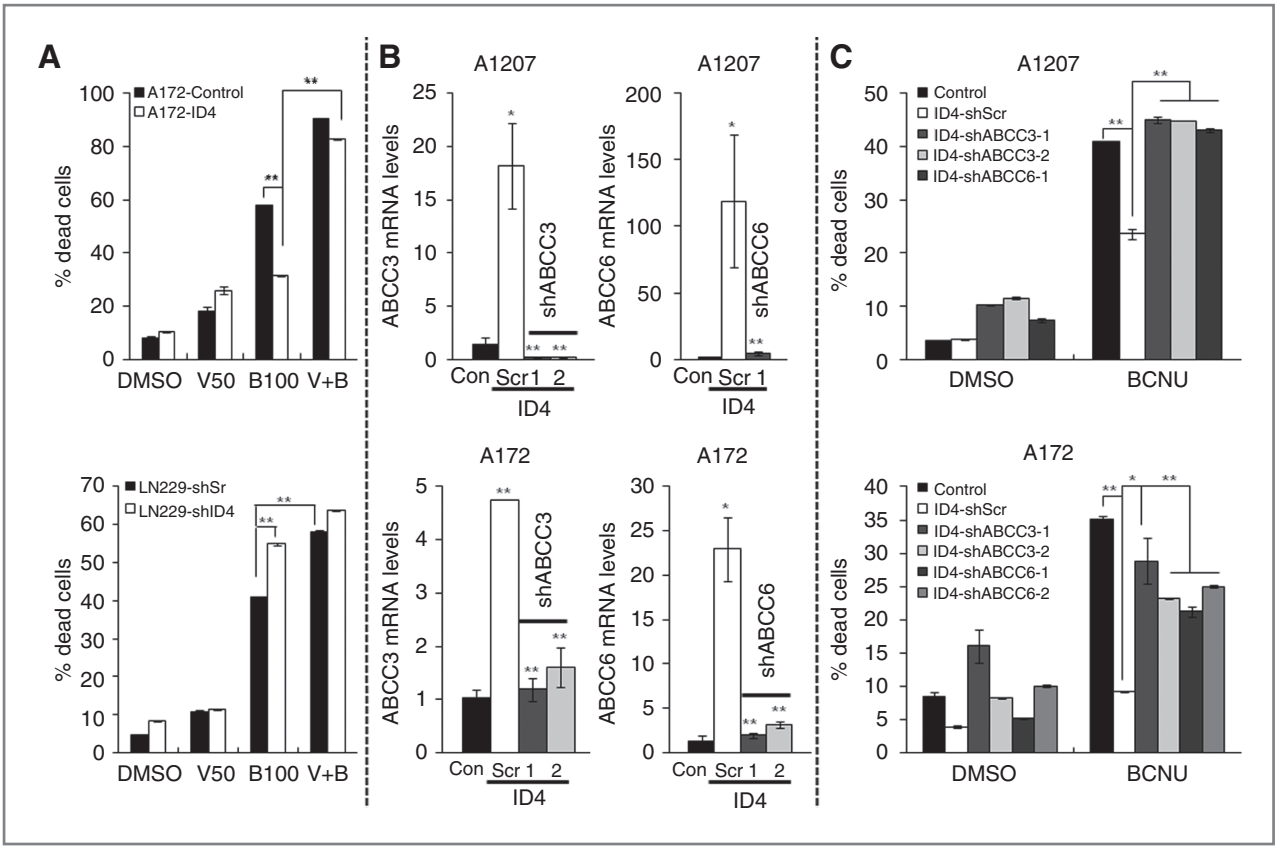


**Figure 1.** ID4 induces anticancer drug resistance in glioma cells by inducing ABCC3 and ABCC6 transporters. A, percentage of dead cells in A172-ID4, LN229-shID4, and their controls treated with BCNU (100 μg/mL; B100) in the presence or absence of verapamil (50 μmol/L; V50) was determined by AnnexinV/propidium iodide (PI)-mediated FACS analysis. Verapamil + BCNU (V + B). B, ABCC3 and ABCC6 mRNA levels in A1207-ID4 and A172-ID4, and in ABCC3- or ABCC6-depleted A1207-ID4 and A172-ID4, together with their controls, were examined by qRT-PCR. The A1207-ID4-shABCC6-1 cell line was used in the qRT-PCR analysis because we failed to establish additional A1207-ID4-shABCC6 cell lines. C, apoptosis rate of cells described in (B) treated with or without BCNU (100 μg/mL); *, $P < 0.05$; **, $P < 0.01$.

a dominant-negative transcriptional repressor (26). During the investigation of regulatory mechanisms pertaining to ID4-driven ABCC3 and ABCC6 expression, we found that the ID4 level correlated with SOX2 protein expression—that is, increased SOX2 protein levels were observed in A1207-ID4 and A172-ID4, whereas decreased SOX2 protein levels were observed in LN229-shID4 (Fig. 2A; Supplementary Fig. S4; ref. 23). In addition, SOX2 protein levels correlated with ABCC3 and ABCC6 protein levels in these cells. Interestingly, qRT-PCR analysis revealed that ID4-induced SOX2 protein levels were not correlated with its mRNA levels (Fig. 2A), indicating that ID4 might regulate SOX2 expression at the posttranscriptional level. Emerging evidence suggests that various biological processes are regulated by miRNAs that act as negative regulators of gene expression at the posttranscriptional level (15). Therefore, we attempted to explore miRNAs based on 3 criteria: (i) miRNAs that can target the 3′-untranslated region (UTR) of SOX2 (examined using bioinformatics programs such as PicTar, TargetScan, miRBase, and Miranda); (ii) miRNAs that are enriched in brain tissues and are involved in differentiation of neural stem cells (NSC) or progenitors; and (iii) miRNAs that possess E-boxes—ID4-responsive elements—in their promoters. Consequently, we found that one such candidate is miR-9*, known to be specifically expressed during brain neurogenesis (27, 28). To validate our bioinformatics criteria, we initially carried out a TaqMan microRNA detection assay and found that miR-9* expression was significantly decreased in A1207-ID4 and A172-ID4 but enhanced in LN229-shID4 (Fig. 2B). The luciferase activity of the reporter construct containing 3 copies of the miR-9*–binding motif (27) was dose-dependently elevated in 293T cells cotransfected with ID4 (Fig. 2B) and, in addition, significantly elevated in A1207-ID4 and A172-ID4 (Fig. 2B), indicating that miR-9* activity was lower in ID4-overexpressing cells. A 6-bp sequence in the 5′-ends of miR-9* was perfectly complementary to SOX2–3′-UTR (Supplementary Fig. S5A). To determine whether this predicted binding site was responsible for SOX2 repression, we created luciferase reporter constructs containing either wild-type (WT) or miR-9*-binding site-mutated SOX2–3′-UTR (Supplementary Fig. S5A; ref. 17). WT, but not mutated SOX2–3′-UTR, repressed its reporter activity in LN229-shID4 but increased in A1207-ID4 (Fig. 2C), indicating that ID4 enhances SOX2 expression, in part, by suppressing miR-9*. The miR-9* transcripts are located in 3 loci of human chromosomes: chromosomes

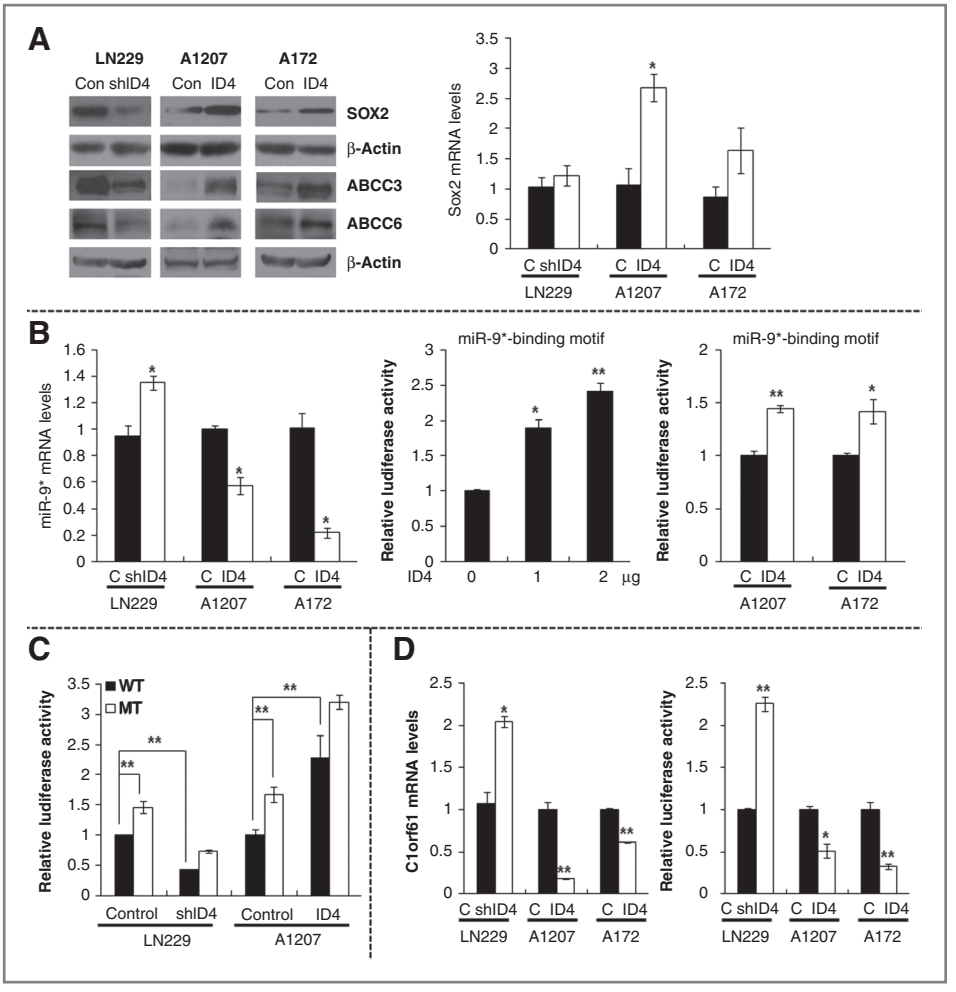


Figure 2. ID4 upregulates SOX2 protein by inhibiting miR-9* expression. A, Western blot analysis showed that SOX2, ABCC3, and ABCC6 protein levels were decreased in LN229-shID4 compared with control LN229-shScrambled and increased in A1207-ID4 and A172-ID4 as compared with their controls. qRT-PCR was carried out to examine SOX2 mRNA levels in these cells. B, miR-9* levels increased in A1207-ID4 and A172-ID4 and decreased in LN229-shID4. The luciferase activity of the reporter gene containing 3 copies of the miR-9*–binding motif was dose-dependently elevated in 293T cells after transfecting different concentrations of ID4. Furthermore, this activity was significantly increased in ID4-overexpressing A1207 and A172 compared with their controls. C, the luciferase activity of the reporter gene containing WT SOX2–3′-UTR, but not miR-9*-binding site-mutated SOX2–3′-UTR, in A1207-ID4, LN229-shID4, and their controls. D, qRT-PCR analysis revealed that expression of the *c1ORF61* gene encoding miR-9* was decreased in ID4-overexpressing A1207 and A172 and increased in ID4-depleted LN229. Luciferase reporter assays revealed that the transcriptional activity of the reporter gene containing the *c1ORF61* promoter was elevated in LN229-shID4 but decreased in A1207-ID4 and A172-ID4; *, $P < 0.05$; **, $P < 0.01$.

1 (hsa-miR-9-1), 5 (hsa-miR-9-2), and 15 (hsa-miR-9-3). In particular, the hsa-miR-9-1 transcript is located in the intron region of the *C1orf61* gene whereas hsa-miR-9-2 and hsa-miR-9-3 transcripts are located in the intergenic region. Because the *C1orf61* gene has 3 conserved "E-box" elements in its promoter (Supplementary Fig. S5B), we carried out qRT-PCR to measure C1orf61 mRNA expression and found that its mRNA level was markedly diminished in A1207-ID4 and A172-ID4 but significantly increased in LN229-shID4 (Fig. 2D). Furthermore, ID4 overexpression decreased the luciferase activity of the reporter gene containing the *C1orf61* gene promoter and ID4 knockdown enhanced *C1orf61* promoter–luciferase activity (Fig. 2D), indicating that ID4 suppresses miR-9* expression through transcriptional repression of the *C1orf61* gene.

### SOX2 promotes anticancer drug resistance through ABCC3 and ABCC6 expression

We wondered whether SOX2 was associated with ID4-mediated chemoresistance to anticancer drugs. Therefore, we established several SOX2-overexpressing glioma cells (A1207-SOX2, A172-SOX2, LN18-SOX2, and LN229-SOX2) by transducing the retroviral vector-encoding *SOX2* gene. In addition, we employed shRNAi to downregulate SOX2 in LN229 (LN229-shSOX2), which expresses a high level of endogenous SOX2. All SOX2-overexpressing glioma cells showed a marked decrease in apoptosis when treated with BCNU (Fig. 3A). On the contrary, LN229-shSOX2 was significantly sensitive to anticancer-drug-induced apoptosis when treated with BCNU, etoposide, and staurosporine (Fig. 3A, far right graph). SOX2, a member of the SRY-related

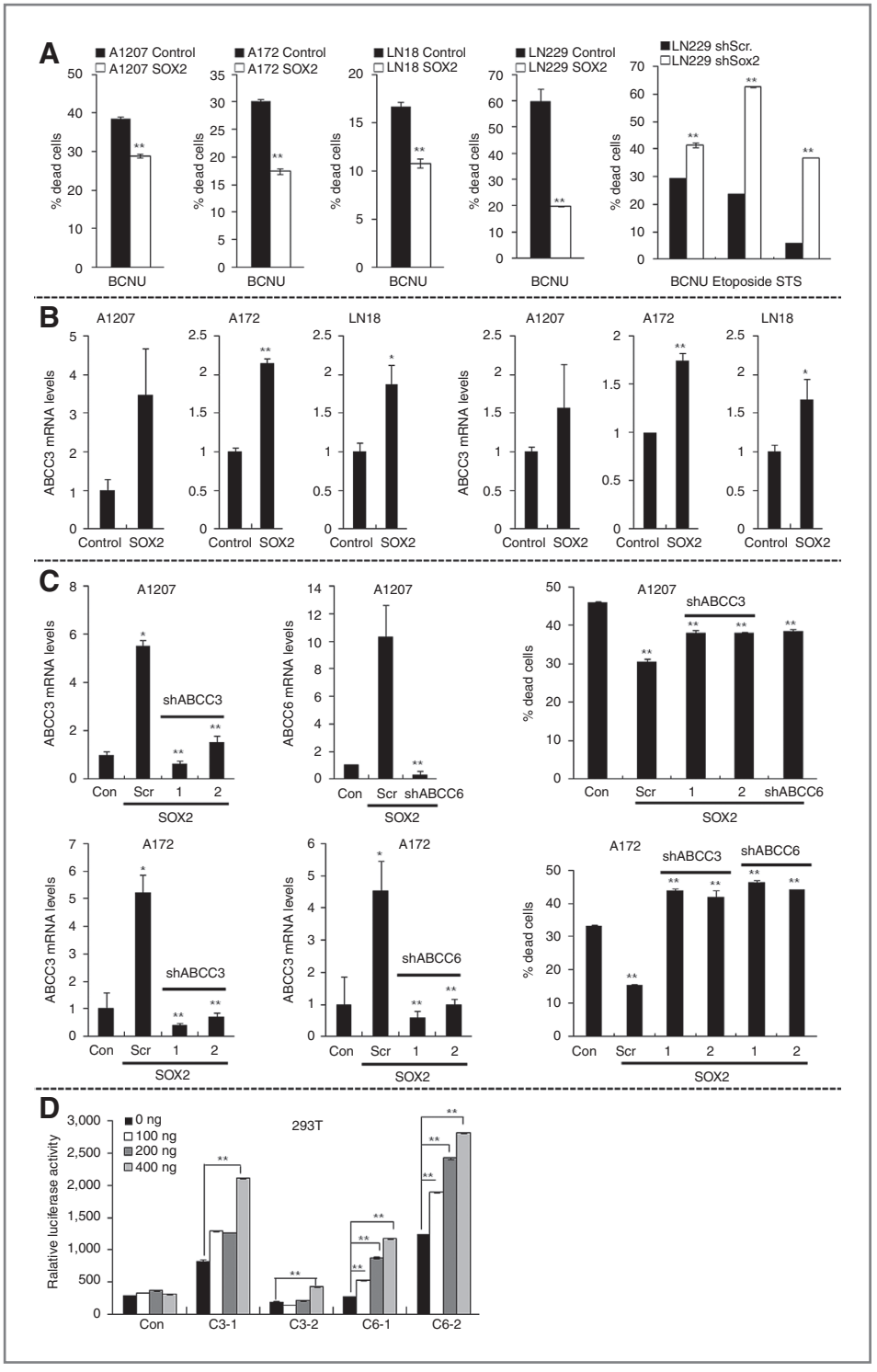


**Figure 3.** SOX2 induces anticancer drug resistance by upregulating ABCC3 and ABCC6 transporters. A, percentage of dead cells in SOX2-overexpressing glioma cells (A1207, A172, LN18, and LN229) and their controls treated with BCNU (80–100 μg/mL), and percentage of dead cells in control LN229-shScrambled and LN229-shSOX2 treated with BCNU (80 μg/mL), etoposide (180 μmol/L), and staurosporine (100 nmol/L; STS). B, qRT-PCR analysis revealed that ABCC3 and ABCC6 mRNA levels increased in all SOX2-overexpressing glioma cell lines that were tested. C, ABCC3 and ABCC6 mRNA levels were markedly reduced in ABCC3- or ABCC6-depleted A1207-SOX2 and A172-SOX2 compared with their controls. Percentage of dead cells in these cells treated with BCNU (100 μg/mL) was determined by AnnexinV/PI-mediated FACS analysis. D, cotransfection of luciferase reporter genes containing *ABCC3* (C3-1 and C3-2) or *ABCC6* promoters (C6-1 and C6-2) at different concentrations of SOX2 in 293T cells revealed that SOX2 increases the transcription of *ABCC3* (C3-1) and *ABCC6* (C6-1 and C6-2) in a dose-dependent manner; *, $P < 0.05$; **, $P < 0.01$.

high mobility group (HMG) box family, regulates expression of various target genes that contain a consensus SOX2-binding motif (A/TA/TCAAA/TG) in their promoters (29) and is upregulated in several human malignancies, including GBM (22, 30); however, its role in tumorigenesis is poorly understood. Initially, qRT-PCR was conducted to examine the expression of ABCC3 and ABCC6 and showed that their expression levels were relatively increased in all SOX2-overexpressing cells (Fig. 3B). To determine whether SOX2 exerted an antiapoptotic effect through induction of ABCC3 and ABCC6, we used shRNAi to knock down ABCC3 and ABCC6 expression in SOX2-overexpressing cells

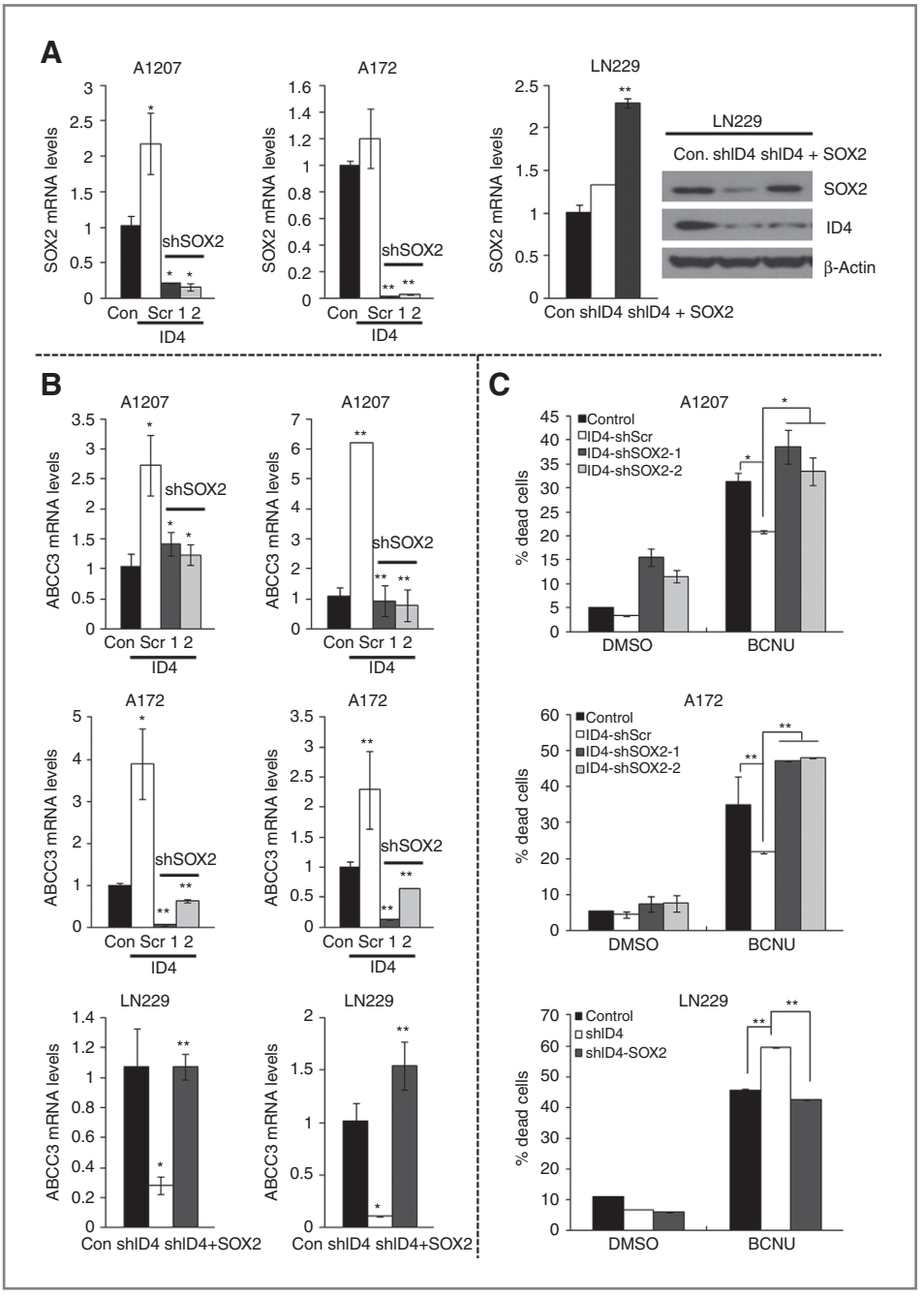


**Figure 4.** SOX2 regulates ABCC3/ABCC6-dependent chemoresistance in ID4-overexpressing glioma cells. A, SOX2 mRNA levels were reduced in A1207-ID4 and A172-ID4 when transduced with 2 SOX2-specific shRNAi constructs. The SOX2 mRNA level was increased by reconstitution of a miR-9*–resistant version of SOX2 in LN229-shID4 (LN229-shID4-SOX2), and Western blot analysis revealed induction of SOX2 protein level in these cells. B, ABCC3 and ABCC6 mRNA levels were diminished in A1207-ID4 and A172-ID4 when transduced with 2 SOX2-specific shRNAi constructs but increased in LN229-shID4-SOX2. C, percentage of dead cells in 2 A1207-ID4-shSOX2 cell lines, 2 A172-ID4-shSOX2 cell lines, and LN229-shID4-SOX2, and their controls treated with or without BCNU (100 μg/mL); *, $P < 0.05$; **, $P < 0.01$.

(A1207-SOX2-shC3 and A1207-SOX2-shC6; A172-SOX2-shC3 and A172-SOX2-shC6) and found that complete knockdown occurred (Fig. 3C). The depletion of these mRNAs in A1207-SOX2 and A172-SOX2 resulted in significant resistance to BCNU (Fig. 3C), indicating that SOX2 causes anticancer drug resistance by inducing ABC transporters. Among several putative binding sites for SOX2 in their promoter regions, 2 sites in each *ABCC3* and *ABCC6* promoter were highly conserved and contained a predicted SOX2-binding consensus motif (Supplementary Fig. S5C). We created luciferase reporter genes containing the promoter regions of *ABCC3* [C3-1 (−7,489 to −7,114) and C3-2 (−1,376 to −881); upstream from the transcription start site] and *ABCC6* [C6-1 (−6,482 to −5,986) and C6-2 (−4,246 to −3,792)], with each harboring a predicted SOX2-binding motif, and conducted promoter–luciferase reporter assays to examine the transcriptional activity of *SOX2*. Transfection of different concentrations of the *SOX2* gene in 293T cells resulted in a dose-dependent increase in all reporter gene activities tested (Fig. 3D). Together, our results suggest that SOX2 promotes anticancer drug resistance through direct activation of ABCC3 and ABCC6 expression.

### ID4 regulates anticancer drug resistance via SOX2 activation

To investigate whether ID4 endows chemoresistance to glioma cells by SOX2-mediated induction of ABCC3 and ABCC6, we stably depleted SOX2 in ID4-overexpressing cells (A1207-ID4-shSOX2 and A172-ID4-shSOX2), using shRNAi, or reconstituted SOX2 in ID4-depleted cells (LN229-shID4-SOX2) by transduction of a retroviral vector-encoding *SOX2* gene that lacks its own 3′-UTR (to avoid a possible miR-9*–driven repression) and confirmed SOX2 expression in these cells using qRT-PCR (Fig. 4A). Notably, Western blot and immunofluorescence analyses revealed that SOX2 expression in LN229-shID4-SOX2 was similar to that in control LN229 (Fig. 4A; Supplementary Fig. S6). Next, we conducted qRT-PCR to determine expression levels of ABCC3 and ABCC6 in A1207-ID4-shSOX2 and A172-ID4-shSOX2 and found that, as expected, ABCC3 and ABCC6 in these cells were diminished to levels similar to those of their parental cells (Fig. 4B). Furthermore, reconstitution of SOX2 in LN229-shID4 resulted in increases in ABCC3 and ABCC6 expression at levels similar to those of control cells (Fig. 4B). We then examined the chemoresistance of A1207-ID4-shSOX2 and A172-ID4-shSOX2 to BCNU and found that SOX2 knockdown in these cells led to a marked increase in apoptosis (Fig. 4C). Conversely, reconstitution of SOX2 in LN229-shID4 rescued the chemoresistance to BCNU at a level similar to that of parental LN229 (Fig. 4C). In summary, these data indicate that SOX2-mediated induction of ABCC3 and ABCC6 plays a crucial role in the acquisition of anticancer drug resistance of ID4-overexpressing cells.

### SOX2 promotes tumorigenicity and self-renewal of glioma cells

SOX2 plays pivotal roles in maintaining stemness properties of NSCs and GSCs (22, 31, 32). Furthermore, ectopic overexpression of SOX2, along with other reprogramming factors, including OCT4, KLF4, and c-MYC, reprograms somatic cells into iPSCs (16). However, the functional role of SOX2 in gliomagenesis, particularly in GSC biology, has not been studied. Therefore, we subcutaneously inoculated SOX2-overexpressing glioma cells in immunocompromised mice and compared their tumorigenic potentials with control cells. We found that A1207-SOX2 and A172-SOX2 displayed a marked increase in tumor growth (Fig. 5A and B). Moreover, SOX2 overexpression caused *Ink4a/Arf*$^{-/-}$ astrocytes to have tumorigenic potential (Supplementary Fig. S7A), indicating an oncogenic effect of SOX2. A1207-SOX2, A172-SOX2, and *Ink4a/Arf*$^{-/-}$ astrocyte-SOX2 accelerated tumor sphere formation—a hallmark of GSCs (Fig. 5A and B; Supplementary Fig. S7B; refs. 6, 7)—when grown in serum-free medium supplemented with EGF and bFGF, which is a reliable suspension culture condition for NSCs and GSCs to form neurospheres and maintain their stemness (33). Furthermore, these results imply that SOX2 might be causally connected to ID4-mediated switching of glioma cells to iGSCs. To address this possibility, we examined the tumor sphere–forming ability of ID4-overexpressing glioma cells with or without SOX2 knockdown. Consistent with our previous study (23), tumor sphere numbers were significantly increased in A1207-ID4 and A172-ID4 compared with their controls. However, SOX2 depletion in these cells dramatically repressed their tumor sphere–forming abilities, partially attributed to a decreased proliferation as determined by BrdU incorporation assay (Fig. 5C; Supplementary Fig. S8A and B).

To further investigate the functional role of SOX2 in the ID4-driven acquisition of stemness of iGSCs, we carried out qRT-PCR to examine stem cell markers and their differentiated progeny markers in ID4-overexpressing glioma cells with or without SOX2 knockdown. Consequently, we observed that stem cell markers (Nestin, CD133, and CD15) were markedly increased in A1207-ID4 and A172-ID4 compared with their controls (Fig. 5C). However, SOX2 knockdown in these cells dramatically repressed stem cell marker expression and, instead, induced differentiated lineage marker expression, including GFAP and S100β (astrocyte), Tuj1 (neuron), as well as NG2 and Olig2 (oligodendrocyte; Fig. 5C). Notably, GFAP expression was predominantly elevated by SOX2 depletion in A1207-ID4 whereas Tuj1, Olig2, and NG2 levels were dramatically increased in A172-ID4-shSOX2 (Fig. 5C), suggesting that aberrant differentiation of GSCs into a particular cell type is not likely to be determined by loss of SOX2. A serial passage analysis revealed that the tumor sphere–forming ability of A1207-SOX2 is gradually increased after 3 consecutive passages, although it is less effective than that in A1207-ID4. Furthermore, FACS analysis showed that, compared with controls, stem cell marker–positive cell populations increased in A1207-ID4 and A1207-SOX2 grown in NSC culture conditions (NBE medium supplemented with EGF and bFGF) whereas differentiation marker-positive populations increased in these cells grown in differentiation culture conditions (NBE medium supplemented with 5% FBS) for 7 days (Fig. 5D). In addition, we found that differentiation marker-positive cell populations decreased in A1207-ID4 and A1207-SOX2 in NSC cell culture conditions (Supplementary Fig. S9). Tumors derived from A1207-ID4 and A1207-SOX2 contained increased stem cell marker–positive cells compared with tumors derived from controls (Supplementary Fig. S10A–C). Together, these data indicate that SOX2 regulates, at least in part, ID4-driven iGSC genesis and maintenance and that SOX2 can reprogram glioma cells into iGSCs.

### The ID4-miR-9*-SOX2 regulatory axis primes ABCC3/6-mediated chemoresistance in hGSCs derived from GBM patients

Consistent with previous observations that serum-free medium supplemented with EGF and bFGF facilitates maintenance of GSC features whereas media supplemented with FBS robustly differentiates GSCs into several differentiated lineages (33), we found that the 5% FBS-containing medium allowed our hGSCs (hGSC1, hGSC2, and hGSC3; derived from GBM patients; ref. 24) to be cells expressing various differentiated lineage markers (Fig. 6A; Supplementary Fig. S11). Furthermore, ID4 and SOX2 protein were markedly reduced in these differentiated hGSCs (Fig. 6A), indicating a plausible connection between loss of ID4 and SOX2 and hGSC differentiation. To investigate whether the ID4-miR-9*-SOX2

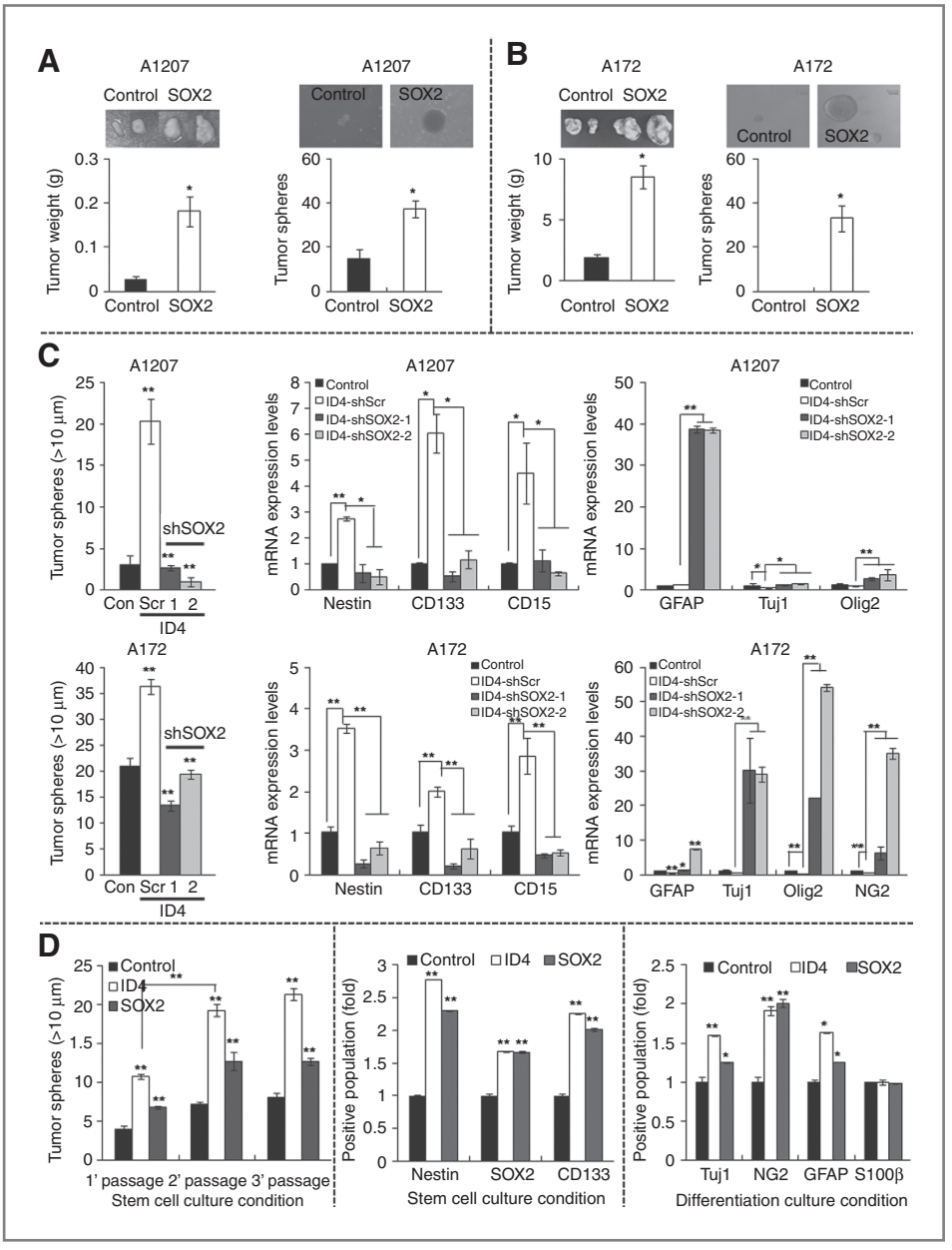


**Figure 5.** SOX2 induces self-renewal and tumorigenicity of glioma cells. A and B, representative tumor images and tumor weights obtained from nude mice ($n = 6$) injected subcutaneously with A1207-control and A1207-SOX2 (A) and A172-control and A172-SOX2 (B). Representative tumor sphere images and their numbers (>10 μm) attained from these cells grown for 14 days in NBE supplemented with EGF and bFGF. C, tumor spheres (>10 μm) were markedly reduced in 2 A1207-ID4-shSOX2 cell lines and 2 A172-ID4-shSOX2 cell lines compared with A1207-ID4 and A172-ID4, respectively. The mRNA levels of GSC and their differentiated lineage markers were examined by qRT-PCR analysis. D, number of tumor spheres (>10 μm) derived from 3 consecutive serial passages of A1207-control, A1207-ID4, and A1207-SOX2 grown for 14 days in NBE medium supplemented with EGF and bFGF. FACS analysis revealed GSC and their differentiated progeny populations of A1207-control, A1207-ID4, and A1207-SOX2 grown in the stem cell and differentiation-culture conditions, respectively; *, $P < 0.05$; **, $P < 0.01$.

regulatory axis is also required for ABCC3 and ABCC6 expression in these hGSCs, we depleted ID4 in hGSC1 and hGSC2 and found that ID4 knockdown resulted in a significant increase in miR-9* levels but a marked reduction in ABCC3 and ABCC6 expression (Fig. 6B). Similar to verapamil treatment, ID4 depletion led to decrease in side population of hGSCs (Fig. 6C). Both hGSC1-shID4 and hGSC2-shID4 showed substantial increases in BCNU-induced apoptosis as compared with their controls (Fig. 6D). Together, these findings suggest that the ID4-miR-9*-SOX2 regulatory axis plays a crucial role in controlling ABC transporter–mediated chemoresistance in both patient-derived hGSCs and human iGSCs.

### Tumor growth and chemoresistance in ID4- and SOX2-overexpressing glioma cells *in vivo*, and correlation of ID4 and ABCC3/ABCC6 expressions in human GBM patients

Compared with the A1207-control, both A1207-ID4 and A1207-SOX2 showed an increased tumor growth and chemoresistance *in vivo* when tumors were intratumorally injected with BCNU (Fig. 7A). Furthermore, immunofluorescence analysis revealed that proliferating cell nuclear antigen (PCNA)-positive cells (proliferating cells) were increased in A1207-ID4 and A1207-SOX2–driven tumors whereas cleaved caspase-3–positive cells (apoptotic cells) were evidently

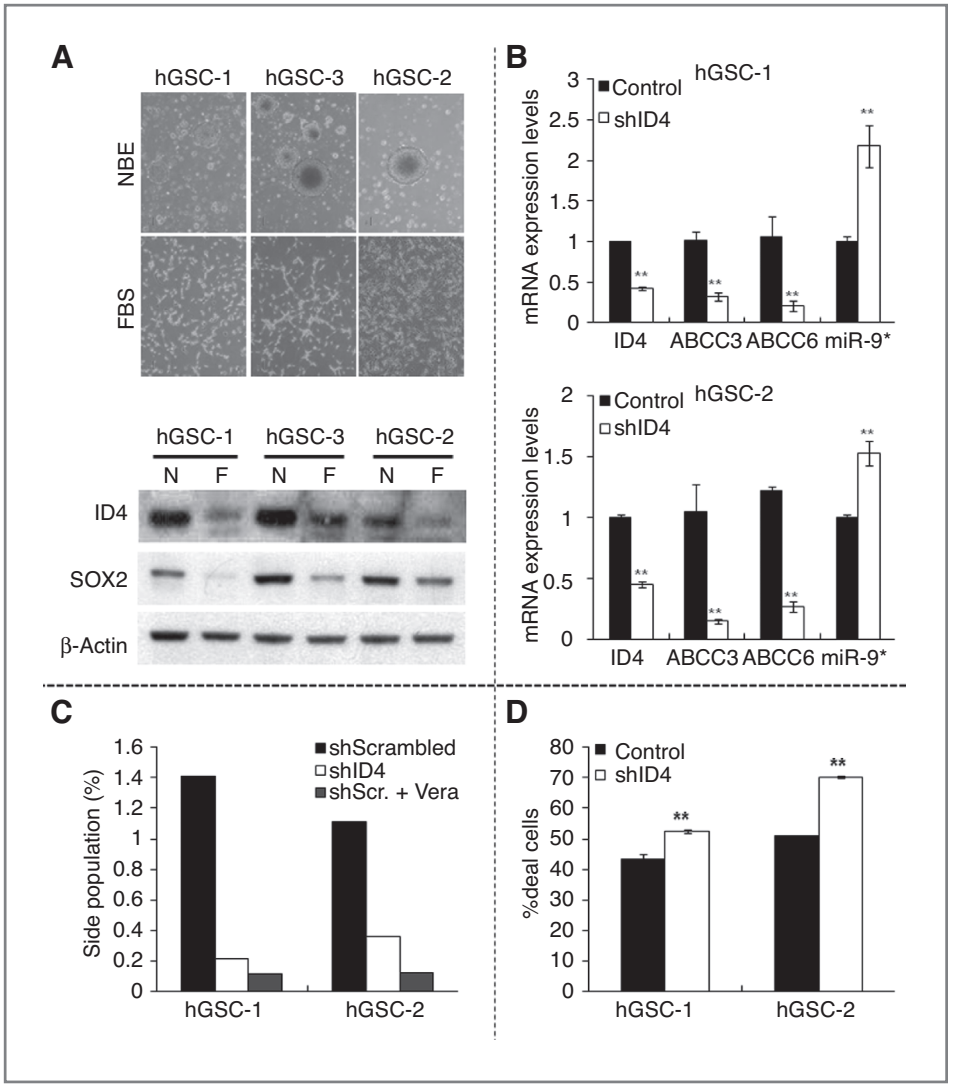


**Figure 6.** Significance of the ID4-miR-9*-SOX2-ABCC3/ABCC6 regulatory pathway in chemoresistance of hGSCs derived from patients with glioma. A, representative images showing morphology of 3 hGSCs grown under NBE medium supplemented with EGF and bFGF (NBE; N) and NBE medium supplemented with 5% FBS (FBS; F). ID4 and SOX2 protein levels decreased in 3 hGSCs grown in NBE medium supplemented with 5% FBS. B, qRT-PCR analysis revealed that ID4 knockdown in hGSC1 and hGSC2 reduced ABCC3 and ABCC6 mRNA levels but increased miR-9* expression. C, percentages of side population in hGSC-1 and hGSC-2. Vera, verapamil. D, percentage of dead cells in the hGSCs described in (B) treated with BCNU was determined by AnnexinV/PI-mediated FACS analysis; *, $P < 0.05$; **, $P < 0.01$.

elevated in A1207-control–driven tumors that were treated with BCNU every 3 days for a total of 9 days (Fig. 7B; Supplementary Fig. S12). In addition, we found that ABCC3 and ABCC6 mRNA levels significantly correlated with ID4 levels in human GBM specimens (Fig. 7C).

## Discussion

In the present study, we show a novel ID4-miR-9*-SOX2 regulatory pathway, where SOX2 plays crucial roles in controlling (i) the chemoresistance of GSCs by inducing ABCC3 and ABCC6, (ii) acquisition of self-renewal potential in glioma cells, and (iii) maintenance of stemness in ID4-driven iGSCs and GSCs derived from GBM patients (Fig. 7D).

In contrast to most ABC transporters (ABCC1, ABCC2, ABCC4, and ABCC5) that are abundantly expressed in various anatomic regions of the normal brain, ABCC3 and ABCC6 expressions are rarely detectable in normal brain tissues (34). A few studies have reported that ABCC3 and ABCC6 diminished the sensitivity of cancer cells to anticancer drugs, such as etoposide, doxorubicin, and daunorubicin, in a cell-type–dependent manner. However, no previous report has described the substrate specificity and role of ABCC3 and ABCC6 in the anticancer drug resistance of GSCs. miRNAs determine fundamental biological processes and human cancer initiation and progression through negative regulation of gene expression (15, 35). In particular, several miRNAs are involved in the development of anticancer drug resistance of many human malignancies by repressing ABC transporters. For example, miR-328, hsa-miR-519c, and hsa-miR-520h can repress ABCG2 expression by directly targeting its 3′-UTR in breast and pancreatic cancer cells (36–38). However, our findings revealed that ABCC3 and ABCC6 are not direct targets for miR-9*; instead, their expression is transcriptionally upregulated by SOX2, which is elevated by ID4-mediated suppression of miR-9*. Moreover, we found that ABCG2 is upregulated in several ID4-driven iGSCs and GSCs derived from patients. Thus, ABCG2 might probably be involved in

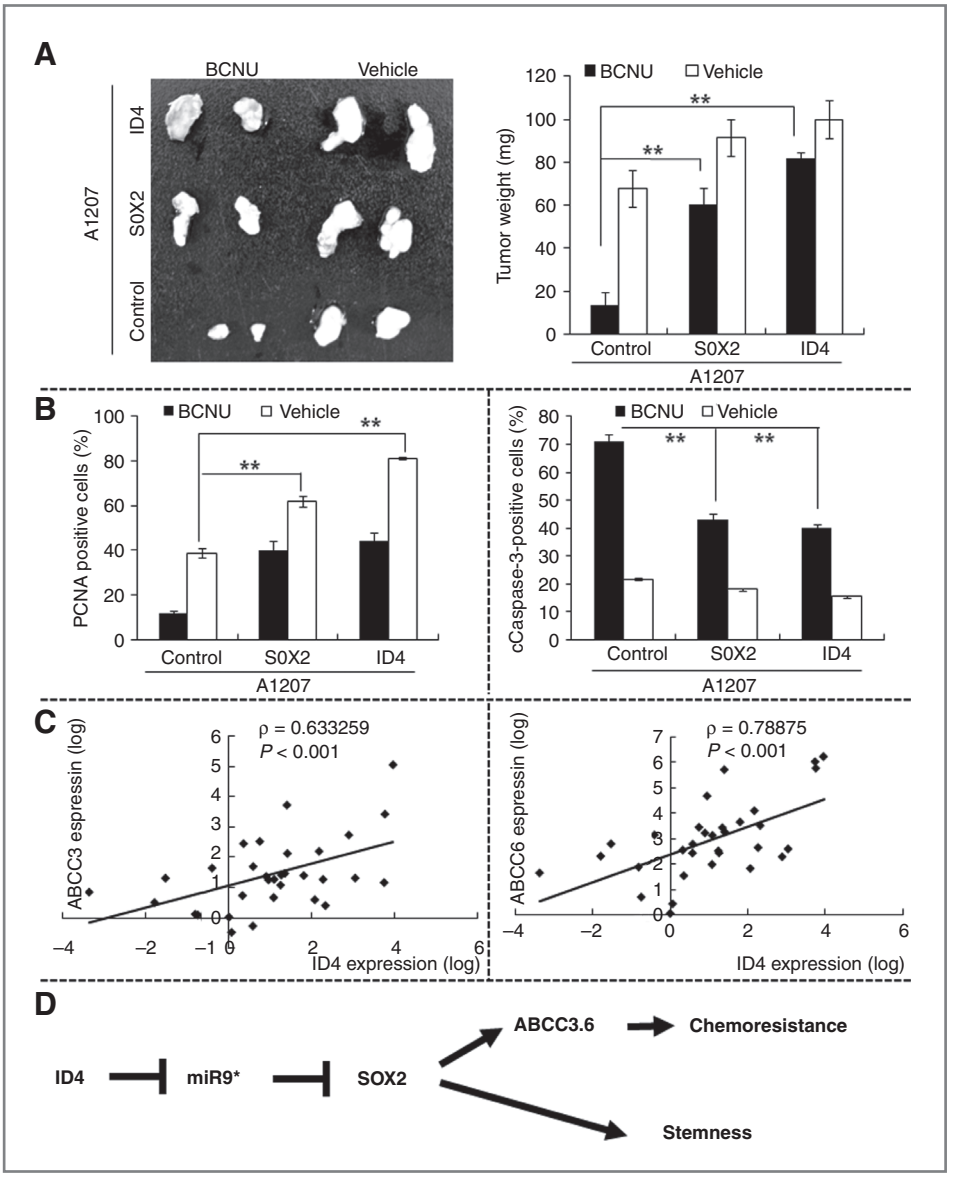


**Figure 7.** Acceleration of tumorigenicity and chemoresistance *in vivo* by ID4 and SOX2, and expression of ID4 and ABCC3/ABCC6 in human GBM patients. A, representative tumor images (left) and tumor weights (right). As subcutaneously transplanted in nude mice ($n = 18$), A1207-ID4 and A1207-SOX2 showed marked increases in tumorigenicity and chemoresistance compared with A1207-control. B, cell proliferation (PCNA-positive ones) was significantly elevated in A1207-ID4 and A1207-SOX2–driven tumors. Compared with control tumors, A1207-ID4 and A1207-SOX2–driven tumors possessed reduced apoptotic cells (cleaved caspase-3–positive ones) as treated with BCNU. C, ABCC3 and ABCC6 mRNA levels correlated with ID4 mRNA levels in human GBM specimens ($n = 32$). D, a schematic diagram showing ID4-miR-9*-SOX2-ABCC3/ABCC6 signaling axis responsible for GSC genesis and chemoresistance; *, $P < 0.05$; **, $P < 0.01$.

ID4-mediated chemoresistance of iGSCs and GSCs to BCNU. However, the expression of ABCG2 in primary GSCs and ID4-driven iGSCs tested in this study differs from that in a previous report showing that ABCG2 activity in GSCs is determined by its cell-membrane localization and not by its expression levels (14). Our findings suggest that ABCC3 and ABCC6 may be *bona fide* GSC-relevant ABC transporters that are associated with chemoresistance because their expression was found to be elevated in most ID4- and SOX2-driven iGSCs and patient-driven GSCs tested but not in glioma cell lines.

SOX2 and other reprogramming factors, such as Nanog, OCT4, and c-MYC, are frequently upregulated in poorly differentiated tumors (39) and have a high correlation with tumor regrowth and metastatic recurrence (40). SOX2 depletion in GSCs promotes differentiation and losses of stemness and tumorigenicity (22). TGF-β signaling enhances GSC stemness and tumorigenicity by inducing SOX4-mediated SOX2 expression (32). Our findings revealed that SOX2 can reprogram *Ink4a/Arf*$^{-/-}$ astrocytes and glioma cells to cells displaying GSC features and plays a crucial role in the maintenance of cancer stemness in ID4-driven iGSCs and GSCs derived from GBM patients. Furthermore, similar to a previous study revealing that the *EWS-FLI-1* oncogene can reprogram primary cells to display the cancer stem cell phenotype by derepressing miR-145–mediated inhibition of SOX2 (41), our findings elucidate the induction of SOX2 by ID4-mediated suppression of miR-9* in GSCs and the sufficiency of SOX2 in reprogramming glioma cells to generate GSCs. Although several studies have reported that miR-145 is ubiquitously expressed in many tissues and functions as a potent SOX2 repressor in ESCs and iPSCs (17, 42, 43), we believe that miR-9* is a *bona fide* SOX2 repressor in NSCs and GSCs, because miR-9* expression is predominantly restricted in differentiated brain cells *in vivo* and *in vitro*, and is inversely associated

with SOX2 expression in NSCs and differentiated neuronal cells (27, 28, 44).

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank Kenneth S. Kosik (University of California, Santa Barbara, CA) for providing the pMir-Report-SOX2–3′-UTR plasmid.

## Grant Support

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST; no. 2008-0058785). H-M. Jeon is a recipient of the Best Graduate Student Scholarship from Korea University.

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.

Received September 10, 2010; revised February 17, 2011; accepted March 3, 2011; published online April 29, 2011.

## References

1. Wen PY, Kesari S. Malignant gliomas in adults. N Engl J Med 2008;359:492–507.
2. Khasraw M, Lassman AB. Advances in the treatment of malignant gliomas. Curr Oncol Rep 2010;12:26–33.
3. Reithmeier T, Graf E, Piroth T, Trippel M, Pinsker MO, Nikkhah G. BCNU for recurrent glioblastomas multiforme: efficacy, toxicity and prognostic factors. BMC Cancer 2010;10:30.
4. Stupp R, Mason WP, Van Den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987–96.
5. Recinos VR, Tyler BM, Bekelis K, Sunshine SB, Vellimana A, Li KW, et al. Combination of intracranial temozolomide with intracranial carmustine improves survival when compared with either treatment alone in a rodent glioma model. Neurosurgery 2010;66:530–7.
6. Vescovi AL, Galli R, Reynolds BA. Brain tumour stem cells. Nat Rev Cancer 2006;6:425–36.
7. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003;63:5821–8.
8. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5:275–84.
9. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006;444:756–60.
10. Das S, Srikanth M, Kessler JA. Cancer stem cells and glioma. Nat Clin Pract Neurol 2008;4:427–35.
11. Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. Mol Cancer 2006;5:67.
12. Shervington A, Lu C. Expression of multidrug resistance genes in normal and cancer stem cells. Cancer Invest 2008;26:535–42.
13. Nakai E, Park K, Yawata T, Chihara T, Kumazawa A, Nakabayashi H, et al. Enhanced MDR1 expression and chemoresistance of cancer stem cells derived from glioblastoma. Cancer Invest 2009;27:901–8.
14. Bleau AM, Hambardzumyan D, Ozawa T, Fomchenko EI, Huse JT, Brennan CW, et al. PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. Cell Stem Cell 2009;4:226–35.
15. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215–33.
16. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76.
17. Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell 2009;137:647–58.
18. Judson RL, Babiarz JE, Venere M, Blelloch R. Embryonic stem cell-specific microRNAs promote induced pluripotency. Nat Biotechnol 2009;27:459–61.
19. Gidekel S, Pizov G, Bergman Y, Pikarsky E. Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell 2003;4:361–70.
20. Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat Rev Cancer 2008;8:976–90.
21. Tian Y, Luo A, Cai Y, Su Q, Ding F, Chen H, et al. MicroRNA-10b promotes migration and invasion through KLF4 in human esophageal cancer cell lines. J Biol Chem 2010;285:7986–94.
22. Gangemi RM, Griffero F, Marubbi D, Perera M, Capra MC, Malatesta P, et al. SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. Stem Cells 2009;27:40–8.
23. Jeon HM, Jin X, Lee JS, Oh SY, Sohn YW, Park HJ, et al. Inhibitor of differentiation 4 drives brain tumor-initiating cell genesis through cyclin E and notch signaling. Genes Dev 2008;22:2028–33.
24. Joo KM, Kim SY, Jin X, Song SY, Kong DS, Lee JI, et al. Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. Lab Invest 2008;88:808–15.
25. Villerbu N, Gaben AM, Redeuilh G, Mester J. Cellular effects of purvalanol A: a specific inhibitor of cyclin-dependent kinase activities. Int J Cancer 2002;97:761–9.
26. Perk J, Iavarone A, Benezra R. Id family of helix–loop–helix proteins in cancer. Nat Rev Cancer 2005;5:603–14.
27. Ko MH, Kim S, Hwang do W, Ko HY, Kim YH, Lee DS. Bioimaging of the unbalanced expression of microRNA9 and microRNA9* during the neuronal differentiation of P19 cells. FEBS J 2008;275:2605–16.
28. Yoo AS, Staahl BT, Chen L, Crabtree GR. MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. Nature 2009;460:642–6.
29. Kamachi Y, Uchikawa M, Kondoh H. Pairing SOX off: with partners in the regulation of embryonic development. Trends Genet 2000;16:182–7.
30. Phi JH, Park SH, Kim SK, Paek SH, Kim JH, Lee YJ, et al. Sox2 expression in brain tumors: a reflection of the neuroglial differentiation pathway. Am J Surg Pathol 2008;32:103–12.
31. Pevny LH, Nicolis SK. SOX2 roles in neural stem cells. Int J Biochem Cell Biol 2010;42:421–4.
32. Ikushima H, Todo T, Ino Y, Takahashi M, Miyazawa K, Miyazono K. Autocrine TGF-beta signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors. Cell Stem Cell 2009;5:504–14.
33. Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, 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.
34. Nies AT, Jedlitschky G, König J, Herold-Mende C, Steiner HH, Schmitt HP, et al. Expression and immunolocalization of the multidrug resistance proteins, MRP1–MRP6 (ABCC1–ABCC6), in human brain. Neuroscience 2004;129:349–60.
35. Zhang B, Pan X, Cobb GP, Anderson TA. MicroRNAs as oncogenes and tumor suppressors. Dev Biol 2007;302:1–12.
36. Pan YZ, Morris ME, Yu AM. MicroRNA-328 negatively regulates the expression of breast cancer resistance protein (BCRP/ABCG2) in human cancer cells. Mol Pharmacol 2009;75:1374–9.
37. To KK, Robey RW, Knutsen T, Zhan Z, Ried T, Bates SE. Escape from hsa-miR-519c enables drug-resistant cells to maintain high expression of ABCG2. Mol Cancer Ther 2009;8:2959–68.

38. Wang F, Xue X, Wei J, An Y, Yao J, Cai H, et al. hsa-miR-520h downregulates ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations. Br J Cancer 2010;103:567–74.
39. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 2008;40:499–507.
40. Saigusa S, Tanaka K, Toiyama Y, Yokoe T, Okugawa Y, Ioue Y, et al. Correlation of CD133, OCT4, and SOX2 in rectal cancer and their association with distant recurrence after chemoradiotherapy. Ann Surg Oncol 2009;16:3488–98.
41. Riggi N, Suvà ML, De Vito C, Provero P, Stehle JC, Baumer K, et al. EWS-FLI-1 modulates miRNA145 and SOX2 expression to initiate mesenchymal stem cell reprogramming toward Ewing sarcoma cancer stem cells. Genes Dev 2010;24:916–32.
42. Iio A, Nakagawa Y, Hirata I, Naoe T, Akao Y. Identification of non-coding RNAs embracing microRNA-143/145 cluster. Mol Cancer 2010;9:136.
43. Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, et al. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 2009;460:705–10.
44. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol 2004;5:R13.