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Original Article

Sox2, a stemness gene, regulates tumor-initiating and drug-resistant properties in CD133-positive glioblastoma stem cells

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Abstract

Background: Glioblastoma multiforme (GBM) is the most lethal type of adult brain cancer and performs outrageous growth and resistance regardless of adjuvant chemotherapies, eventually contributing to tumor recurrence and poor outcomes. Considering the common heterogeneity of cancer cells, the imbalanced regulatory mechanism could be switched on/off and contribute to drug resistance. Moreover, the subpopulation of GBM cells was recently discovered to share similar phenotypes with neural stem cells. These cancer stem cells (CSCs) promote the potency of tumor initiation. As a result, targeting of glioma stem cells has become the dominant way of improving the therapeutic outcome against GBM and extending the life span of patients. Among the biomarkers of CSCs, CD-133 (prominin-1) has been known to effectively isolate CSCs from cancer population, including GBM; however, the underlying mechanism of how stemness genes manipulate CSC-associated phenotypes, such as tumor initiation and relapse, is still unclear.

Methods: Tumorigenicity, drug resistance and embryonic stem cell markers were examined in primary CD133-positive ($CD133^+$) GBM cells and $CD133^+$ subpopulation. Stemness signature of $CD133^+$ GBM cells was identified using microarray analysis. Stem cell potency, tumorigenicity and drug resistance were also tested in differential expression of SOX2 in GBM cells.

Results: In this study, high tumorigenic and drug resistance was noticed in primary CD-133^+ GBM cells; meanwhile, plenty of embryonic stem cell markers were also elevated in the CD-133+ subpopulation. Using microarray analysis, we identified *SOX2* as the most enriched gene among the stemness signature in $CD133^+$ GBM cells. Overexpression of *SOX2* consistently enhanced the stem cell potency in the GBM cell lines, whereas knockdown of *SOX2* dramatically withdrew CD133 expression in $CD133^+$ GBM cells. Additionally, we silenced *SOX2* expression using RNAi system, which abrogated the ability of tumor initiation as well as drug resistance of $CD133^+$ GBM cells, suggesting that *SOX2* plays a crucial role in regulating tumorigenicity in $CD133^+$ GBM cells.

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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Conclusion: *SOX2* plays a crucial role in regulating tumorigenicity in $CD133^+$ GBM cells. Our results not only revealed the genetic plasticity contributing to drug resistance and stemness but also demonstrated the dominant role of *SOX2* in maintenance of GBM CSCs, which may provide a novel therapeutic target to overcome the conundrum of poor survival of brain cancers.

Keywords: CD133-positive cell; glioblastoma; Sox-2

1. Introduction

Glioblastoma multiforme (GBM) is a common, malignant form of high-grade glioma. Despite treatment, prognosis is still very poor within 12 months from diagnosis of GBM.[1] In addition, neurological symptoms such as epilepsy and neural dysfunction have serious consequences for patients' quality of life. Understanding the molecular mechanisms of disease progression and chemoradioresistance in GBM is crucial for developing therapeutic approaches and improving patient survival.[2,3] Currently, the first-line treatment of newly diagnosed GBM is surgical resection to the greatest extent followed by adjuvant chemo- or radiotherapy. However, tumor relapse commonly occurs within months following adjuvant therapy. Some studies suggested that cancer stem cells (CSC) are key contributors to therapeutic resistance and are thought to be responsible for GBM progression as well as recurrence after conventional therapy. Accumulated evidence indicates only a subset of GBM cancer cells are able to proliferate extensively, whereas most cancer cells only show a limited ability for proliferation.[4,5] Also, some studies further demonstrate that the characteristics of stem cells originating from CSCs exhibit a self-renewing capacity and are suggested to be responsible for tumor heterogeneity, maintenance and metastasis.[6,7] It has been recently shown that CSCs are responsible for the formation and growth of neoplastic tissue and are resistant to chemotherapy, explaining why traditional chemotherapies fail to fully eradicate the tumor mass and recurrence occurs. The method of CSC isolation from solid tumors has been well-established through three distinct methodologies based on the properties of CSC.[8,9] CD133 (also known as prominin-1), a membrane protein, has long been linked to glioblastoma CSC isolation. Despite CD133 having first been identified from hematopoietic stem cells and the function of CD133 not yet having been uncovered, isolation via CD133 is still broadly used for the isolation of CSCs due to its rapid and efficient advantages using magnetic-activated cell sorting (MACS). Some reports mentioned that chemotherapy as well as radiotherapy increased the proportion of CSCs.[10,11] The results demonstrated that CD133-expressing CSCs from brain tumors play a critical role not only in the restoration of tumor cells and CSCs but also in resistance to radiotherapy.[12,13] Although the role of CD133 in brain tumor cells is not fully characterized, early data suggest that the CD133-positive ($CD133^+$) subpopulation of brain tumor cells may be an important target for optimizing brain tumor treatment through radiotherapy.

Sox2, a well-known marker of embryonic stem cells, is critical for maintaining the self-renewing properties of stem cells and is used for somatic cell reprogramming.[14,15] Abundant Sox2 expression has been linked to the maintenance of CSC properties in glioma and medulloblastoma.[16,17] Remarkably, glioblastoma stem cells have high Sox2 expression, while Sox2 knockdown forfeits the tumorigenicity and stemness in glioma CSCs.[16] Sox2 has been proven to regulate self-renewal and proliferation in neural stem cells, and is crucial in embryonic development. Therefore, investigation of the regulation of Sox2-associated drug resistance is important.

In this study, we had three different but interconnected aims: (1) elucidation of the role of Sox2 targeting pathways in GBM and GBM-CSC; (2) identification of the novel CD133/Sox2 regulatory axis; and (3) investigation of Sox2-mediated oncogenic, cytoprotective signaling pathway, as well as the drug resistance mechanism. We have identified the significant role of CD133 in regulating GBM malignancy, and further identified Sox2 as one of CD133 downstream targets which promote self-renewal in GBM-derived stem cells. Thus, the results of this study could be an interpretation of the role of CSCs in radio-resistance and the oncogenic properties of GBM, and could provide important information for developing novel therapeutic strategies against GBM-CSCs.

2. Methods

2.1. Cell lines and treatment

Primary GBM cells, clone 1 and clone 2, were isolated from GBM patients following surgery and written informed consent was provided. Briefly, sterile scissors were used to cut the tumor into small pieces and dissociate tissue using collagenase IV for 1 hour. Then the primary cells were seeded on 75 cm^2 flask in DMEM/F12 medium plus glutamate (5 mmol/L) and 10% fetal bovine serum. Cells were replaced with fresh medium twice a week and incubated at 37°C in 5% CO_2. Both GBM primary clones were cultured over 10 selective passages.

2.2. Isolation and culture of glioblastoma-derived $CD133^+$ cells

This study was conducted according to the tenets of the Declaration of Helsinki, and all samples were acquired after patients provided informed consent. All cells in samples were dissociated using trypsin and labeled with 1 mL CD133/l micromagnetic beads per 1 million cells following the

CD133 cell isolation kit protocol (Miltenyi Biotech, Auburn, CA, USA). The sorted $CD133^+$ CSCs were maintained in a serum-free medium culture system, which contained DMEM/F12 (Gibco-BRL, Gaithersburg, MD), N2 supplement (R&D Systems, Inc., Minneapolis, MN, USA), 20 ng/ml human recombinant bFGF (R&D Systems) and 20 ng/ml EGF (R&D Systems). CD133-negative ($CD133^-$) cells were cultured in DMEM medium with 10% fetal bovine serum and glutamate (5 mmol/L) added. The expression of CD133 surface markers was determined by flow cytometry. Briefly, 1×10^5 cells were suspended in 100 μL of phosphate-buffered saline (PBS) with 5μL anti-human CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) and chilled on ice for 15 minutes. After washing two times with PBS, cells were resuspended in 100 μL of PBS, and analyzed with a FACSCalibur (Becton-Dickinson, San Jose, CA, USA).[18–21]

2.3. Lentiviral-mediated RNA interference

Lentiviral backbone-Sox2 short hairpin RNA (shRNA) was purchased from the National RNA interference Core Facility, Academia Sinica, Taiwan. All the plasmids were separately transfected into 293T cells, and the validation of expression of Sox2 in protein level was estimated by Western blotting. The shSox2-lentivirus was generated by transfection of 293T cells using Lipofectamine 2000 (LF2000, Invitrogen, Carlsbad, CA, USA), with supernatants obtained after 48 hours transfection. This resulted in 80% of the confluent cells being infected with lentivirus in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA) in the virus soup.

2.4. Immunofluorescent staining

Samples were fixed in 4% paraformaldehyde for 15 minutes on ice then washed three times with PBS. Blocking and antibody dilution buffer used PBS containing 5% normal goat serum. The anti-Sox2 and anti-CD133 antibodies were added at a dilution of 1:200 in antibody dilution buffer. After incubation overnight at 4°C, samples were washed with PBS three times. The fluorescein isothiocyanate-labeled and allophycocyanin-labeled secondary antibodies were added to the samples for 1 hour and washed twice with PBS. Then, Hoechest 33342 was treated for 10 minutes to label the nuclear and mounted samples by an antifade solution (Dako Corp., Carpinteria, CA, USA). Slides were observed and analyzed using a confocal fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Images were captured using a Photometrics Sensys CCD camera (Roper Scientific, Tucson, AZ, USA).

2.5. Quantitative real-time reverse-transcriptase-polymerase chain reaction

The quantitative reverse-transcriptase-polymerase chain reaction (RT-PCR) followed previously described procedures, and cDNA was obtained from each sample which was reverse-transcribed by Superscript II RT (Invitrogen). The primer sequences used in this study were Oct4: F: GTGGAGAGCAACTCCGATG, R: TGCTCCAGCTTCTCCTTCTC; Klf4: F: CCGCTCCATTACCAAGAGCT, R: ATCGTCTTCCCCTCTTTGGC; Nanog: F: ATTCAGGACAGCCCTGATTCTTC, R: TTTTTGCGACACTCTTCTCTGC; Sox2: F: CGAGTGGAAACTTTTGTCGGA, R: TGTGCAGCGCTCGCAG. The amplification was performed using LightCycler–FastStart DNA Master SYBR green I (Roche Molecular Systems, Alameda, CA, USA). PCRs were incubated at 95°C for 10 minutes, then denatured at 95°C for 10 seconds, annealed at 55°C for 5 seconds, and extended at 72°C for 20 seconds followed by 40 cycles. All reactions followed the rule of standard curves of cycle threshold values versus template concentrations, then each target gene was compared with the endogenous reference (GAPDH) in each sample. Data analysis was quantified by LightCycler Relative Quantification Software version 3.3 (Roche Molecular Systems).[22]

2.6. Western blot assay

In this study, protein extraction and Western blot procedure were done as previously reported.[20] Briefly, the prepared protein samples were added to protein sample buffer and heated at 95°C for 5 minutes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used by loading 40 μg of protein extracted from each sample, then the wet-transfer system was used to transfer the protein to Hybond-electrochemiluminescence (ECL) nitrocellulose paper (Amersham, Arlington Heights, IL, USA). The primary antibodies used were rabbit anti-human Sox2 (1: 2000) and mouse anti-ß-actin (1:5000) (Chemicon, Temecula, CA, USA). ECL chromatography was detected by the ECL detection system (Amersham Biosciences Co., Piscataway, NJ, USA).

2.7. In vivo tumor model

Six-week-old female nude mice were used in this research. The mice were caged in groups of five or less. Animals were fed a diet of animal food and water *ad libitum*. All surgical procedures followed the institutional animal welfare guidelines of Taipei Veterans General Hospital. $CD133^+$, $CD133^-$ and other treated cells were dissociated by trypsin, and cells were counted in different cell dosages separately. Cells were then suspended in 5 μL PBS and injected at 3 mm to the right of the bregma in the brain using a Hamilton syringe. Further, we cleaned the surface of the wound with sterile cotton and filled the injection hole with bone wax after injection. All mice with orthotopic brain tumors were measured for tumor size every week.

2.8. Statistical analysis

All results are shown as mean ± SD, and statistical analysis was performed using a Student *t* test, one- or two-way analysis of variance test followed by Tukey's test, as appropriate. A p value <0.05 was considered statistically significant.

3. Results

3.1. Isolation and characterization of CD133$^+$ cells from GBM cell lines

Using magnetic beads,[18] we successfully isolated CD133$^+$ cells from GBM cell lines (Fig. 1A). We subsequently examined the mRNA levels of "stemness" genes (*Oct-4/Nanog/Sox-2/KLF-4*) by real-time RT-PCR and found that these "stemness" genes were up-regulated in GBM-CD133$^+$ as compared to GBM-CD133$^-$ (Fig. 1C). It has been reported that tumor initiating cells can be cultured in suspension to generate floating spheroid bodies (SB) that maintain their self-renewal capabilities in serum-free media with bFGF and EGF.[23,24] We further evaluated the chemo- and radiation-resistant abilities of CD133$^+$ and CD133$^-$. When compared with CD133$^-$, CD133$^+$ was significantly resistant to cisplatin (Fig. 1D; $p < 0.01$) and VP-16 (Fig. 1E; $p < 0.01$) in the individual clones isolated from GBM cell lines. As shown in Fig. 1F, after 2 Gy ionizing radiation treatment, the survival rate and number of CD133$^+$ were significantly higher than those of CD133$^-$ ($p < 0.01$; Fig. 1F). These data support our observation that GBM-CD133$^+$ tumor initiating cells possess a higher degree of radio-chemoresistance.

3.2. Detection of high Sox2 expression in isolated CD133$^+$ cells from GBM cell lines

To further identify the systemic differential gene expression profile in GBM-CD133$^+$, we performed a literature-based network analysis of all MEDLINE records (title and abstract) and used Cytoscape software (NIGMS, Bethesda, MD, USA) to group the target-linkage genes from our microarray data using a natural language processing regimen for gene and protein names. This analysis successfully identified literature-based network genes that were involved in stemness signatures, and Sox-2-related pathway in GBM-CD133$^+$ as opposed to GBM-CD133$^-$ (Fig. 2A). To further address the regulatory pathways, we dissected the network shared by Sox2 as well as CD133 (also known as PROM1) and demonstrated a close relationship between the duos using Cytoscape. Upon the knowledge-based data mining, we noticed that Sox2 and CD133 are closely connected through a bunch of stemness genes including LIN28B, OCT4 (a.k.a. POU5F1), and several

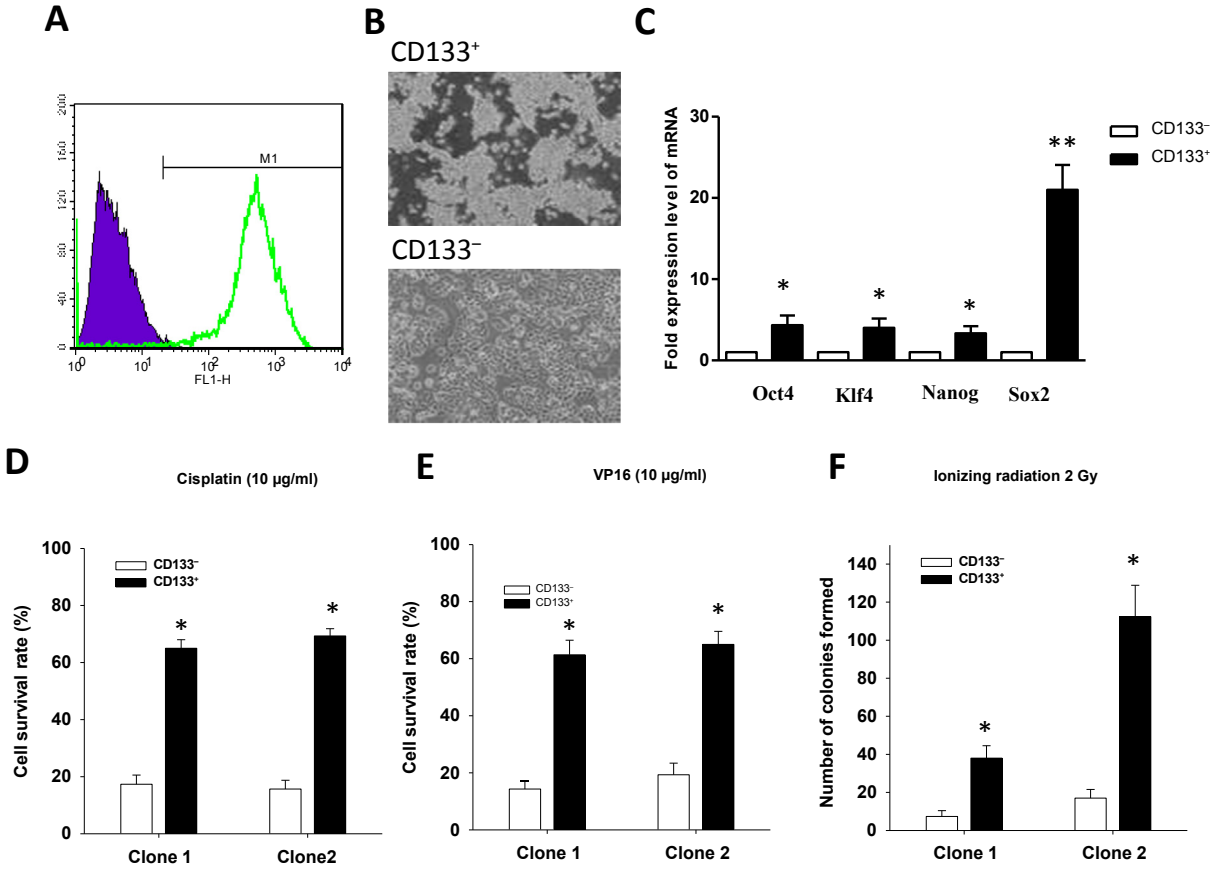

Fig. 1. Isolation and characterization of CD133$^+$ tumor initiating cells from primary GBM cell lines. (A) Population of CD133$^+$ GBM cells was estimated by flow cytometry using magnetic beads. (B) The morphological difference between CD133$^+$ and CD133$^-$ GBM cells are shown (scale bar represents 100 μM). (C) The mRNA expression level of stemness genes (Oct4, Klf4, Nanog and Sox2) in CD133$^+$ GBM cells as compared with CD133$^-$ GBM cells. (D, E) The survival rate of CD133$^+$ GBM cells was increased after cisplatin and VP-16 treatment for 24 hours compared with CD133$^-$ GBM cells. (F) The effect of radio-resistance in CD133$^+$ and CD133$^-$ GBM cells was analyzed by colony formation assay. (Clone 1 and clone 2 were collected from different GBM patients.). CD133$^+$ = CD133-positive; CD133$^-$ = CD133-negative; GBM = glioblastoma multiforme.

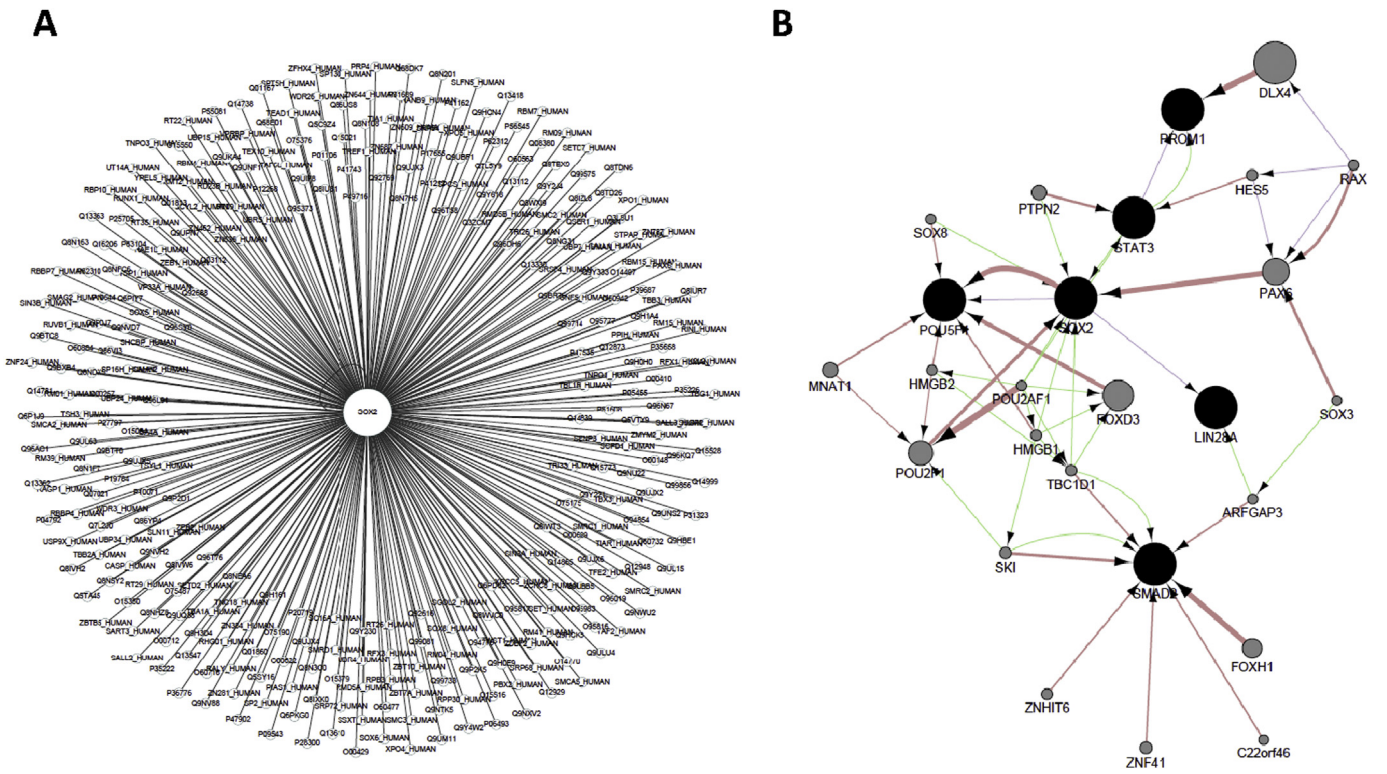

Fig. 2. A simplified scheme of Sox2-related pathway in GBM-CD133$^+$ cells as opposed to GBM-CD133$^-$ with systems biology analysis. (A) Literature-based network analysis to group the target-linkage genes from our microarray data using a natural language processing regimen for gene and protein names. This analysis successfully identified literature-based network genes that were involved in stemness signatures, and Sox-2-related pathway in GBM-CD133$^+$ as opposed to GBM-CD133$^-$. (B) The expression and the knowledge-based data mining presence of GBM-CD133$^+$ cells was directly correlated with LIN28, Sox2, POU5F1, STAT3 and SMAD2/3. Lines indicate co-citation in the literature in more than one article. CD133$^+$ = CD133-positive; CD133$^-$ = CD133-negative; GBM = glioblastoma multiforme.

key regulators including STAT3 and Smad2/3 transcription factors (Fig. 2B). Furthermore, we aligned the literature-based network along a signaling cascade trend, which clearly displayed that Sox2 coordinately regulated the stemness with CD133 (Fig. 2B).

The results of this literature linkage analysis support the microarray data, which suggest that Sox2-axis pathway expression is involved in the activation of anti-apoptosis and cell migration-related gene clusters (Fig. 2). Furthermore, using immunofluorescence assays, we detected high expression levels of Sox-2 protein in GBM-CD133$^+$ (Fig. 3A) as compared to CD133$^-$ cells ($p < 0.01$; Fig. 3B). In accordance with the systemic analysis, our immunofluorescence assay detected high levels of Sox2 in GBM-CD133$^+$ from two patients with GBM, but not in GBM-CD133$^-$ (Fig. 3). Taken together, these data suggested that Sox2, an embryonic stem cell marker, is highly expressed in GBM CSCs, as well as highly correlated with CD133 surface in CSCs.

3.3. The significance of Sox2 in GBM and GBM stem cells

To address whether Sox2 plays a role in maintaining self-renewal or in regulating glioma stem-like properties in GBM, we assessed the CSC-GBM cells' derived SB formation ability and CD133 surface marker expression (Fig. 4). Firstly, we performed the gene silencing method using the short interfering RNA system with lentiviral vector for knockdown of Sox2 expression in GBM-CD133$^+$. We found it important that the treatment of Sox2 shRNA in GBM-CD133$^+$ significantly inhibited the ability for SB formation ($p < 0.001$; Fig. 4A). The result of Western blotting showed the treatment of Sox2-shRNA effectively blocked Sox2-protein expression in GBM-CD133$^+$ (Fig. 4B), confirming the suppressive efficacy of Sox2-shRNA. Furthermore, after 14 days of treatment with Sox2 shRNA, the SB of GBM-CD133$^+$ could not maintain floating spheres, and proliferation ability under stem cell culture medium was significantly suppressed. Moreover, the treatment of control shRNA with lentiviral vector did not affect the Sox2-protein expression (Fig. 4B) and sphere-formation ability in GBM-CD133$^+$ (Fig. 4A and C). Notably, the results of FASC-flow cytometry demonstrated that the expression levels of CD133 were significantly inhibited in Sox2 shRNA-treated GBM-CD133$^+$ and the percentages of GBM-CD133$^-$ were dramatically elevated in GBM-CD133$^+$, which was treated with Sox2 shRNA for 14 days after infection ($p < 0.001$; Fig. 4D). Coordinately, the *in vivo* limited dilution xenograft assay revealed that the knockdown of Sox2 by shRNA dramatically eliminated the tumorigenicity of CD133$^+$ GBM CSCs (Table 1). These data indicated that Sox2 may maintain the self-renewal and further regulate cancer stem-like properties in malignant glioma.

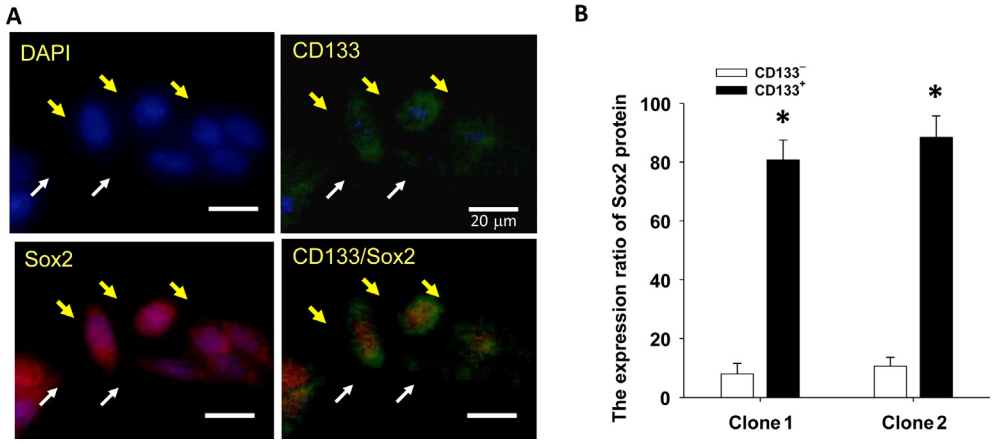

Fig. 3. The significant expression of Sox2 in GBM and GBM stem cells. (A) The localization of CD133 and Sox2 was identified by immunofluorescence in $CD133^+$ GBM cells. (B) The quantitative expression levels of Sox2 protein in $CD133^+$ and $CD133^-$ GBM cells were analyzed by imaging analysis software. $CD133^+$ = CD133-positive; $CD133^-$ = CD133-negative; GBM = glioblastoma multiforme.

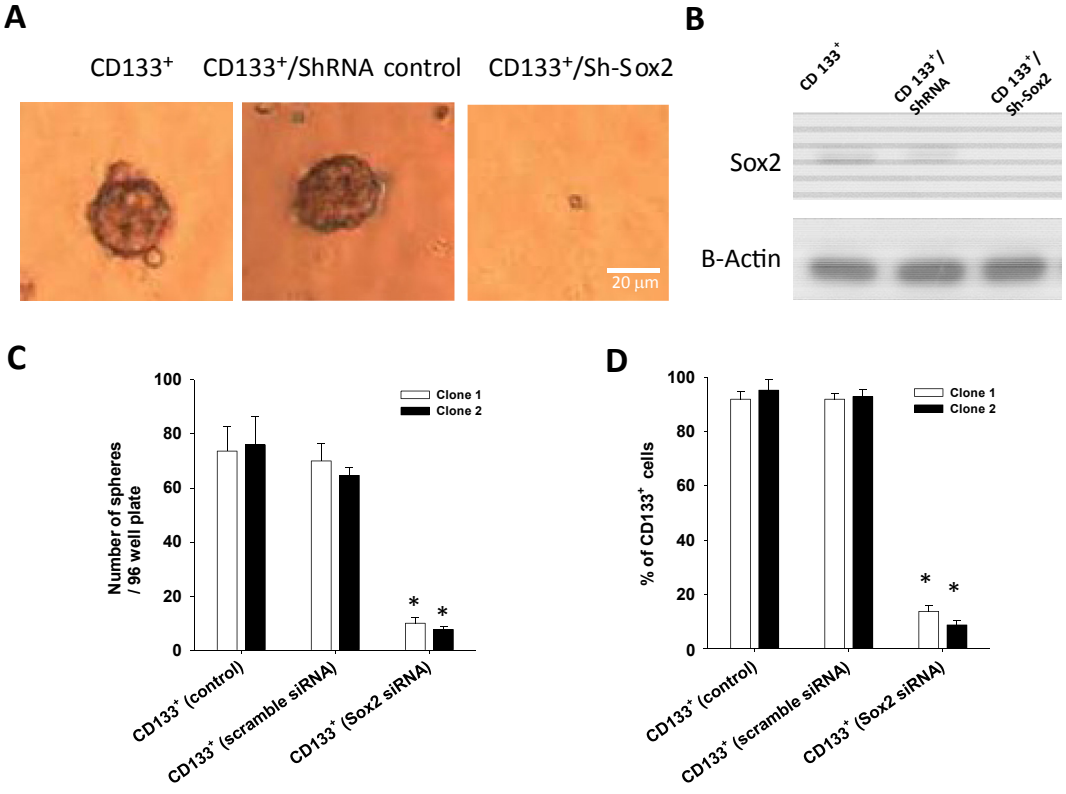

Fig. 4. The regulation of CSC properties by Sox2. (A) Phase-contrast images of tumor sphere in parental, scramble control and Sox2 knockdown $CD133^+$ GBM cells are shown (scale bar means 100 μM). (B) The protein expression of Sox2 and beta-actin in parent, scramble control and Sox2 knockdown $CD133^+$ cells were estimated by Western blot. (C) Sox2 knockdown in $CD133^+$ GBM cells decreased the sphere formation ability as compared with parental and scramble control of $CD133^+$ GBM cells. (D) The CD133 population was decreased as Sox2 knockdown in $CD133^+$ GBM cells.

4. Discussion

CSCs have been largely discussed with regard to their characteristics and the methodologies used to isolate tumor cells from malignant cancers. Considering the heterogeneity of the tumor burden, diverse approaches have been developed to isolate different subpopulations of CSCs. CD133 has been well-characterized as a markable surface marker for several tumor stem cells, including these of leukemia, lung cancer, hepatic cancer, gastric cancer, breast cancer and brain tumor.[25–29] Therefore, techniques using fluorescence-activated cell sorting and MACS have been developed to efficiently isolate CSCs, including $CD133^+$ cancer cells, from a bunch of tumor cells. Secondly, the side population of tumor

Table 1
Analysis of tumorigenic abilities in $CD133^{+/-}$ cells isolating from GBM clones.

	Injected number of cells	$CD133^-$	$CD133^+$	$CD133^+$/si RNA	$CD133^+$/si Sox2
Clone 1	100,000	0/3	3/3	3/3	0/3
	10,000	0/3	3/3	3/3	0/3
	3000	0/3	2/3	3/3	0/3
Clone 2	100,000	0/3	3/3	3/3	0/3
	10,000	0/3	3/3	3/3	0/3
	3000	0/3	3/3	2/3	0/3

$CD133^+$ = CD133-positive; $CD133^-$ = CD133-negative; GBM = glioblastoma multiforme.

tissues or cancer cell lines, which may cause chemoresistance, significantly displays intracellular Hoechst 33342 exclusion in isolated CSCs.[30] Expression of ABC transporter, including ABCG2, an ATPase transporter, is closely associated with the side population phenotype and drug-resistant abilities.[31,32] Thirdly, sphere formation is enriched in CSCs under the inoculation of defined serum-free medium with growth factors from individual solid tumors, primary tumor cells, or cancer cell lines.[33–36] Each method for CSC isolation contributes to a different phenotype, but are all prone to tumor initiation, tumor metastasis and drug resistance. In this study, we demonstrated that MACS was used to quickly obtain GBM CSCs via CD133 expression, which may acquire a different subpopulation compared to side population, which can directly indicate drug resistance regarding the rapid drug efflux through ABC transporters. Indeed, our results concomitantly showed that $CD133^+$ GBM CSCs demonstrate a high ability for drug-resistance. In addition, activation of the Hedgehog-Gli1 pathway has been identified in a population of chemoradioresistant glial tumor cells, known as CSCs-$CD133^+$.[37] These CSCs-$CD133^+$ are resistant to current treatment modalities, such as radiation and temozolomide-based chemotherapy.[38] Xenografts in immunocompromised mice were significantly blocked by shRNA targeting Sox2. Coherently, knockdown of Sox2 significantly suppressed the sphere formation ability and side population percentage (Fig. 4). These data suggested that Sox2 is important for both tumorigenicity and drug resistance in CD133-isolated GBM stem cells.

Sox2, one of the neural stem cell markers, is a key member of the transcriptional regulatory network that regulates pluripotency and self-renewal of stem cells. Sox2 has also been found to express in various tumor tissues, including lung cancers, colorectal cancers, and pediatric brain tumors.[39,40] Importantly, Gangemi et al demonstrated that tumor initiating ability could be diminished through silencing Sox2 expression in freshly derived glioblastoma tumor-initiating cells. They further suggested that silenced Sox2 GBM tumor-initiating cells would stop proliferating and lose tumorigenicity in immunosuppressant mice.[15] Furthermore, Yang et al showed that the knockdown of the *SOX2* gene in the GBM cell line (LN229) reduces cellular proliferation and colony formation.[41] Interestingly, we found a putative connection between CD133 and Sox2 using bioinformatics analysis (Fig. 2). Notably, several stemness gene signatures were involved, as well as proliferation-related genes, suggesting that crosstalk between the duos is required for CSC properties.

In conclusion, our results suggest that Sox2 plays a critical role in the carcinogenesis and maintenance of GBM stem cells, which greatly contribute to resistance to therapies including chemotherapy and radiotherapy. Here, we have illustrated a new regulatory axis of CD133-Sox2 coordinates to the enrichment of CSCs from brain tumors. Thus, CD133-Sox2 can be a suitable target for new therapeutics in a curative intent aimed at GBM in the coming era.

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