Selective enrichment of CD133+/SOX2+ glioblastoma stem cells via adherent culture

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Abstract. Most of the brain tumors are malignant with an extremely poor survival rate. Recent progress in identifying cancer stem cells (CSCs) within the brain tumors is starting to revolutionize our understanding in the imitation and progression of tumors as well as relapse and the development of therapeutic strategies. Suspension spheroid body culture paradigm is a routine method in enriching CSCs. While, it was reported recently that CSCs within the brain tumor may also be enriched through adherent monolayer culture with optimized properties. In the present study, 18 surgically resected brain tumors were used for analyzing the feasibility of adherent enrichment of CSCs. The results indicated that 50% of glioblastomas were able to generate adherent CSCs, which were uniformly positive for Sox2, CD133, GFAP and Nestin. However, adherent culture paradigm failed to yield CSCs in secondary brain tumors, including neurocytomas, ependymomas, germ cell tumors or low-grade astrocytomas, which is most likely due to a lack of CD133+/Sox2+ cells within the original biopsies. Therefore, it was concluded that the adherent culture paradigm may serve as a reliable method in enriching brain CSCs, but this method is more suitable for enriching CD133+/Sox2+ CSCs in glioblastomas.

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Introduction

According to the World Health Organization (WHO), central nervous system (CNS) tumors can be subdivided into primary and secondary tumors. Secondary tumors develop from cells that spread to the brain from a cancer in another part of the body. Primary brain tumors, including medulloblastoma, choroid plexus papilloma, pituitary tumor, neurocytoma, germ cell tumor and glioma in the majority of cases start from local cells of the brain (1). Given the ability of brain tumors to infiltrate and a high rate of recurrence, the prognosis is usually poor even though multimodal therapies have been applied (2). For example, it was reported that the median survival is ≤15 months for patients with grade IV glioma (glioblastoma, GBM) (2).

The cancer stem cell (CSC) hypothesis originates from the hierarchical cellular organization within a tumor. Indeed, CSCs could be enriched and expanded *in vitro*, and more importantly they could initiate the formation of new tumors following xenotransplantation (3-7). An increasing number of studies suggested that purified CSCs grown *in vitro* could be applied for studying the initiation and progression of tumor as well as chemo- or radiotherapy resistance, tumor relapse and testing for new therapeutic drugs (3,5). In general, suspension spheroid-formation culture protocols are used for CSC enrichment. Solid brain tumor mass is disassociated into single cells and then grown in a non-adherent serum-free culture with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) to trigger spheroid formation (6). By using this suspension culture technique, CSCs have been successfully cultured from various brain tumors, including glioblastoma, medulloblastoma and oligodendroglioma (4,7-9). However, the efficiency for initial growing tumorspheres from brain tumors may be low and variable (10-12). Furthermore, the spheroid culture method tends to enrich CSCs with specific molecular profiles, including phosphatase and tensin homolog deficiency, wild-type isocitrate dehydrogenase 1, amplified chromosome 7 and deleted chromosome 10q (4,13).

Previously, two groups reported that they developed a new adherent culture protocol for growing brain CSCs (14,15). In this protocol, freshly resected glioblastomas from patients were digested into single cells and then seeded onto laminin-coated culture surface under standard neural stem

cell culture conditions (14,15). Through this advanced cell culture protocol, brain CSCs could be enriched as an adherent monolayer, which displayed a great potential for initiating the formation of new tumors *in vivo* (14,15).

In the present study, the efficiency of deriving brain CSCs was investigated by using this adherent culture method. Whether the adherent culture method may be applied to a wide spectrum of brain tumors or specifically to glioblastomas was also examined. The results indicated that CSCs from glioblastomas but not other types of brain tumors could be grown through adherent culture and the derivation efficiency was ~50%. It was also found that $CD133^+/Sox2^+$ cells within the glioblastomas might be the original cells, which were enriched under adherent culture conditions.

Materials and methods

Ethics statement. The entire study was approved by the Institutional Review Board of the Shanghai Tenth People's Hospital (Shanghai, China). All patients provided written informed consent to the surgical procedures and gave permission for the use of resected tissue specimens. All animal protocols were approved by the Institutional Animal Care and Use Committee of Tongji University (Shanghai, China). A total of 20 male Balb/c nude mice (18-20 g, 6 weeks old) purchased from Shanghai Slaccas Experimental Animal Limited Company (Shanghai, China), were housed in specific pathogen-free conditions throughout the experiments that were automatically maintained at a temperature of 23±2˚C, a relative humidity of 45-65%, and a controlled 12/12 h light/dark cycle. Animals had *ad libitum* access to food and water.

Brain tumor samples. Tumor specimens were obtained from 18 patients with CNS tumors who underwent surgical procedures at the neurosurgery department, the Shanghai Tenth People's Hospital. Tumors were classified by histopathologic examination according to the WHO classification, including the cytokeratin AE1/AE3, epithelial tumor marker (16). The clinical profiles of the patients were obtained from medical records, and patient characteristics are listed in Table I.

Isolation of primary brain CSCs. The standard CSC enrichment protocols (14,15) were followed, which resemble the culture conditions for normal neural stem cells. The tumor tissues were minced with a scissor and then digested with accutase (A11105; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 20 min. The cells were then triturated into single cells and plated on culture surfaces precoated with laminin for ≥3 h at 37˚C. The formula for the culture medium (DMEM/F12/N2/B27) was, DMEM/F12 (catalog no. 11330; Gibco; Thermo Fisher Scientific, Inc.), 1X N2 (catalog no. 17502; Gibco; Thermo Fisher Scientific, Inc.), 1X B27 supplement (catalog no. 17504; Gibco; Thermo Fisher Scientific, Inc.) and 10 ng/ml EGF (catalog no. E9644; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 10 ng/ml bFGF (catalog no. 100-18B; PeproTech, Inc., Rocky Hill, NJ, USA). The cells were attached overnight, and cell debris together with floating red blood cells and tumor cells were removed on the next day. The established CSC lines were regularly passaged every 5-6 days by using accutase for digestion.

Mice brain tissue collection. Male C57BL/6 nude mice (6 weeks) were purchased from Slaccas Experimental Animals LLC, Shanghai Slaccas (Shanghai, China). The mice were deeply anesthetized with avertin (Sigma-Aldrich; Merck KGaA) and fixed with 4% paraformaldehyde in PB for 15 min via transcardial perfusion. The brains were excised and dehydrated in gradient sucrose. The frozen sections were cut at 40 μm in thickness using a freezing microtome (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Immunostaining. The expression levels of CD133, Nestin, Sox2 and GFAP in brain tumor tissues, normal mice brain or cultured cells were analyzed by immunofluorescence staining. The resected brain tumors were fixed with 4% paraformaldehyde (PFA) for 3 h, dehydrated by sucrose and sectioned using a microtome. The cultured cells were plated on laminin-coated coverslips and subsequently were used for immunostaining. The sections or 4% PFA fixed-coverslips with cultured cells were blocked with 10% normal donkey serum (Beijing Biodee Biotechnology Co., Ltd., Beijing, China) and 0.1% Triton X-100/PBS for 1 h at room temperature followed by incubation with primary antibodies overnight at 4˚C. On the next day, after adequate washing, the tissue sections or coverslips were incubated with the appropriate fluorescence-labeled secondary antibodies for 40 min at room temperature. The antibodies used in this study were: Anti-CD133 (1:50; catalog no. ARH4033; Antibody Revolution Inc., San Diego, USA), anti-Nestin (1:500; catalog no. MAB5326; EMD Millipore, Billerica, MA, USA), anti-Sox2 (1:500; catalog no. AF2018; R&D Systems, Inc., Minneapolis, MN, USA), anti-GFAP (1:1,000; catalog no. z0334; Dako A/S, Glostrup, Denmark), Peroxidase-conjugated donkey anti-goat (1:1,000, catalog no. 705035003; Jackson ImmunoResearch Inc., West Grove, PA, USA), Peroxidase-conjugated goat anti-mouse (1:1,000, catalog no. 115035003; Jackson ImmunoResearch Inc.) and Peroxidase-conjugated goat anti-rabbit (1:1,000, catalog no. 111035003; Jackson ImmunoResearch Inc.). The nuclei were counterstained with Hoechst solution for 5 min at room temperature. The images were taken with a confocal microscope (SP-5; Leica Microsystems, Inc.).

Statistical analysis. The data were statistically analyzed using Statistical Package for the Social Sciences (SPSS) statistical (version 21) software from SPSS, Inc., (Chicago, IL, USA) and using one-way analysis of variance followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Enrichment of CSCs from clinically resected glioblastomas through adherent culture. A total of 18 surgically resected brain tumors were mechanically grinded followed by digestion with accutase. Single cell mixtures were plated down onto laminin-coated surface in DMEM/F12/N2/B27 medium supplied with bFGF and EGF. A total of 4 adherent lines were then successfully established from these 18 tumors. As indicated in Table I, all 4 tumors that were able to generate adherent cultures were primary glioblastomas (grade IV

Table I. Adherent CSCs could only be enriched from glioblastomas.

Patient no.	Age	Sex	Tumor localization	Clinical diagnosis	Growth of CSCs	Pathological examination (paraffin section/immunohistochemistry/immunofluorescence)										
						GFAP	p53	Vim	S100	Ki67 (%)	CGA	SYN	AE1/AE3	EMA	EGFR	CD34
1	17	M	Right insula	Germ cell tumors	No	-	++	N/A	+	90	-	-	-	-	N/A	+
2	64	M	Cerebellum	Astrocytoma (WHO II)	No	+	N/A	+	+	2	N/A	N/A	-	N/A	N/A	+
3	71	M	Left temporal-occipital	Glioblastoma (WHO IV)	Yes	+	N/A	+	+	30	-	-	-	N/A	N/A	+
5	61	M	Left temporal lobe	Astrocytoma (WHO III)	No	+	N/A	+	+	30	N/A	N/A	-	-	N/A	+
6	62	M	Frontal lobe	Glioblastoma (WHO I)	Yes	+	N/A	+	+	5	N/A	N/A	-	-	N/A	+
7	46	M	Frontal lobe	Glioblastoma (WHO III)	No	+/-	+	+	+	50	-	+/-	N/A	+	N/A	+
8	14	F	Lateral ventricle	Ependymoma (WHO II)	No	+	+	+	+	4	N/A	+/-	-	N/A	N/A	N/A
9	21	F	Bilateral ventricle	Neurocytoma (WHO II)	No	+	+	N/A	+	15	+	+	N/A	N/A	-	+
10	46	F	Left frontoparietal lobe	Glioblastoma (WHO IV)	No	+	N/A	N/A	+	25	-	N/A	-	-	N/A	+
11	74	M	Corpus callosum	Glioblastoma (WHO IV)	No	+	++	N/A	-	30	-	-	N/A	-	+	N/A
12	32	M	Corpus callosum	Astrocytoma (WHO III)	No	+	++	N/A	N/A	10	N/A	N/A	N/A	N/A	N/A	N/A
13	44	F	Thalamus	Glioblastoma (WHO IV)	No	+	+	+	+	20	-	-	-	-	++	N/A
14	37	M	Right frontal lobe	Astrocytoma (WHO III)	No	+	++	+	+	3	N/A	N/A	-	+	+	+
15	61	M	Left frontal lobe	Glioblastoma (WHO IV)	Yes	+	N/A	+	+	30	-	-	-	-	+	+
16	60	M	Right temporal lobe	Glioblastoma (WHO IV)	No	+	N/A	N/A	+	40	-	-	-	-	N/A	+
17	39	M	Frontal lobe	Astrocytoma (WHO I)	No	+	+/-	+	+	1	-	-	-	-	N/A	+
18	54	M	Right frontal lobe	Glioblastoma (WHO IV)	Yes	+	+	+	+	25	N/A	N/A	N/A	-	N/A	+
19	65	F	Right temporal lobe	Metastatic lung adenocarcinoma	No	-	+/-	-	+	1	-	-	+	+	N/A	+

A total of 18 human brain tumors were mechanically and enzymatically digested into single cells, and plated onto laminin-coated surface in neural culture medium. Among these tumors, adherent CSC lines were only generated from 4 glioblastomas. The detailed information of the patients, pathology and main molecular signatures of the tumors are listed. CGA, chromogranin A; EGFR, epidermal growth factor receptor; CSC, cancer stem cell; EMA, epithelial membrane antigen; GFAP, glial fibrillary acidic protein; SYN, synaptophysin; AE1/AE3, cytokeratin AE1/AE3; WHO, World health Organization.

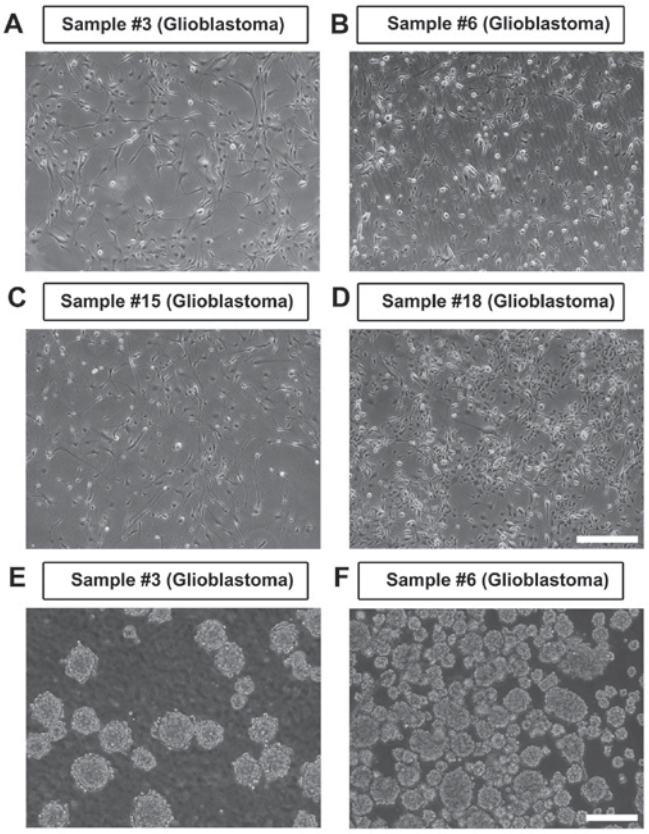

Figure 1. Adherently cultured CSCs can be normally passaged and retain the capability to form tumorspheres. Glioblastoma CSCs in samples (A) no. 3; (B) no 6; (C) no. 15; (D) no. 18 have the typical morphology of neural progenitors. The adherent cells form tumorspheres when re-suspended using spheroid culture conditions (E and F). Scale bars, 300 μm for A-D; 100 μm for E and F. CSC, cancer stem cell.

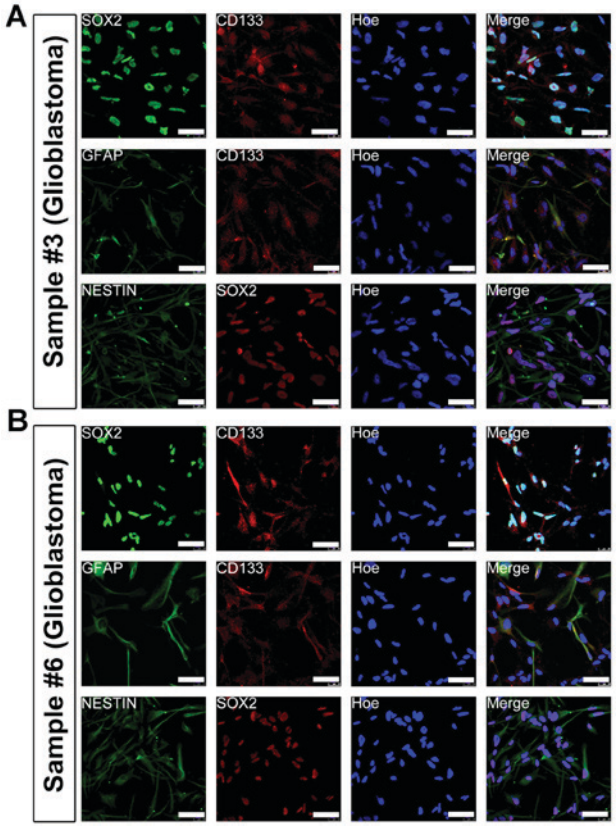

Figure 2. *In vitro* cultured adherents CSCs uniformly express Sox2, CD133, Nestin and GFAP. The CSCs generated from samples (A) no. 3 and (B) no. 6 glioblastomas are homogenously positive for progenitor markers, Sox2, CD133, Nestin and GFAP. Scale bar, 50 μm. CSC, cancer stem cell; GFAP, glial fibrillary acidic protein.

astrocytoma). While other tumor subtypes, including germ cell tumor (patient no. 1), ependymoma (patient no. 8), neurocytoma (patient no. 9), low-grade astrocytoma (patient nos. 2, 5, 12, 14 and 17), and secondary brain tumor metastasized from lung adenocarcinoma (patient no. 19), were not able to generate adherent cultures. This suggests that the adherent culture paradigm favors enriching CSCs from glioblastomas. A total of 4 out of 8 glioblastomas generated adherent CSCs, and therefore the derivation efficiency was ~50% (Fig. 1A-D). The derivation efficiency may be more closely associated with the molecular signatures of CSCs rather than the proliferation rate of the tumor cells, since there is no difference in the percentage of $Ki67^+$ cells in the tumor mass between the groups that succeeded and failed (Table I). A total of 3 out of 4 adherently cultured CSCs were able to be for >50 passages, while cell line 15 gradually stopped growing and died within 3 passages. All derived cell lines exhibited typical progenitor morphology, and tumorspheres were easily formed from adherent cells when re-suspended in a petri dish (Fig. 1E and F). In addition, the cultured cells uniformly expressed CSC markers-Nestin, Sox2, CD133 and GFAP (Fig. 2). Together, these data indicated that the adherent culture paradigm is suitable for enriching homogenous CSCs. In addition, this adherent culture method is more suitable for enriching CSCs from glioblastomas and is less efficient for other brain tumor subtypes.

Secondary brain tumor does not contain $Sox2^+$/$CD133^+$ cells and fails to generate adherent CSCs. Next, whether different types of brain tumors might harbor various molecular signatures were examined, which may account for the differences in the efficiency of CSC enrichment. Nestin, Sox2, CD133 and GFAP are key progenitor markers used for labeling brain CSCs (17). To test the specificity of the antibodies, normal brain tissues that were adjacent to tumors and adult mouse brain tissues were used for immunostaining. As expected, only GFAP-positive astrocytes were detected in normal brain tissues that were adjacent to tumors and adult mouse brain sections. By contrast, no immunostaining were detected for Nestin, Sox2 or CD133 (Fig. 3A, B, D and E). The tumor from patient no. 19 was a secondary brain tumor that was metastasized from lung adenocarcinoma. The results indicated that the metastasized tumor only had infiltrated $GFAP^+$ astrocytes, whereas it was negative for Nestin, Sox2 or CD133, confirming that the metastasized tumor cell origin was not of a neural lineage (Fig. 3C and F). Furthermore, enriching CSCs from this tumor by using the adherent culture paradigm failed, suggesting that the adherent culture method may favor tumors of a neural lineage.

Sox2-negative brain tumors fail to generate adherent CSCs. The tumors from patients with pilocytic astrocytoma (WHO grade I, patient no. 17) and CNS germ cell tumor (patient no. 1) failed to yield adherent CSCs. The

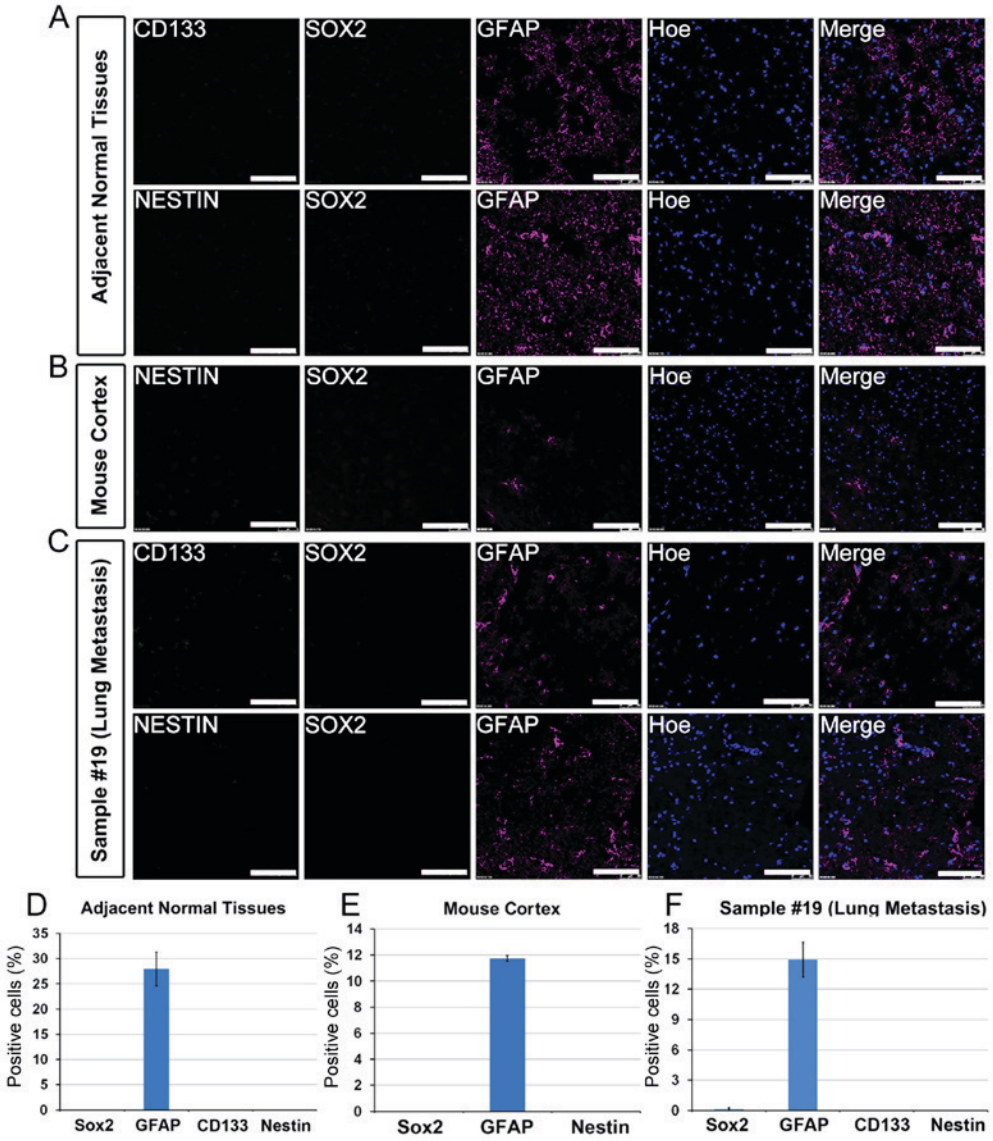

Figure 3. Secondary brain tumor fails to grow adherent CSCs. (A) Only GFAP$^+$ astrocytes are present in normal brain tissues, which are located adjacent to tumor specimens. CD133$^+$, Nestin$^+$ or Sox2$^+$ progenitors were all absent in normal brain tissues. (B) Mouse cortex tissue exhibits GFAP-positive staining but not positive for Nestin or Sox2. (C) A secondary brain tumor metastasized from lung adenocarcinoma only infiltrates with GFAP-positive astrocytes but does not contain CD133$^+$, Sox2$^+$ or Nestin$^+$ progenitor cells with a neural fate. Quantitative data from (D) panels A, (E) B and (F) C. Data are presented as the mean ± standard error of the mean. Scale bar, 100 μm. CSC, cancer stem cell; GFAP, glial fibrillary acidic protein.

immunostaining results showed that there was a significant number of GFAP$^+$, Nestin$^+$, CD133$^+$, GFAP$^+$/Nestin$^+$ and GFAP$^+$/CD133$^+$ cells within the original biopsy compared with Sox2$^+$ cells, which were rarely identified (Fig. 4A-F). These data indicated that Sox2$^+$ cells may be from cells that were enriched on laminin-coated culture surface and neither GFAP$^+$/Nestin$^+$/Sox2$^-$ nor GFAP$^+$/CD133$^+$/Sox2$^-$ cells were able to be cultured under the adherent culture conditions.

CD133-negative brain tumors fail to generate adherent CSCs. The tumors from patients with astrocytoma (WHO grade II, patient no. 2) and ependymoma (WHO grade II, patient no. 8) also failed to generate adherent CSCs. The immunostaining studies indicated that these tumors did contain large populations of Sox2$^+$ cells (24-33%). Moreover, there were also a large amount of GFAP$^+$, Netstin$^+$, Sox2$^+$/GFAP$^+$, Sox2$^+$/Nestin$^+$ and GFAP$^+$/Nestin$^+$ cells within the tumor mass. However, these tumors almost completely lacked CD133 expression (Fig. 5A-F). It was therefore hypothesized that CD133 expression may be another prerequisite for enrichment of adherent CSCs.

Sox2$^+$/CD133$^+$ glioblastomas were able to generate adherent CSCs. Adherent CSCs were successfully enriched from glioblastomas (WHO grade IV, patient nos. 3, 6, 15 and 18). The immunostaining of these tumor samples indicated a high level of Sox2 expression (30%) as well as a high level of CD133 expression (from 10 to 14%) (Fig. 6D-F). The percentage of GFAP$^+$ and Nestin$^+$ cells were similarly detected within the tumor samples (Fig. 6D-F). The glioblastomas comprised 4-5% Sox2$^+$/CD133$^+$ cells as quantified in Fig. 6G-I. Combined with aforementioned data that Sox2$^-$ or CD133$^-$ tumors failed to enrich CSCs, it was concluded that Sox2$^+$/CD133$^+$ cells may represent CSCs within the tumor biopsy, and these double positive cells may be adherently enriched.

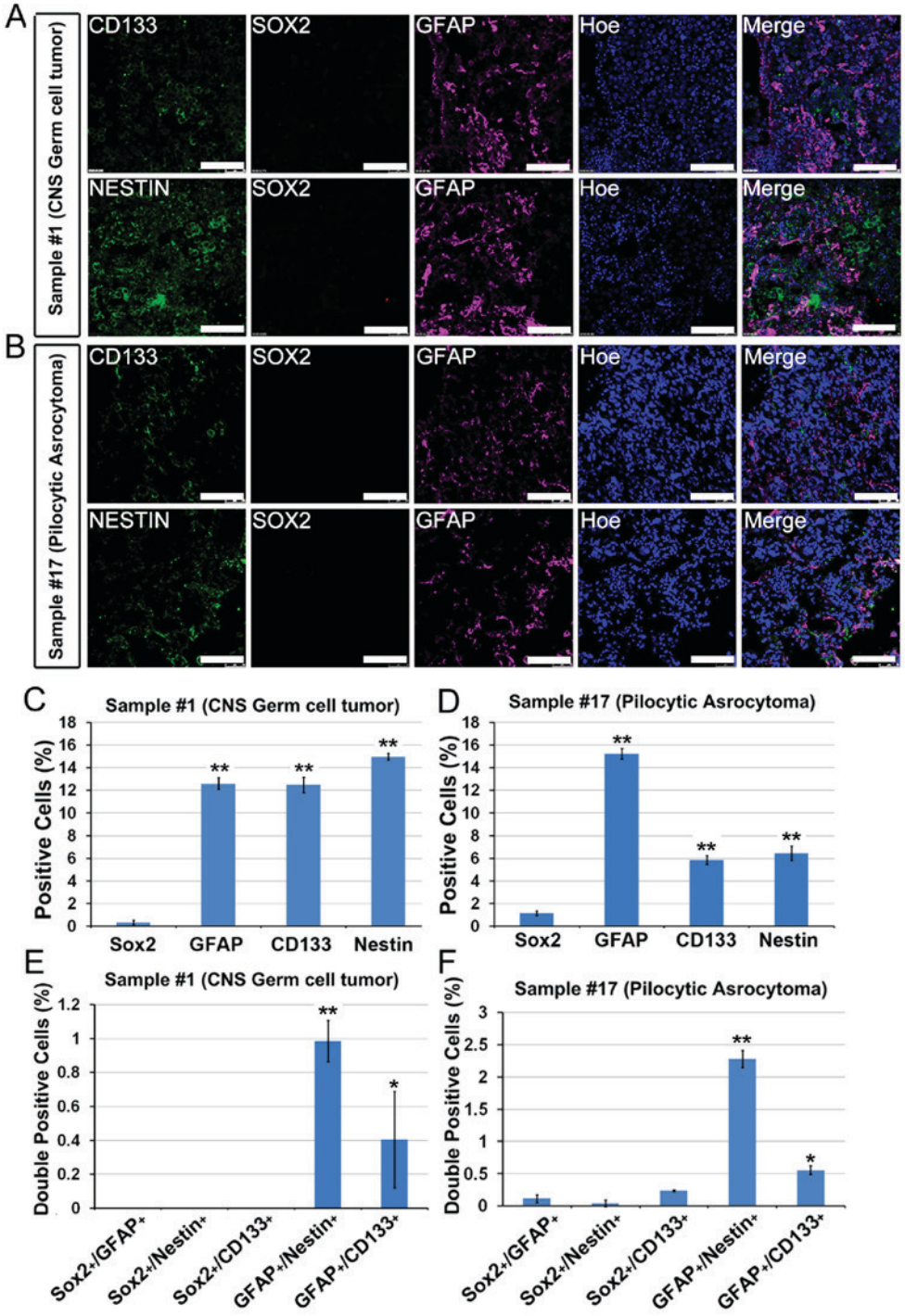

Figure 4. Sox2$^-$ brain tumors fail to yield adherent CSCs. (A) A central nervous system germ cell tumor and (B) a pilocytic astrocytoma fail to generate adherent CSCs. Immunostaining for Sox2, CD133, Nestin and GFAP in the tumors indicates that both lack Sox2$^+$ cells. Positive cells (%) in sample (C) nos. 1 and (D) 17. Double positive cells (%) in sample (E) nos. 1 and (F) 17. Data are presented as the mean ± standard error of the mean. Scale bar, 100 μm. *P<0.05, **P<0.01. CSC, cancer stem cell; GFAP, glial fibrillary acidic protein.

Discussion

In the present study, the feasibility of the adherent culture paradigm in enriching brain CSCs was analyzed. A total of 4/9 glioblastomas were able to successfully yield CSCs with a high purity, which was positive for GFAP, Nestin, Sox2 and CD133. It was also revealed that Sox2$^+$/CD133$^+$ cells might be originated from cells that were enriched under the adherent culture conditions.

CD133, Nestin, GFAP and Sox2 are frequently used as key markers to characterize CSCs in human glioblastomas (17). Among these key markers, CD133 is the most widely used for identification and enrichment of CSCs. CD133 may also be used to predict prognosis of individual glioblastoma of patient and allow specific therapies (18-20). At the cellular level, the knockdown of CD133 in CSCs significantly compromised their self-renewal ability and tumorigenic potential (21).

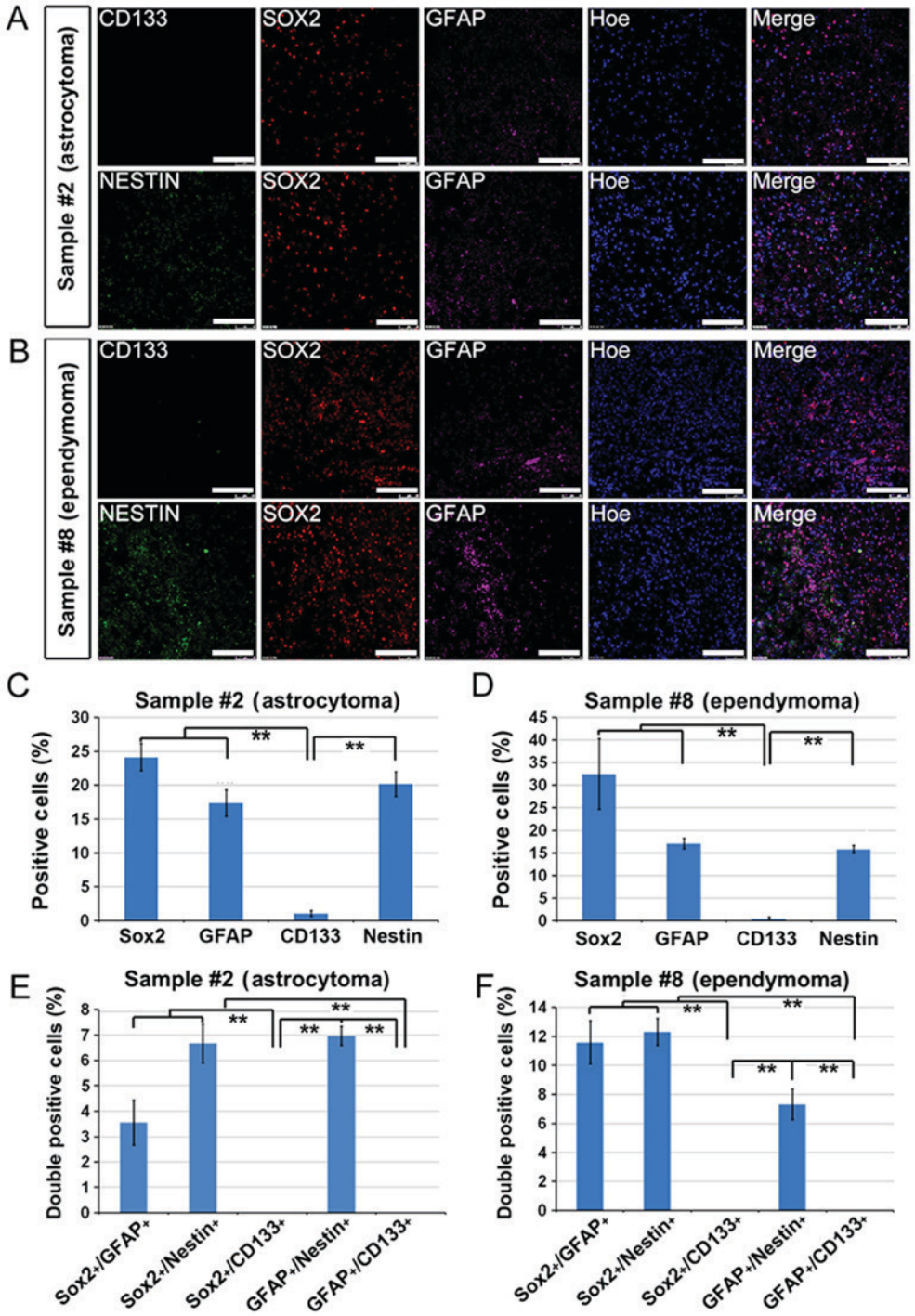

Figure 5. CD133- brain tumors fail to yield adherent CSCs. (A) A World Health Organization grade II astrocytoma and (B) an ependymoma fail to generate adherent CSCs. Immunostaining for Sox2, CD133, Nestin and GFAP shows both tumors lack CD133+ cells. Positive cells (%) in sample (C) nos. 2 and (D) 8. Double positive cells (%) in sample (E) nos. 2 and (F) 8. Data are presented as the mean ± standard error of the mean. Scale bar, 100 μm. **P<0.01. CSC, cancer stem cell; GFAP, glial fibrillary acidic protein.

Sox2 is a transcription factor involved in pluripotent stem cell or neural stem cell maintenance and lineage reprogramming (22-24). Sox2 is also another CSC marker for glioblastomas due in part to its association with initiating gliomas as well as its role in multipotency and cell cycle progression of progenitor cells (9,25,26). Silencing of Sox2 in glioblastomas results in cell cycle arrest (9,25-29). Moreover, high level of Sox2 expression could also serve as a reliable prognostic indication for accelerated disease progression and poor clinical outcomes (26,29,30).

In the present study, Sox2+/CD133+ cells may be easily enriched through adherent culture. There is not a single marker, which has been shown to be sufficient to confer stem cell-like properties. Therefore, a combination of different markers is used to identify and isolate CSCs in glioma, including Nestin, Sox2 and GFAP (4,30-35). Despite CD133-positive glioma cells exhibiting CSC properties, there is a subset of CD133-negative cells with apparent stem cell-like characteristics (17-20). On the other hand, Sox2 is

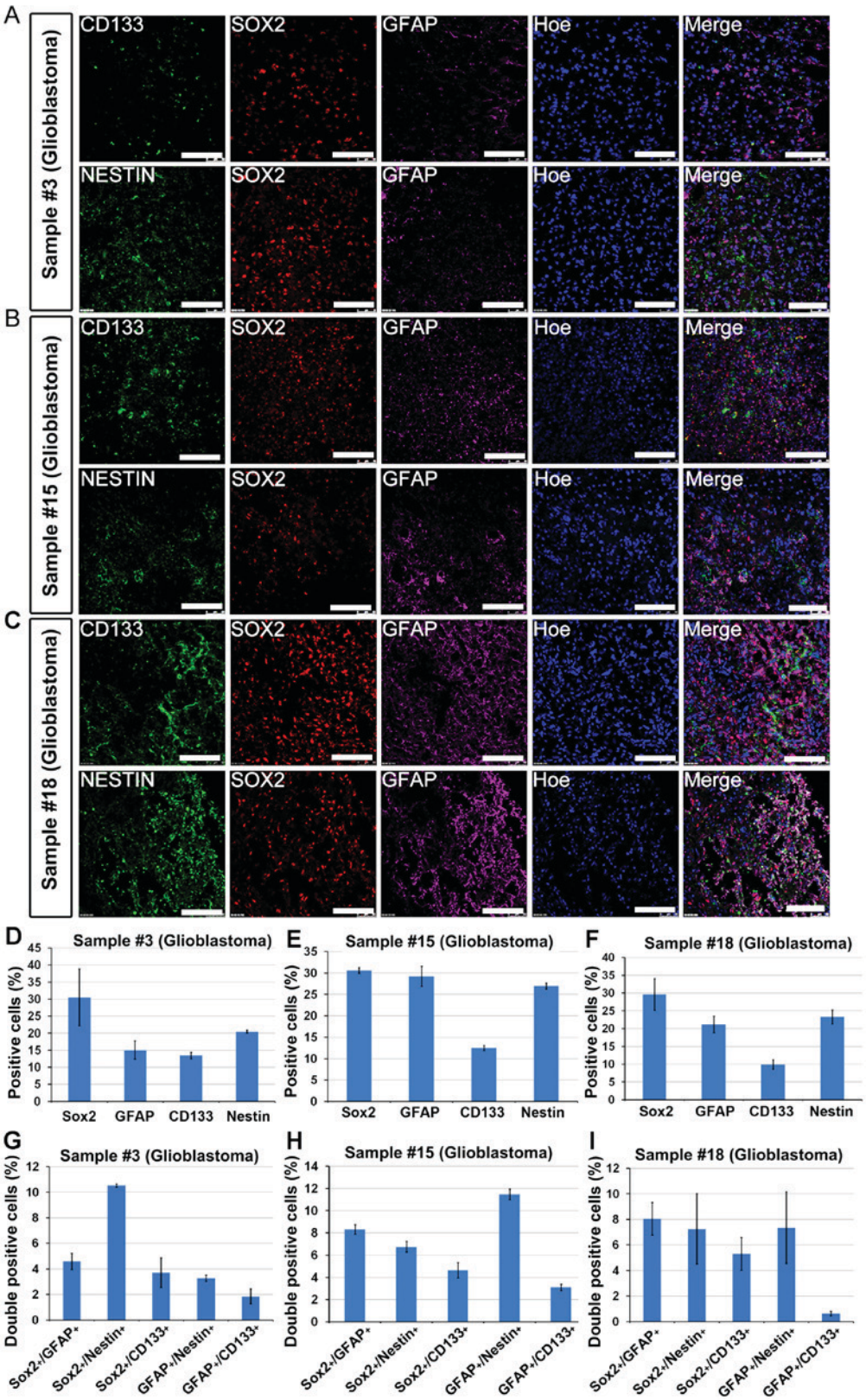

Figure 6. Sox2$^+$/CD133$^+$ glioblastomas were able to generate adherent CSCs. A total of 3 WHO grade IV glioblastomas, sample nos. (A) 3; (B) 15 and (C) 18, successfully yielded adherent CSCs. Immunostaining indicated the presence of Sox2$^+$, CD133$^+$, Nestin$^+$, GFAP$^+$ and most importantly Sox2$^+$/CD133$^+$ cells within the original biopsy. Positive cells (%) in sample (D) nos. 3, (E) 15 and (F) 18. Double positive cells (%) in sample (G) nos. 3, (H) 15 and (I) 18. Data are presented as the mean ± standard error of the mean. Scale bar, 100 μm. CSC, cancer stem cell; GFAP, glial fibrillary acidic protein.

also expressed in more differentiated neoplastic cells within the glioblastoma (9,25-27,29). These findings are in line with our conclusion that Sox2$^+$/CD133$^+$ cells rather than those single positive cells represent real CSCs and the cells were efficiently enriched under adherent culture conditions *in vitro*.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

All authors discussed the experiments and contributed to the text of the manuscript. KL and ZC conducted most of the experiments and wrote the draft of the manuscript. LL, QZ and XZ conceived, discussed the project and modified the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the Shanghai Tenth People's Hospital (Shanghai, China).

Patient consent for publication

All patients or parents of patients under 16 provided written informed consent for publication according to the ethical principles of Shanghai Tenth People's Hospital.

Competing interests

The authors declare that they have no competing interests.

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