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# Autocrine TGF-β Signaling Maintains Tumorigenicity of Glioma-Initiating Cells through Sry-Related HMG-Box Factors

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## SUMMARY

**Despite aggressive surgery, radiotherapy, and chemotherapy, treatment of malignant glioma remains formidable. Although the concept of cancer stem cells reveals a new framework of cancer therapeutic strategies against malignant glioma, it remains unclear how glioma stem cells could be eradicated. Here, we demonstrate that autocrine TGF-β signaling plays an essential role in retention of stemness of glioma-initiating cells (GICs) and describe the underlying mechanism for it. TGF-β induced expression of Sox2, a stemness gene, and this induction was mediated by Sox4, a direct TGF-β target gene. Inhibitors of TGF-β signaling drastically deprived tumorigenicity of GICs by promoting their differentiation, and these effects were attenuated in GICs transduced with Sox2 or Sox4. Furthermore, GICs pretreated with TGF-β signaling inhibitor exhibited less lethal potency in intracranial transplantation assay. These results identify an essential pathway for GICs, the TGF-β-Sox4-Sox2 pathway, whose disruption would be a therapeutic strategy against gliomas.**

## INTRODUCTION

Glioblastoma multiforme (GBM), the most malignant form of glioma, is one of the most aggressive human cancers with a 5 year survival rate of less than one out of ten (Surawicz et al., 1998). Despite past huge efforts, this statistic has not markedly improved over the past years. Excessive proliferation, diffuse infiltration into surrounding brain tissue, and suppression of antitumor immune surveillance contribute to the malignant phenotype of glioblastomas.

Cancer-initiating cells (cancer stem cells, CSCs) are rare tumor cells characterized by their ability to induce tumorigenesis and to self-renew. Recent concepts for cancer suggest that a minority population of CSCs may determine the biological and pathological characters of tumors. Similar to other tumors, glioma-initiating cells (glioma stem cells, GSCs) have been isolated from human glioma tissues and several glioma cell lines (Singh et al., 2004; Kondo et al., 2004; Hirschmann-Jax et al., 2004). GSCs are characterized by the expression of neural stem cell (NSC) antigens and the ability to grow as nonadherent spheres termed "neurospheres" or "glioma spheres" when cultured in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) under serum-free condition. Thus, GSCs share several characteristics with normal NSCs (Vescovi et al., 2006).

According to the concept of CSCs, failure to cure cancer may be attributed to the current therapeutic strategies, which have been aimed at the tumor bulk without significantly affecting CSCs. Like other CSCs, GSCs have been reported to be resistant to conventional radiation and pharmacological therapies (Bao et al., 2006; Liu et al., 2006a). Although elimination of CSCs has been regarded as a prerequisite for the development of successful therapeutic strategies, it has not still been fully elucidated how their stemness is maintained. To establish therapeutic strategies against glioma, in vitro and in vivo models that faithfully recapitulate the stem cell component of gliomas have been developed. Among these models, glioma spheres cultured in serum-free media supplemented with EGF and bFGF are considered to reflect biological and pathological characters of primary glioma tissues, have ability to self-renew, and mimic original tumors after intracranial transplantation (Singh et al., 2004; Lee et al., 2006).

Although transforming growth factor (TGF)-β suppresses proliferation of certain carcinoma cells and is well known to be a tumor suppressor, it promotes proliferation of tumors of non-epithelial origin, including glioma and osteosarcoma, through induction of PDGF-BB (Bruna et al., 2007; Matsuyama et al., 2003). TGF-β binds to type I and type II serine/threonine kinase receptors and transduces intracellular signals principally through Smad proteins (Derynck and Zhang, 2003; Massagué, 2008; Miyazawa et al., 2002). Upon phosphorylation by type I receptors, receptor-regulated Smads (R-Smads; Smad2 and -3) form heteromeric complexes with common-partner Smad (Co-Smad; Smad4), translocate into the nucleus, and regulate expression of various target genes. In addition to induction of proliferation, TGF-β pathway has also been implicated in invasion, metastasis, and intratumoral angiogenesis of glioma. These multiple roles of TGF-β in glioma progression have promoted the development of therapeutic agents based on the inhibition of the TGF-β pathway (Golestaneh and Mishra, 2005).

Here, we report that autocrine TGF-β signaling induces Sox2 expression, one of the crucial factors for maintenance of NSCs,

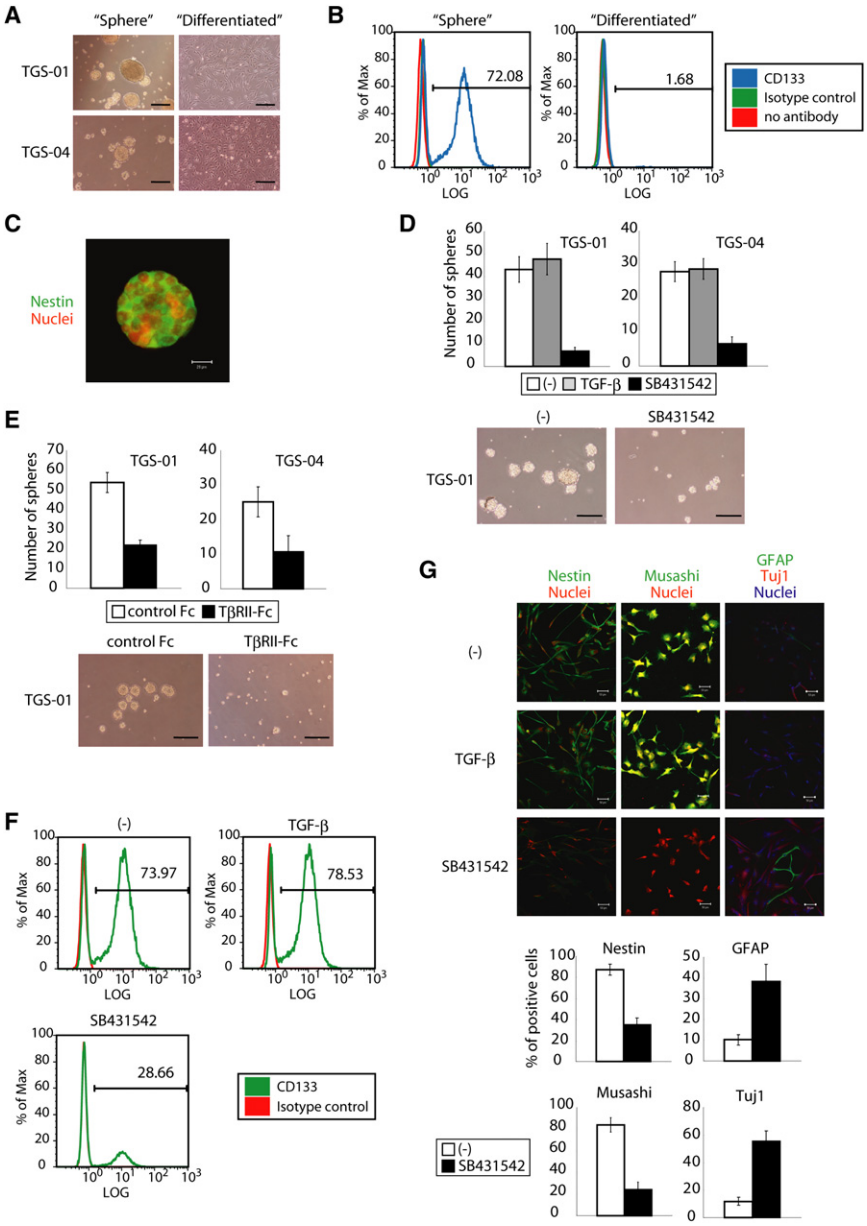


**Figure 1. TGF-β Signaling Maintains Stemness of Glioma-Initiating Cells**

(A) Representative images of glioma spheres TGS-01 and TGS-04 cultured in serum-free neurobasal media with EGF and bFGF ("Sphere"), and glioma cells derived from the same pathological samples as spheres but cultured in media containing 10% fetal bovine serum ("Differentiated"). Scale bars, 100 μm.

(B) $CD133^+$ ratio of "Sphere" cells (TGS-01) and "Differentiated" cells was determined by flow cytometry.

(C) TGS-01 spheres were stained with Nestin. Scale bars, 20 μm.

(D) TGF-β inhibitor deprives glioma-initiating cells of sphere-forming ability. Glioma-initiating cells were dissociated into single-cell populations and cultured with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 7 days. The data are presented as the number of glioma spheres formed (means ± SEM of five fields). Scale bars, 100 μm.

(E) Glioma-initiating cells were dissociated into single-cell populations and cultured with human TGF-β RII/Fc chimera (1 μg/ml) or control $IgG_1$ Fc (1 μg/ml) for 7 days. The data are presented as the number of glioma spheres formed (means ± SEM of five fields). Scale bars, 100 μm.

(F) Effects of TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) on $CD133^+$ subpopulation of TGS-01 cells were determined by flow cytometry.

(G) Immunostaining of TGS-01 cells. Spheres were disaggregated, seeded on poly-L-ornithine and fibronectin-coated slide glasses, and cultured in serum-free medium with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 7 days. Quantification of Nestin-, Musashi-, Tuj1-, or GFAP-positive cells was shown in the bottom graphs. Scale bars, 50 μm.

and plays essential roles in maintenance of stemness of the glioma-initiating cells. We also demonstrate that another Sry-related high-mobility group (HMG) box-containing gene, Sox4, is a crucial mediator of TGF-β-induced Sox2 expression. Notably, glioma-initiating cells pretreated with TGF-β signaling inhibitor were less aggressive and showed less lethal potency in intracranial transplantation assay. These findings open the way to depriving GSCs of the tumorigenic activity and will offer new therapeutic possibilities.

## RESULTS

### Inhibition of TGF-β Signaling Deprives Glioma-Initiating Cells of Tumorigenic Activity

To study the mechanisms of how stemness of glioma-initiating cells is maintained, we have used glioma tissues obtained from two patients with GBM. They were cultured in serum-free medium and termed TGS-01 and TGS-04 (Figure 1A), both of which have the ability to self-renew and mimic the original tumor after transplantation into the brains of immunocompromised mice (Lee et al., 2006). Profiles of the patients and properties of the GBM cells are described in Figure S1 (in Supplemental Data available online). Expression of phosphatase and tensin homolog (Pten) was lost in TGS-01 and TGS-04 "sphere" cells. CD133 (Prominin-1) was reported to be a marker for GSCs (Singh et al., 2004). We confirmed that the $CD133^+$ subpopulation is enriched in these glioma spheres compared to cells derived from the same patient but cultured in media containing 10% fetal bovine serum ("Differentiated" or "Adherent") (Sphere; 72.0%, Differentiated; 1.6%, Figure 1B). In addition, tumor spheres derived from each of tissue samples could be passaged serially and expressed Nestin (neural precursor cell marker), confirming that these are clonogenic and self-renewing cells (Figure 1C). We also validated enrichment of glioma-initiating cells in TGS-01 and TGS-04 "sphere" cells by an intracranial transplantation assay (M.T., Y.I., and T.T., unpublished data).

To test a possible role of TGF-β signaling in glioma-initiating cells, we first examined the effect of inhibition of TGF-β signaling

on their biological characters. After treatment with TGF-β type I receptor (ALK5) kinase inhibitor SB431542 (Inman et al., 2002), glioma-initiating cells were drastically deprived of sphere-forming ability (Figure 1D). Similar results were obtained using three other glioma-initiating cells from patients with GBM (Figures S1 and S2). As SB431542 can inhibit TGF-β type I receptor (ALK5) signaling, as well as activin/nodal type I receptor (ALK4 and ALK7) signaling, we also examined the effect of TGF-β receptor II/Fc chimera (TβRII-Fc) on glioma-initiating cells to assess the role of TGF-β signaling definitely. Glioma-initiating cells treated with TβRII-Fc formed glioma spheres with lower efficiency (Figure 1E). Similar results were obtained with other TGF-β signaling inhibitors, A-78-03 (Tojo et al., 2005) or LY364947 (Sawyer et al., 2003) (Figure S3A), as well as infection of adenovirus carrying cDNA of Smad7, an endogenous negative regulator of TGF-β signaling (Figure S3B). Moreover, preformed sphere cells lost their spherical growth pattern and became attached in the presence of SB431542 (Figure S3C). Decreased number of glioma spheres formed by glioma-initiating cells with depleted TGF-β signaling suggests impaired self-renewal. In agreement with the suggested effect of TGF-β signaling depletion, treatment of glioma-initiating cells with SB431542 for 7 days prior to the sphere-forming assay without the inhibitor also reduced the number of spheres (Figure S4). We also investigated the effects of TGF-β inhibitor on the $CD133^+$ subpopulation. SB431542 decreased the size of $CD133^+$ pool in glioma-initiating cells (Figure 1F). Next, to examine the expression of neural precursor or differentiation markers in each cell, spheres in serum-free medium were disaggregated and seeded on poly-L-ornithine and fibronectin-coated slide glasses. Inhibition of TGF-β signaling decreased the number of cells positive for Nestin or Musashi (neural precursor cell markers) and increased that for GFAP (astrocyte differentiated marker) or Tuj1 (βIII-tubulin, neuronal marker) (Figure 1G). Taken together, these findings suggest endogenous TGF-β signaling maintains tumorigenicity and stemness of glioma-initiating cells. Conversely, we failed to observe striking effects of addition of TGF-β ligand on sphere-forming ability, $CD133^+$ ratio, or marker expression of glioma-initiating cells (Figures 1D, 1F, and 1G). It may be because glioma-initiating cells express all the major components of TGF-β signaling pathway and secrete TGF-β1 and -β2 proteins (Figures S5A and S5B), producing sufficient autocrine TGF-β signaling to maintain their stemness (Figure S5C).

TGF-β is reported to work as a proapoptotic or an antiapoptotic factor in a cell-context-dependent manner (Sánchez-Capelo, 2005; Ehata et al., 2007), but we failed to observe any significant effect of TGF-β ligand or inhibitor on apoptosis of glioma-initiating cells (Figure S6A). TGF-β is also known to control cell proliferation via regulating $p15^{INK4b}$, $p21^{WAF1}$, $p27^{KIP1}$, and *c-myc* (Massagué, 2008), but in glioma-initiating cells, stimulation or inhibition of TGF-β signaling did not markedly affect their expression levels at 3 or 24 hr except for only a slight increase of $p21^{WAF1}$ 24 hr after inhibition of TGF-β signaling (Figure S6B).

## Sox2 Expression Is Induced by TGF-β in Glioma-Initiating Cells to Maintain Their Stemness

To elucidate the mechanism by which stemness of glioma-initiating cells is maintained by TGF-β signaling, we next examined the effect of TGF-β or SB431542 on expression of various markers for stemness. mRNA expression of Sox2, a member of HMG-box factors, was induced by TGF-β but suppressed by SB431542 after 24 hr treatment (Figure 2A) and kept at the low levels for at least 7 days (data not shown). In contrast, expression levels of Oct4, Nanog, LIF, or other pluripotent stem cell-related molecules were not significantly affected by TGF-β ligand or inhibitor in our glioma-initiating cells in TGS-01 and -04 cells (Figure S7), although Nanog and LIF were reported to be induced by TGF-β stimulation in some types of cells (Xu et al., 2008; Bruna et al., 2007). Induction of Sox2 by TGF-β was clearly suppressed in the presence of siRNA against Smad2 and Smad3 (Figure 2B), indicating that Sox2 expression is regulated by TGF-β-Smad signaling. We also confirmed regulation of Sox2 protein expression by TGF-β and SB431542 (Figure 2C). Knockdown of Sox2 expression by siRNA (Figure S8) resulted in drastic reduction of sphere-forming ability and self-renewal capacity of glioma-initiating cells (Figures 2D and 2E) and decreased size of $CD133^+$ subpopulation (75.1% to 29.3% or 35.9%; Figure 2F). Drastic reduction of sphere-forming ability by knockdown of Sox2 was also observed in four other glioma cells (Figure S9). Moreover, the number of Nestin-positive cells was reduced and that of GFAP-positive cells was increased by Sox2 knockdown (Figure 2G). These findings indicate that Sox2 is an essential factor for maintenance of stemness of glioma-initiating cells and that downregulation of Sox2 expression as early as 24 hr after treatment with SB431542 appears to be the cause, rather than the result, of deprivation of stemness of glioma-initiating cells.

## Downregulation of Sox2 Is a Crucial Step for Differentiation of Glioma-Initiating Cells Induced by TGF-β Inhibitor

For further study of the role of Sox2 in maintenance of stemness by TGF-β, we examined the effect of TGF-β inhibitor on glioma-initiating cells infected with adenovirus encoding Sox2 cDNA. SB431542 only weakly deprived Sox2-overexpressed glioma-initiating cells of sphere-forming ability compared to LacZ-overexpressed cells (Figure 3A). Moreover, SB431542 failed to reduce the number of Nestin-positive cells or to increase the number of GFAP-positive cells in Sox2-overexpressed glioma-initiating cells (Figure 3B). These data suggest that deprivation of stemness of glioma-initiating cells by TGF-β inhibitor is due to downregulation of Sox2, which maintains stemness of glioma-initiating cells.

## Sox4 Is a TGF-β Target Gene, which Is Highly Expressed in Glioma-Initiating Cells

Induction of Sox2 expression by TGF-β was observed 24 hr, but not 3 hr, after stimulation (Figure 2A) and attenuated in the presence of cycloheximide, an inhibitor of protein synthesis (Figure 4A). These findings indicate that Sox2 expression is not directly induced by TGF-β but regulated through other factor(s) that are induced by TGF-β. We, thus, searched candidate genes that mediate TGF-β-induced Sox2 expression to play important roles in retention of stemness of glioma-initiating cells. For this purpose, we used microarray data of public resources (Beier et al., 2007; Bruna et al., 2007; Günther et al., 2008; Lee et al., 2006; Tso et al., 2006). Criteria for selection were as follows: (1) genes with higher expression in glioma-initiating cells compared

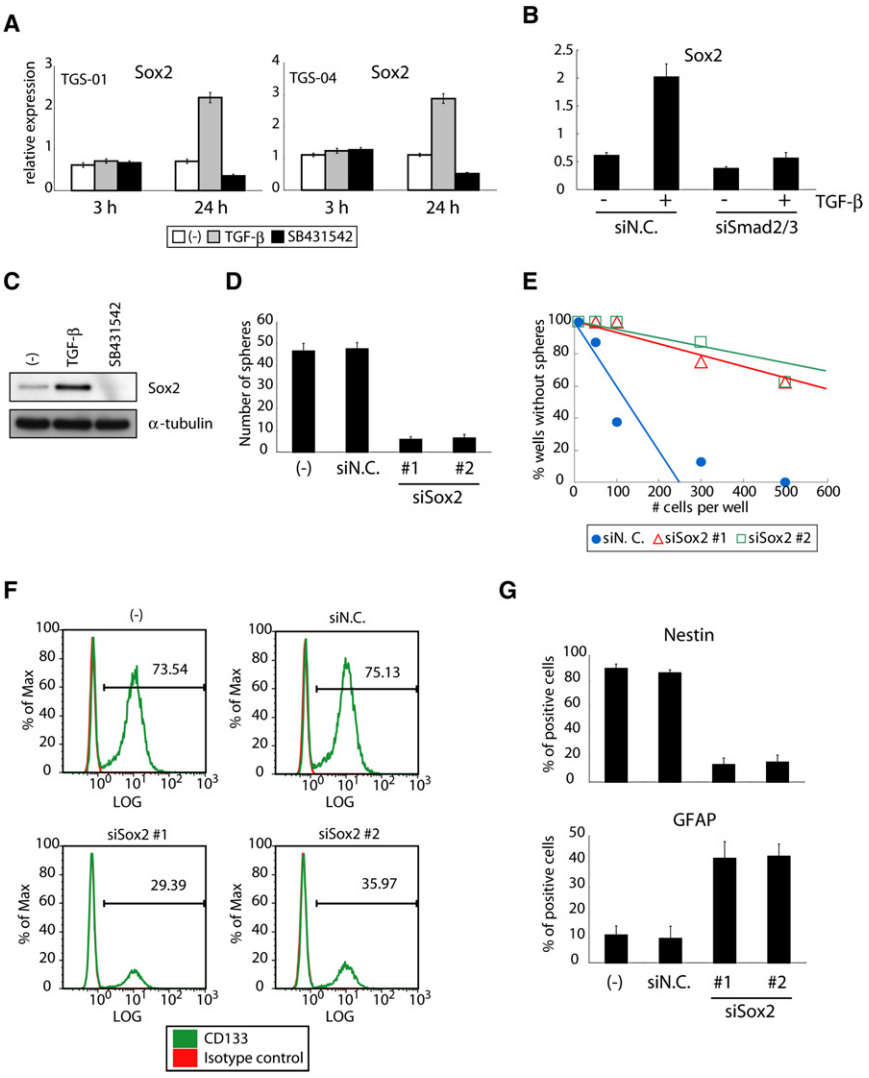


**Figure 2. TGF-β Induces Expression of Sox2, an Essential Factor for Retention of Stemness of Glioma-Initiating Cells**

(A) Expression of Sox2 mRNA was determined after treatment with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 3 or 24 hr. Values were normalized to that of GAPDH mRNA. Error bars represent SEM.

(B) TGS-01 cells were transfected with siRNA oligonucleotides and incubated for 24 hr. Cells were treated with TGF-β ligand (100 pM) for 24 hr. Values were normalized to that of GAPDH mRNA. Error bars represent SEM.

(C) Expression of Sox2 protein in TGS-01 cells was determined after treatment with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 24 hr. α-tubulin was used as a loading control.

(D) TGS-01 cells were dissociated into single-cell populations, transfected with control (N.C.) or Sox2 siRNA duplex, and cultured for 7 days. The data are presented as the number of glioma spheres formed (means ± SEM of five fields).

(E) Knockdown of Sox2 expression by siRNA in TGS-01 cells resulted in decrease of self-renewal capacity in limiting dilution assay.

(F) Effects of Sox2 knockdown on $CD133^+$ subpopulation of TGS-01 cells were determined by flow cytometry.

(G) Quantification of Nestin-positive or GFAP-positive cells among total cells. Differentiation of TGS-01 cells by Sox2 knockdown was analyzed 7 days after transfection of control (N.C.) or Sox2 siRNA duplex. Error bars represent SEM.

to bulk tumor cells; (2) genes directly induced by TGF-β and suppressed by TGF-β inhibitor in glioma cells; and (3) genes whose expression levels are correlated with that of Sox2 in glioma cells. Among genes highly expressed in glioma-initiating cells, we identified a transcription factor Sox4 as a TGF-β target gene.

We observed higher expression levels of Sox4 in TGS-01 and TGS-04 cells than in matched "differentiated" cells (Figure 4B). We checked whether Sox4 expression is regulated by TGF-β signaling (Figures 4C and 4D). Sox4 mRNA expression was immediately induced after TGF-β stimulation and inversely downregulated by TGF-β inhibitor in TGS-01 and TGS-04 cells. To examine whether Sox4 is a direct target gene of TGF-β, we performed chromatin immunoprecipitation assay using antibody against Smad2/3, DNA-binding mediators of TGF-β signaling. Smad complexes directly bound to Sox4 promoter in response to TGF-β stimulation, and this binding was clearly suppressed by SB431542 (Figure 4E). Moreover, induction of Sox4 by TGF-β was not significantly affected by cycloheximide (Figure 4F). These findings indicate that Sox4 is a direct target gene of TGF-β signaling.

## Sox4 Associates with the Sox2 Enhancer Region and Promotes Its Expression

Next, we studied the effect of Sox4 on Sox2 expression. Sox4 overexpression in glioma-initiating cells resulted in upregulation of Sox2 expression (Figure 5A). In contrast, Sox2 expression was suppressed by Sox4 knockdown (Figure 5B). We confirmed that Sox2 mRNA expressed under the control of cytomegalovirus (CMV) promoter was not downregulated by siSox4 (Figure S10), indicating that Sox2 mRNA is not a direct target of siSox4. These results indicate that Sox2 expression is positively regulated by Sox4 at the transcriptional level. To examine whether this regulation is direct, we performed chromatin immunoprecipitation assay using antibody against Sox4. It has been demonstrated that the enhancer element located at the 3′ flanking region of Sox2 gene is important for regulation of Sox2 expression (Chew et al., 2005; Tomioka et al., 2002). The region contains the consensus binding motif for Sox4, "CATTGTA" (Liao et al., 2008). Recruitment of Sox4 to the enhancer element was increased 24 hr after TGF-β stimulation, and such recruitment was clearly suppressed by SB431542 treatment (Figure 5C). These results appear to be due to regulation of Sox4 expression by TGF-β ligand or inhibitor. In addition, TGF-β could induce Sox2 expression only weakly under the condition that Sox4 was knocked down (Figure 5D). Altogether, we concluded that Sox4 directly induced by TGF-β

upregulates Sox2 expression through association with its enhancer region.

### Sox4 Is An Essential Factor for Maintenance of Stemness of Glioma-Initiating Cells

So far, Sox4 has not been reported to have any role in maintenance of stem cell properties. To study the role of Sox4 in glioma-initiating cells, we examined the effect of Sox4 knockdown on stemness of glioma-initiating cells. Sox4-knocked-down glioma-initiating cells showed less sphere-forming ability and self-renewal capacity (Figures 6A and 6B), and the size of $CD133^+$ pool was decreased (72.6% to 41.5% or 41.1%; Figure 6C). Moreover, the number of Nestin-positive cells was decreased, while that of GFAP-positive cells was increased by Sox4 knockdown (Figure 6D). These results indicate that Sox4 is involved in a crucial pathway for maintenance of stemness of glioma-initiating cells.

### Sox4 Plays an Essential Role in Maintenance of Stemness of Glioma-Initiating Cells by TGF-β

From these results, we hypothesized that Sox4 expression directly induced by TGF-β promotes Sox2 expression and maintains stemness of glioma-initiating cells. To test this hypothesis, first, we examined the effect of TGF-β inhibitor on glioma-initiating cells infected with adenovirus carrying cDNA of Sox4. Sox4-overexpressing glioma-initiating cells showed resistance against deprivation of sphere-forming ability by SB431542 (Figure 7A). Moreover, the number of Nestin-positive cells and that of GFAP-positive cells of Sox4-overexpressed glioma-initiating cells were minimally affected by SB431542 (Figure 7B). These data suggest that direct induction of Sox4 expression by TGF-β plays essential roles in maintenance of stemness of glioma-initiating cells.

### TGF-β Inhibitor Deprives Glioma-Initiating Cells of In Vivo Tumorigenic Activity through Downregulation of Sox4 Expression

To study the role of TGF-β-induced Sox4 in maintenance of stemness in vivo, we examined the effects of TGF-β inhibitor and Sox4 on intracranial growth of glioma-initiating cells (Figure 7C). As TGF-β is well known to promote proliferation of bulk glioma cells, SB431542 was used in pretreatment, rather than successive treatment, to distinguish the effect on differentiation from that on proliferation. The survival of the mice inoculated with SB431542-pretreated glioma-initiating cells was significantly prolonged compared to mice injected with control glioma-initiating cells. All mice bearing control glioma-initiating cells developed neurological signs and displayed large tumors. In contrast, all mice bearing SB431542-pretreated glioma-initiating cells did not develop neurological signs and the brains showed no evidence of tumor by pathological examination with H&E staining. Furthermore, the survival advantage effect of SB431542 was not observed for Sox4-transduced glioma-initiating cells (Figure 7C). Altogether, we concluded that TGF-β upregulates Sox2 expression through direct induction of Sox4 to sustain stemness of glioma-initiating cells and that TGF-β inhibitor blocks this pathway to deprive them of aggressiveness (Figure 7D).

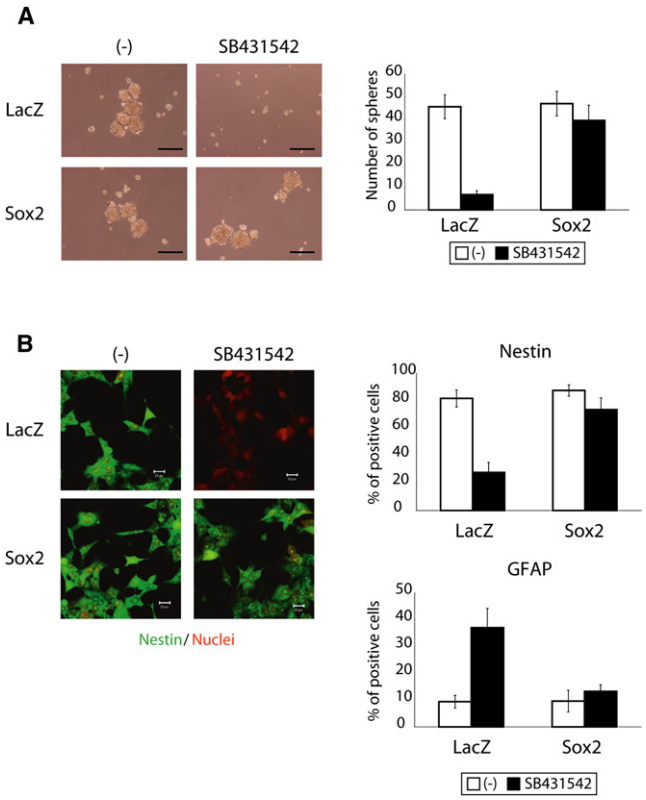


**Figure 3. Downregulation of Sox2 Expression by TGF-β Inhibitor Is a Critical Step for Differentiation of Glioma-Initiating Cells**

(A) Representative images of TGS-01 cells infected with adenovirus-Sox2 or LacZ (negative control) and cultured with or without SB431542 (1 μM) for 7 days. The data are presented as the number of glioma spheres formed (means ± SEM of five fields). Scale bars, 100 μm.

(B) Marker expression of TGS-01 cells infected with adenovirus-Sox2 or LacZ and cultured with or without SB431542 (1 μM) for 7 days. Images of Nestin-positive cells are shown in the left panels. Quantification of Nestin-positive or GFAP-positive cells among total cells is shown in the right graphs. Error bars represent SEM. Scale bars, 20 μm.

## DISCUSSION

TGF-β was reported to induce epithelial-mesenchymal transition (EMT) in immortalized human mammary epithelial cells and increase the ability to form mammospheres (Mani et al., 2008). These findings suggest a direct link between EMT and the gain of epithelial stem cell properties of carcinoma. Such a link could be applied to other carcinoma stem cells than breast CSCs. However, since glioma cells per se have nonepithelial characters, the link may not be applied to GSCs.

To analyze roles of TGF-β signaling in glioma stem cells, we have used glioma tissues obtained from patients with GBM and they were cultured in serum-free medium ("sphere"). All of the ten mice injected intracranially with $5 \times 10^3$ TGS-01 "sphere" cells were killed within 70 days after transplantation, while all of the ten mice bearing $5 \times 10^5$ matched "differentiated" cells had survived for more than 140 days without neurological signs (M.T., Y.I., and T.T., unpublished data). These results indicate that populations with a higher tumorigenic activity were enriched in "sphere" cells.

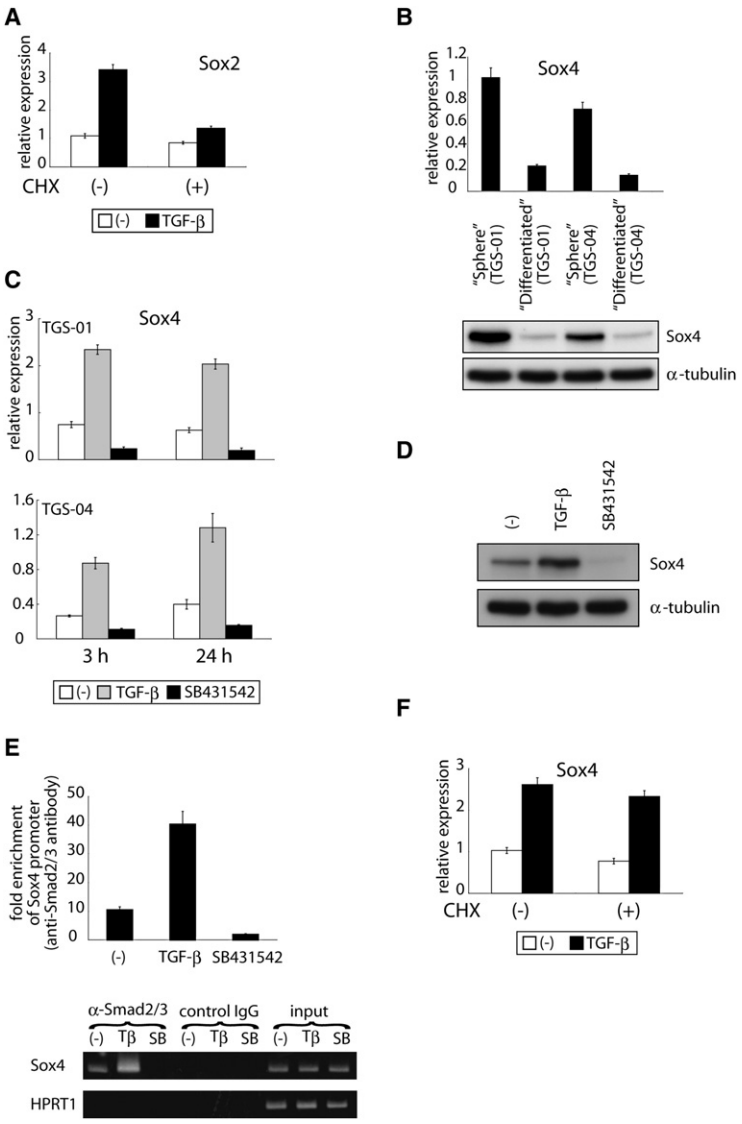


**Figure 4. Sox4 Is a Direct Target Gene of TGF-β Signaling**

(A) Expression levels of Sox2 mRNA after TGF-β (100 pM) treatment for 24 hr. Treatment with cycloheximide (CHX, 3 μg/ml) was started 30 min before stimulation with TGF-β. Values were normalized to amounts of GAPDH mRNA. Error bars represent SEM.

(B) Expression levels of Sox4 mRNA and protein in "Sphere" cells and "Differentiated" cells. α-tubulin was used as a loading control.

(C) Amount of Sox4 mRNA was determined after TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) treatment for 3 or 24 hr. Values were normalized to that of GAPDH mRNA. Error bars represent SEM.

(D) Amount of Sox4 protein was determined after TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) treatment for 24 hr. α-tubulin was used as a loading control.

(E) Association of Smad complex with the Sox4 promoter. Chromatin immunoprecipitation analysis was performed using TGS-01 cells treated with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 1.5 hr. Eluted DNAs were subjected to quantitative real-time PCR analysis (graph) or conventional RT-PCR (figure). In real-time PCR analysis, values were normalized to the amount of the first intron of hypoxanthine phosphoribosyltransferase (HPRT) 1. Error bars represent SEM. Input, 1%.

(F) Expression levels of Sox4 were analyzed using cDNAs, which were prepared in the experiments shown in (A).

We have shown here that inhibition of TGF-β signaling induces differentiation of glioma-initiating cells and that TGF-β maintains stemness of these cells through induction of Sox2 expression. Moreover, we have demonstrated that Sox4 is a direct target of TGF-β and that Sox4 mediates TGF-β-induced Sox2 expression. Although inhibition of TGF-β signaling markedly promoted differentiation of glioma-initiating cells, addition of TGF-β ligand failed to induce substantial change in glioma-initiating cells. It may be because TGF-β ligand is sufficiently produced by glioma-initiating cells for maintenance of their stemness at the basal level (Figure S5) in spite of the findings that TGF-β signaling itself is not saturated (Figures 2A, 2C, 4C, and 4D). Consistent with these results, overexpression of Sox4 or Sox2 in glioma-initiating cells did not affect their sphere-forming ability in the presence of autocrine TGF-β signaling. These results indicate that in glioma-initiating cells Sox4 and Sox2 are present at sufficient levels for retention of their aggressiveness in the presence of autocrine TGF-β signaling.

Bone morphogenetic protein (BMP) 4, another member of the TGF-β family, promotes differentiation and depletes the pool of glioma-initiating cells (Piccirillo et al., 2006). BMP signaling is known to control the activity of normal brain stem cells (Lim et al., 2000), and TGF-β signaling also regulates normal brain development (Golestaneh and Mishra, 2005; Muñoz-Sanjuán and Brivanlou, 2002). Altogether, the roles of TGF-β and BMP signaling in GSCs may reflect those in normal NSCs, supporting the concept that GSCs and NSCs are closely related (Vescovi et al., 2006).

Sox2 is well known to be one of the self-renewal genes, such as Oct4 and Nanog, and to play pivotal roles in maintaining stemness of embryonic stem cells (Kamachi et al., 2000). Sox2 null mutant embryos cannot give rise to embryonic or trophectoderm lineages (Gubbay et al., 1990). Introduction of Sox2 together with Oct4, Klf4, and c-Myc to human or mouse adult fibroblasts results in generation of induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Sox2 also plays essential roles in maintenance of NSCs (Graham et al., 2003), and Sox2 deficiency causes impaired neurogenesis in adult mouse brain (Ferri et al., 2004). Although it was reported that glioma tissues highly express Sox2 mRNA compared to nonmalignant tissues (Ben-Porath et al., 2008; Schmitz et al., 2007), the role of Sox2 in the development of glioma has not yet been determined. In glioma cells, expression levels of Sox2 are linearly and exponentially correlated with those of Nestin and CD133, respectively (Figure S11A, data set from Lee et al., 2006). Here, we demonstrated that knockdown of Sox2 resulted in deprivation of tumorigenic activity of glioma-initiating cells. Our report is the first to show significance of Sox2 in development of glioma and maintenance of the aggressive characters of glioma cells. The results that Sox2 is an essential factor for

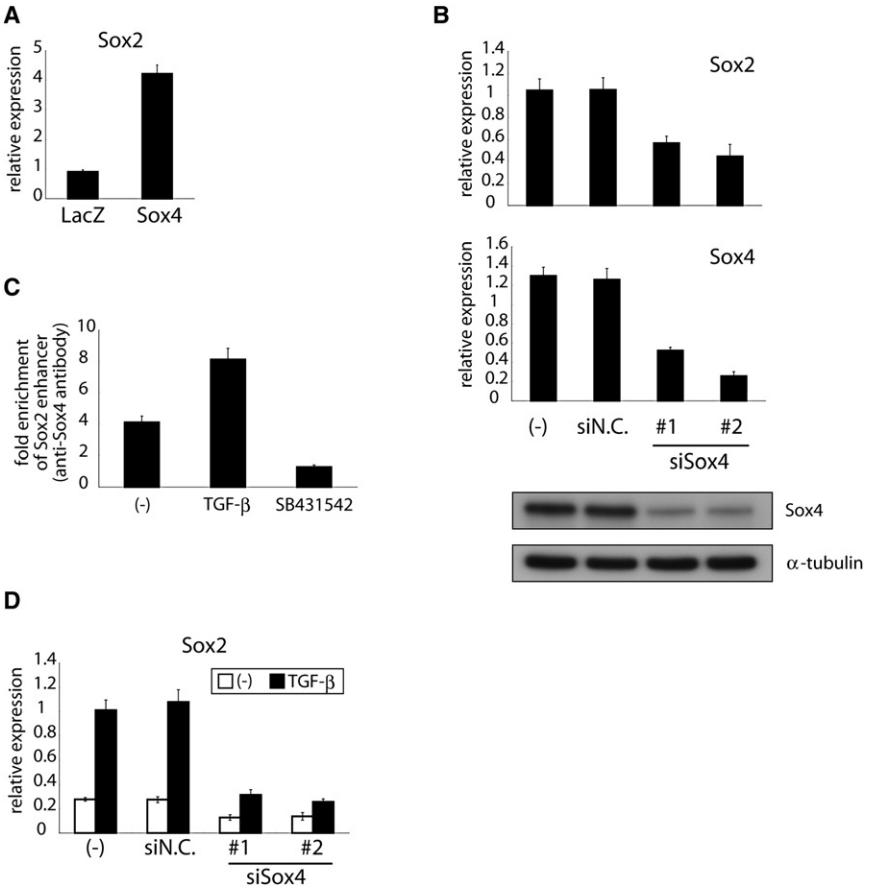


**Figure 5. Sox4 Is a Crucial Mediator for TGF-β-Induced Sox2 Expression**

(A) Effects of Sox4 overexpression on expression of Sox2. TGS-01 cells were infected with adenovirus-Sox4 or LacZ 24 hr before harvest. Values were normalized to the amount of GAPDH mRNA. Error bars represent SEM.

(B) Effects of Sox4 knockdown on expression of Sox2. Upper graphs show mRNA levels of Sox2 and Sox4. RNA levels were normalized to amounts of GAPDH mRNA. Error bars represent SEM. Lower panels show protein levels of Sox4 and α-tubulin.

(C) Association of Sox4 with the Sox2 enhancer. Chromatin immunoprecipitation analysis was performed using TGS-01 cells treated with TGF-β ligand (100 pM) or inhibitor (SB431542, 1 μM) for 24 hr. Eluted DNAs were subjected to quantitative real-time PCR analysis. Values were normalized to the amount of the first intron of HPRT1. Error bars represent SEM.

(D) TGF-β-induced Sox2 expression under the condition of Sox4 knockdown. Expression levels of Sox2 mRNA after TGF-β (100 pM) treatment for 24 hr were determined by real-time PCR. Indicated siRNAs were transfected 24 hr before TGF-β treatment.

maintenance of GSCs as well as NSCs and embryonic stem cells support the concept that malignant cancer cells and normal developing cells are closely associated with each other in their biological properties. Because Sox2 is a crucial factor for maintenance of other tissue stem cells, it can be speculated that Sox2 also plays significant roles in maintenance of other CSCs.

Although Sox2 is already known to play crucial roles to maintain stemness of NSCs, Sox4 has not been reported to have any function in stem cell properties. So far, Sox4 was reported to be overexpressed in glioma tissues compared to normal brain tissues (Tso et al., 2006), but its role in the development of glioma has not been determined. Here, we have demonstrated that Sox4 binds to Sox2 enhancer region and plays important roles in sustaining tumorigenicity of glioma-initiating cells. From the public data sets, we found that glioma-initiating cells show higher expression of Sox2 and Sox4 and that their expression levels significantly correlate with each other (Figures S11B and S11C, data set from Lee et al., 2006), suggesting that the TGF-β-Sox4-Sox2 pathway is widely used in glioma-initiating cells for retention of their stemness. These findings may add a new player, Sox4, to the concept of stemness.

Sox4 contains a DNA-binding domain known as the HMG box and has been shown to be a transcriptional activator involved in the development of the cardiac outflow tract and the central nervous system (Cheung et al., 2000). So far, Sox4 overexpression has been found to be associated with several human cancer types, including not only glioma but also hepatic cellular carcinoma, prostate cancer, lung cancer, bladder carcinoma, and medulloblastoma (Aaboe et al., 2006; Lee et al., 2002; Liao et al., 2008; Liu et al., 2006b; Pramoonjago et al., 2006; Yokota et al., 2004). The mechanism of Sox4 function as an oncogene, however, has not been fully investigated. From our report, Sox4 can be defined as an oncogene with new mechanisms, at least in glioblastoma.

Although we demonstrated that the TGF-β-Sox4-Sox2 pathway is indispensable for the maintenance of stemness of glioma-initiating cells, the possibility cannot be excluded that other signaling pathways are also involved in it. Hedgehog signaling has been implicated in the development of glioma for many years. It was reported that Hedgehog-Gli1 pathway regulates the stemness of glioma-initiating cells and that cyclopamine, a hedgehog inhibitor, could reduce glioma tumor volume (Clement et al., 2007), though the mechanisms by which inhibition of Hedgehog-Gli1 pathway promotes differentiation of glioma-initiating cells were not investigated. Cyclopamine abrogated sphere-forming ability of TGS-01 cells, but this effect was weaker than that of SB431542, and these two agents failed to show additive or synergistic effects (Figure S12). Therefore, it is likely that there are at least two types of GSCs, Hedgehog-Gli1 pathway-dependent cells and TGF-β-Sox4-Sox2 pathway-dependent cells. To apply Hedgehog inhibitor and TGF-β inhibitor to clinical stages, it will be necessary to assess GSCs of each patient for their signal dependency.

While this manuscript was under revision, Peñuelas et al. (2009) independently reported that TGF-β increases glioma-initiating cell self-renewal through induction of LIF in human glioblastoma. Their results agree with our report in terms of the role

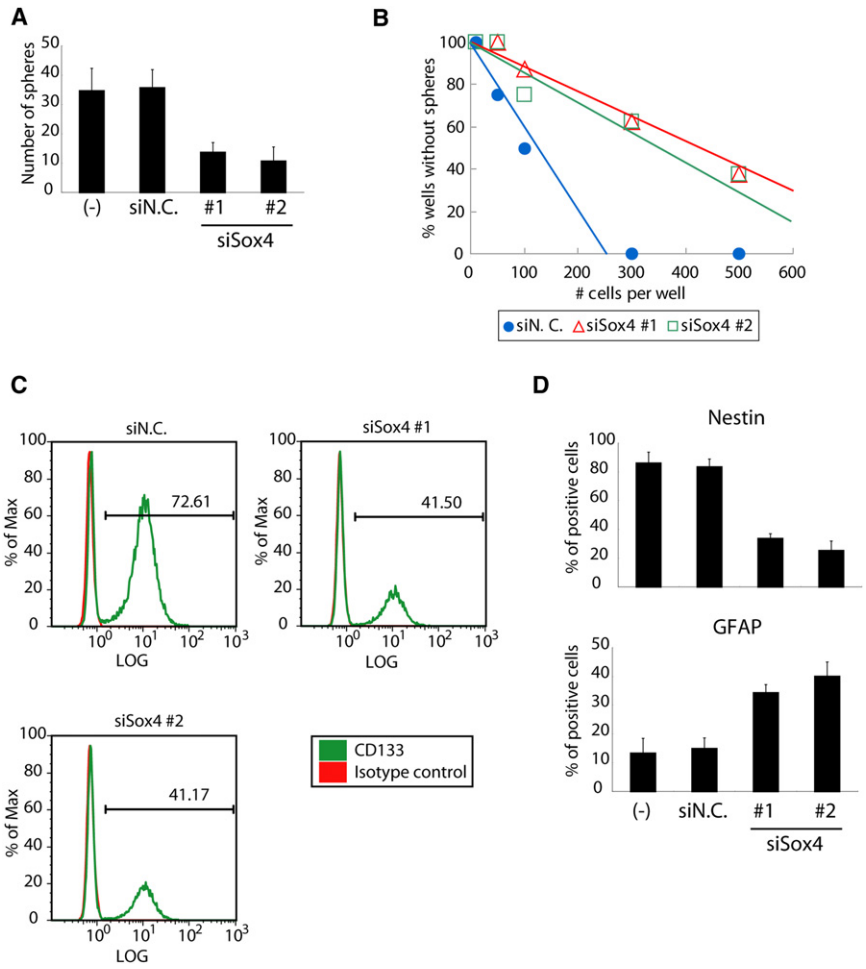


**Figure 6. Sox4 Plays Important Roles in Maintenance of Stemness of Glioma-Initiating Cells**

(A) TGS-01 cells were dissociated into single-cell populations, transfected with control or Sox4 siRNA duplex, and cultured for 7 days. The data are presented as the number of glioma spheres formed (means ± SEM of five fields).

(B) Knockdown of Sox4 expression by siRNA in TGS-01 cells resulted in a decrease of self-renewal capacity in limiting dilution assay.

(C) Effects of Sox4 knockdown on $CD133^+$ subpopulation of TGS-01 cells were determined by flow cytometry.

(D) Quantification of Nestin-positive or GFAP-positive cells among total cells. Differentiation of TGS-01 cells by Sox4 knockdown was analyzed 7 days after transfection of control (N.C.) or Sox4 siRNA duplex. Error bars represent SEM.

of TGF-β signaling in maintenance of glioma-initiating cells. Although glioma-initiating cells used in our study, TGS-01 and -04, were deprived of their tumorigenicity in the presence of anti-LIF neutralizing antibody (Figure S13), TGF-β signaling failed to induce LIF expression (Figure S7). Taken together, TGF-β signaling may maintain tumorigenicity of glioma-initiating cells through multiple (at least two) independent pathways.

To target TGF-β signaling in glioma, clinical studies of a TGF-β2-specific antisense oligonucleotide AP 12009 in recurrent or refractory high-grade glioma are ongoing (Schlingensiepen et al., 2006). Our findings indicate that such TGF-β inhibitors should be delivered to GSCs, not only to the tumor bulk. This indication will thus pursue the realization of new therapeutic strategies, inducing differentiation of GSCs in addition to suppressing bulk tumor growth. Classical pharmacological therapies cannot sufficiently eradicate GSCs (Liu et al., 2006a). Moreover, GSCs were known to be resistant against radiotherapy (Bao et al., 2006). Our results raise the possibility that TGF-β inhibitors can be used in a combination therapy with classical pharmacological therapies and radiation to make glioblastoma less aggressive. To achieve this, we need to develop a method for delivery of TGF-β inhibitor to GSCs. Delivery of TGF-β inhibitor to GSCs without affecting the functions of normal brain tissues may improve prognosis of GBM, one of the most lethal malignant tumors.

## EXPERIMENTAL PROCEDURES

### Cell Culture and Reagents

Primary grade IV glioblastoma samples were obtained during surgery from consenting patients, as approved by the Institutional Review Board at the University of Tokyo Hospital (Characteristics of the patients are listed in Figure S1A). Spheres were cultured in DMEM/F12 serum-free medium (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/ml of EGF, and 20 ng/ml of bFGF (both from PeproTech). The antibodies used were as follows: anti-Musashi (Chemicon), anti-Nestin (Chemicon), anti-GFAP (Dako), anti-Tuj1 (Covance), anti-Sox2 (R&D), anti-Sox4 (Santa Cruz), anti-α-tubulin (Sigma-Aldrich), anti-phospho-Smad2 (Zymed Laboratories), anti-Smad2/3 (BD Transduction Laboratories), and phycoerythrin-conjugated CD133/2 (293C3) antibody (Miltenyi Biotec). SB431542 and cycloheximide were purchased from Sigma-Aldrich. Recombinant human TGF-β RII/Fc chimera was from R&D Systems.

### Immunostaining

Glioma-initiating cells were seeded on poly-L-ornithine (Sigma)- and fibronectin (Sigma)-coated slide glasses and cultured for 7 days with indicated ligands or inhibitors in serum-free medium. Cells were fixed with 3.7% paraformaldehyde, permealized with PBS containing 0.3% Triton X-100, and incubated with indicated antibodies. Subsequently, samples were incubated with secondary antibodies and stained with propidium iodide (Molecular Probes) for nuclear staining. Stained cells were observed with a confocal microscope (LSM510, Carl Zeiss).

### Flow Cytometry

Cells were dissociated into single-cell populations and labeled with a phycoerythrin-conjugated CD133 antibody. The expression level was analyzed using a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software.

### Sphere-Forming Assay

Glioma-initiating cells were cultured in non-tissue-culture-treated flask (BD Biosciences) with vented caps (BD Biosciences) for 7 days. Floated spheres in five fields per each sample were counted under a microscope (magnification, ×40).

### RNA Interference

Small interfering RNAs (siRNAs, see Table S1 for sequences) were purchased from Invitrogen and introduced into cells using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions.

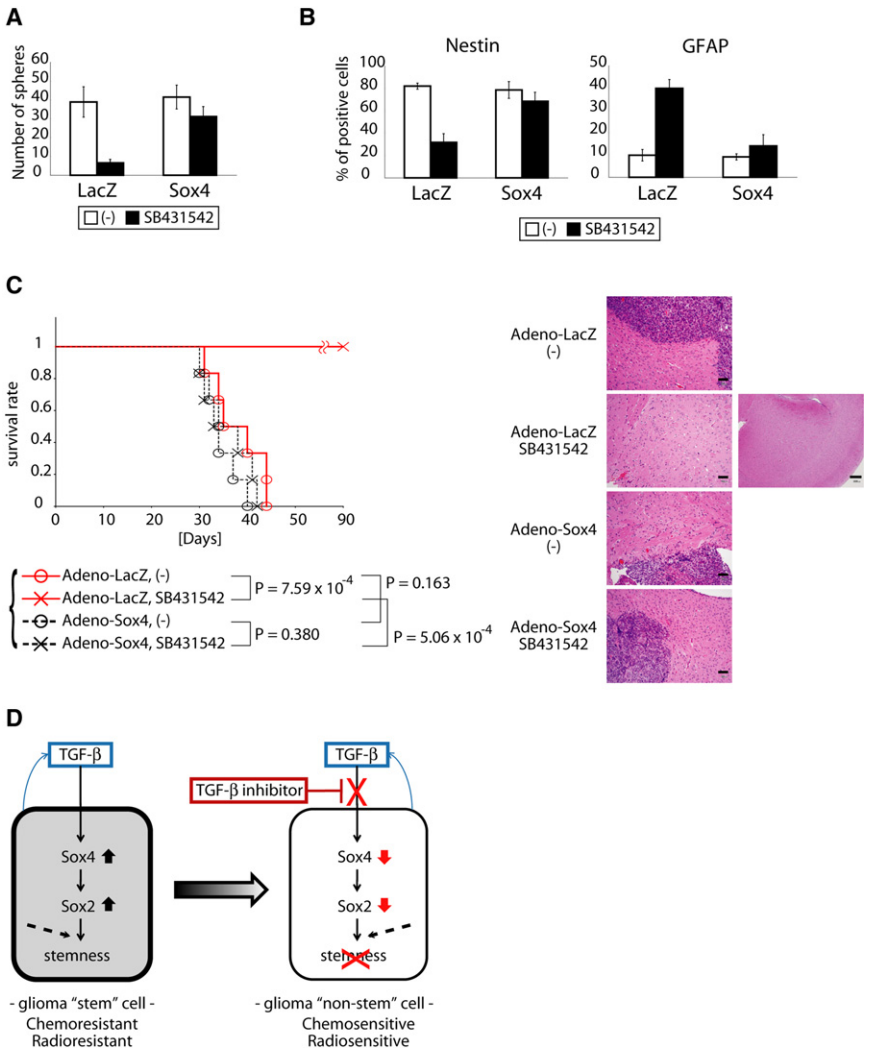


**Figure 7. Direct Suppression of Sox4 Expression by TGF-β Inhibitor Is a Critical Step for Differentiation of Glioma-Initiating Cells In Vitro and In Vivo**

(A) TGS-01 cells were dissociated into single-cell populations, infected with adenovirus-Sox4 or LacZ, and cultured with or without SB431542 (1 μM) for 7 days. The data are presented as the number of glioma spheres formed (means ± SEM of five fields).

(B) Marker expression of TGS-01 cells infected with adenovirus-Sox4 or LacZ and cultured with or without SB431542 (1 μM) for 7 days. Quantification of Nestin-positive or GFAP-positive cells among total cells is shown in graphs. Error bars represent SEM.

(C) Development of gliomas after intracranial transplantation of 5 × $10^4$ TGS-01 cells infected with adenovirus-Sox4 or LacZ (negative control) and treated with or without TGF-β inhibitor (SB431542, 1 μM) for 24 hr. Survival of mice (n = 6 mice for each condition) was evaluated by Kaplan-Meier analysis (left graph). p value was calculated using a log-rank test. Right panels show the results of histological examination of the samples dissected at 30 days after intracranial transplantation. Tissue sections were stained with hematoxylin and eosin. Scale bars, 50 μm in the four left images (×20) or 300 μm in the right image (×4).

(D) Model of maintenance of stemness of glioma-initiating cells by autocrine TGF-β signaling. TGF-β directly induces Sox4 expression. Subsequently, Sox4 promotes expression of Sox2, which plays significant roles in sustaining stemness of glioma-initiating cells, possibly in cooperation with other signaling pathways (dotted arrows). TGF-β inhibitor blocks this "TGF-β-Sox4-Sox2" pathway, promotes differentiation of glioma-initiating cells, and deprives them of aggressiveness.

### Adenoviruses

Flag-tagged full-length Sox2 and Sox4 cDNAs were cloned into a pENTR vector (Invitrogen) and introduced into the adenoviral genome via recombination between pENTR vector and the pAd/CMV/V5-DEST vector using LR Clonase (Invitrogen). HEK293A cells were infected with pAd/CMV/Sox2 or pAd/CMV/Sox4 after linearization of it with *PacI*. Viral particles were isolated by three freeze-thaw cycles and amplified by reinfection to HEK293A cells.

### Cell Lysis and Immunoblotting

Cells were lysed with a buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1% aprotinin, and 5 mM EDTA. Proteins in cleared cell lysates were subjected to SDS-PAGE and transferred to Fluoro Trans W membrane (Pall). Immunoblotting was performed using the indicated antibodies.

### Quantitative Real-Time PCR

Quantitative real-time reverse transcription PCR was performed as described previously (Ikushima et al., 2008). All samples were run in triplicate in each experiment. The primers used are listed in Table S1. Values were normalized to that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### Limiting Dilution Assay

Sphere cells were dissociated and plated in 96-well plates in 200 μl serum-free medium. After a 7 day culture, the percentage of wells not containing spheres for each cell-plating density was calculated and plotted against the number of cells per well.

### Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as described previously (Koinuma et al., 2009). Following reverse crosslinking, DNA was treated with proteinase K and purified using a PCR purification kit (QIAGEN). DNA was eluted in 30 μl of TE and used for PCR analysis or quantitative real-time PCR. PCR primers are listed in Table S1.

### Intracranial Proliferation Assay

Viable glioma-initiating cells (5 × $10^4$) in 5 μl of DMEM/F12 medium were injected stereotactically into the right cerebral hemisphere of 5-week-old female BALB/c *nu/nu* mice at a depth of 3 mm. All animal experimental protocols were performed in accordance with the policies of the Animal Ethics Committee of the University of Tokyo.

### Statistical Analysis of Microarray Data

Microarray data were obtained from GEO (http://www.ncbi.nlm.nih.gov/geo/) and ArrayExpress (http://www.ebi.ac.uk/microarray/). Data were analyzed with statistical software R (http://www.R-project.org).

## SUPPLEMENTAL DATA

Supplemental Data include one table and 13 figures and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00402-0.

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