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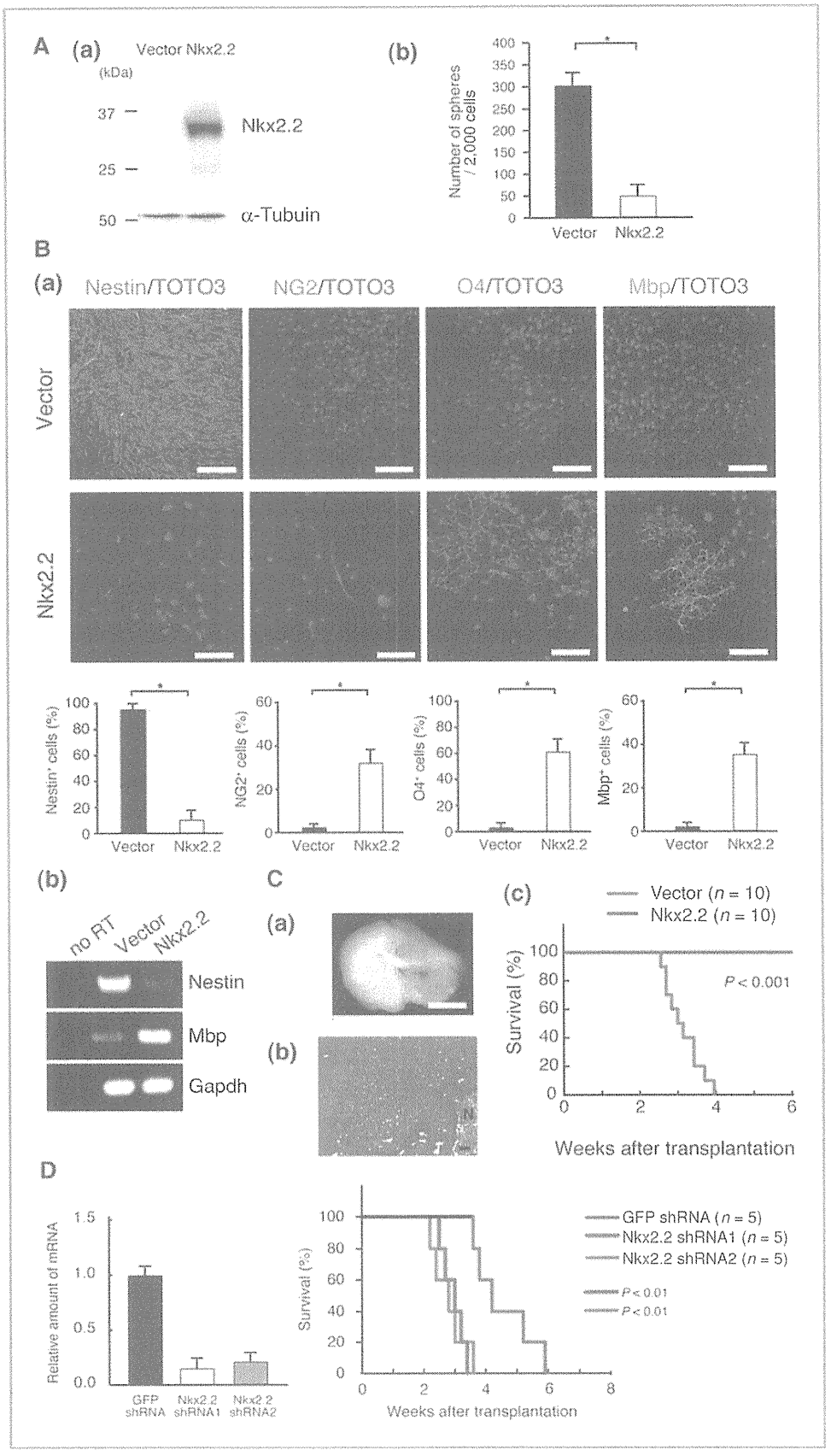

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of mature neurons (NeuN; Supplementary Fig. S5). Expression of Mbp or O4, both of which are expressed in mature oligodendrocytes, was not observed in either type of glioma (Supplementary Fig. S5 and data not shown). However, interestingly, Nkx2.2, a homeodomain transcription factor essential for oligodendroglial differentiation, was strongly expressed in AAs but barely detectable in GBMs (Fig. 2D). These data suggest that mechanisms governing oligodendroglial differentiation may play a critical role in regulating malignant progression of glioma.

Nkx2.2 regulates mouse gliomagenesis

Our observation that malignant glioma progression is inversely correlated with loss of Nkx2.2 expression suggested that presence of Nkx2.2 inhibits GBM formation. To test this hypothesis, we assessed the effects of Nkx2.2 upregulation on the fate of GICs within TNSs derived from GBMs. Nkx2.2 reportedly induces oligodendroglial differentiation of normal NPCs (25, 26). We overexpressed Nkx2.2 in dissociated GBM cells derived from $p53^{-/-}$:$p16^{Ink4a-/-}$/$p19^{Arf-/-}$;$NR^{+/Lumo}$ mice and examined TNS formation. Nkx2.2 overexpression markedly reduced both the numbers of TNSs formed and their expression of Nestin (Fig. 3A and B). Notably, cells within these TNSs showed increased expression of the oligodendrocyte markers, chondroitin sulfate proteoglycan (NG2), O4, and Mbp. These data indicate that Nkx2.2 can induce oligodendroglial differentiation of GICs, decreasing their self-renewal capacity (Fig. 3B). Next we used intracranial injection to introduce vector-transduced murine TNSs into NOD/SCID mice ($n = 10$), lethal, infiltrating gliomas developed in brains of all mice within 1 month. By contrast, all mice (10 of 10) injected with TNSs overexpressing Nkx2.2 survived for more than 2 months (Fig. 3C). Thus, induction of oligodendroglial differentiation of GICs by Nkx2.2 suppresses GBM formation *in vitro*.

To directly examine the effect of Nkx2.2 loss on gliomagenesis, we assessed the ability of NPCs in which Nkx2.2 was downregulated via shRNA knockdown to promote gliomagenesis in a different genetic murine glioma model, the EGFRvIII-induced GBM model (27). Introduction of *Nkx2.2* shRNA into cultured NPCs from these mice efficiently knocked down *Nkx2.2* expression (Fig. 3D). When recipient mice were inoculated with $p16^{Ink4a-/-}$/$p19^{Arf-/-}$ NPCs expressing EGFRvIII plus *Nkx2.2* shRNA, mouse survival was significantly shorter than that of recipients that received $p16^{Ink4a-/-}$/$p19^{Arf-/-}$ NPCs expressing EGFRvIII plus control shRNA (Fig. 3D). Histologic differences were not observed between gliomas arising from NPCs transduced with control versus *Nkx2.2* shRNAs (data not shown). These data suggest that Nkx2.2 downregulation accelerates formation of lethal GBMs.

NKX2.2 suppresses self-renewal of human GICs by induction of oligodendroglial differentiation *in vitro*

To investigate whether our findings are relevant to human gliomas, we first examined NKX2.2 protein expression in 96 human high-grade gliomas: 33 GBMs, 36 AAs, and 27 anaplastic oligodendrogliomas (AO). As previously reported, immunohistochemical analysis revealed that NKX2.2 was expressed at higher levels in AOs (22 positive cases in 27 AOs, 81%) than the levels in AAs (15 positive cases in 36 AAs, 42%; Fig. 4). Interestingly, most GBMs (28 cases in 33 GBMs) did not express NKX2.2 (Fig. 4). Thus, in both humans and mice, NKX2.2 suppression is positively correlated with increased malignancy in astrocytomas.

To assess the relevance of our findings to human GICs, we examined the effect of NKX2.2 overexpression on TNS formation by GICs. To do so, we used early passage, patient-derived GIC lines, termed TGS-01 and TGS-04, which under serum-free conditions retain phenotypes and genotypes more closely mirroring primary tumor profiles (17). NKX2.2 overexpression markedly reduced both the number of TNS formed from GICs and their expression of Nestin (Fig. 5A–D). In addition, cells within these TNSs showed increased O4 expression. These data indicate that NKX2.2 can decrease self-renewal capacity and induce oligodendroglial differentiation of human GICs *in vitro*, suggesting that mechanisms observed in our mouse models are conserved in human GICs. To examine whether NKX2.2 is directly regulated by transforming growth factor β (TGFβ) or bone morphogenetic protein 4 (BMP) signal, which is involved in maintenance of GICs (17, 28, 29), we examined the effects of the TGFβ inhibitor (SB431542, 1 μmol/L) and BMP4 (100 nmol/L) on NKX2.2 expression in TGS-01 by immunoblotting and immunocytochemistry. NKX2.2 expression was not remarkably affected by these treatments (data not shown) nor was phosphorylation of Smad2/3 and Smad1/5/8 altered by NKX2.2 overexpression. These data suggest that NKX2.2 does not directly interact with TGFβ or BMP signaling in human GICs.

Critical roles of Nkx2.2 in mouse gliomagenesis. A, Nkx2.2 overexpression decreases TNS formation. Primary mouse glioma cells isolated from $p53^{-/-}$:$p16^{Ink4a-/-}$/$p19^{Arf-/-}$;$NR^{+/Lumo}$ mice were cultured as TNSs, infected with Vector- or Nkx2.2-expressing retrovirus, and cultured for 7 days. a, Western blot analysis of Nkx2.2 protein in representative samples. b, data shown are the mean number ± SD of TNSs generated per 2,000 cells ($n = 5$/group). *, $P < 0.001$. B, decreased Nestin but increased NG2, O4, and Mbp expression. a, TNSs were cultured on coverslips, infected with Vector- or *Nkx2.2*-expressing retrovirus, selected with blasticidine-S for 4 days, and stained with anti-Nestin (red) plus TOTO3, anti-NG2 (red) plus TOTO3, anti-O4 (red) plus TOTO3, or anti-Mbp (green) plus TOTO3. Data shown represent 5 experiments. Scale bars, 50 μm. Data shown in the bottom are the mean percentage ± SD of $Nestin^+$ or $NG2^+$ or $O4^+$ or Mbp^+ cells among $TOTO3^+$ cells ($n = 5$/group). *, $P < 0.001$. b, RT-PCR of genes encoding proteins indicated in (a). C, secondary tumors. NOD/SCID mice were injected with retrovirus-infected TNSs cultured as in (B). a, image showing gross appearance of a coronal section from forebrain of a mouse injected with Vector-infected TNS. Data shown represent 10 mice. Scale bar, 3 mm. b, a coronal section of the secondary tumor in (a) stained with H&E. "N" indicates an area of palisading with regional necrosis. Scale bar, 100 μm. c, Kaplan–Meier tumor-free survival curves of NOD/SCID mice injected with TNSs expressing Vector alone or *Nkx2.2*. D, confirmation of shRNA-mediated *Nkx2.2* knockdown (left). SVZ cells from neonatal $p16^{Ink4a-/-}$/$p19^{Arf-/-}$ mice were infected with virus expressing control *GFP* shRNA or the indicated *Nkx2.2* shRNAs. *Nkx2.2* mRNA levels were determined by real-time RT-PCR, normalized to *β-actin* expression, and expressed as arbitrary units relative to control samples (defined as equal to 1). Results shown are the mean ratio ± SD of *Nkx2.2* mRNA relative to *β-actin* ($n = 3$/group). Right, decreased mouse survival. SVZ cells derived from neonatal $p16^{Ink4a-/-}$/$p19^{Arf-/-}$ mice were cultured, infected with *EGFRvIII*-expressing virus, cultured for 7 more days, and injected into brains of NOD/SCID mice. Kaplan–Meier tumor-free survival curves are shown.

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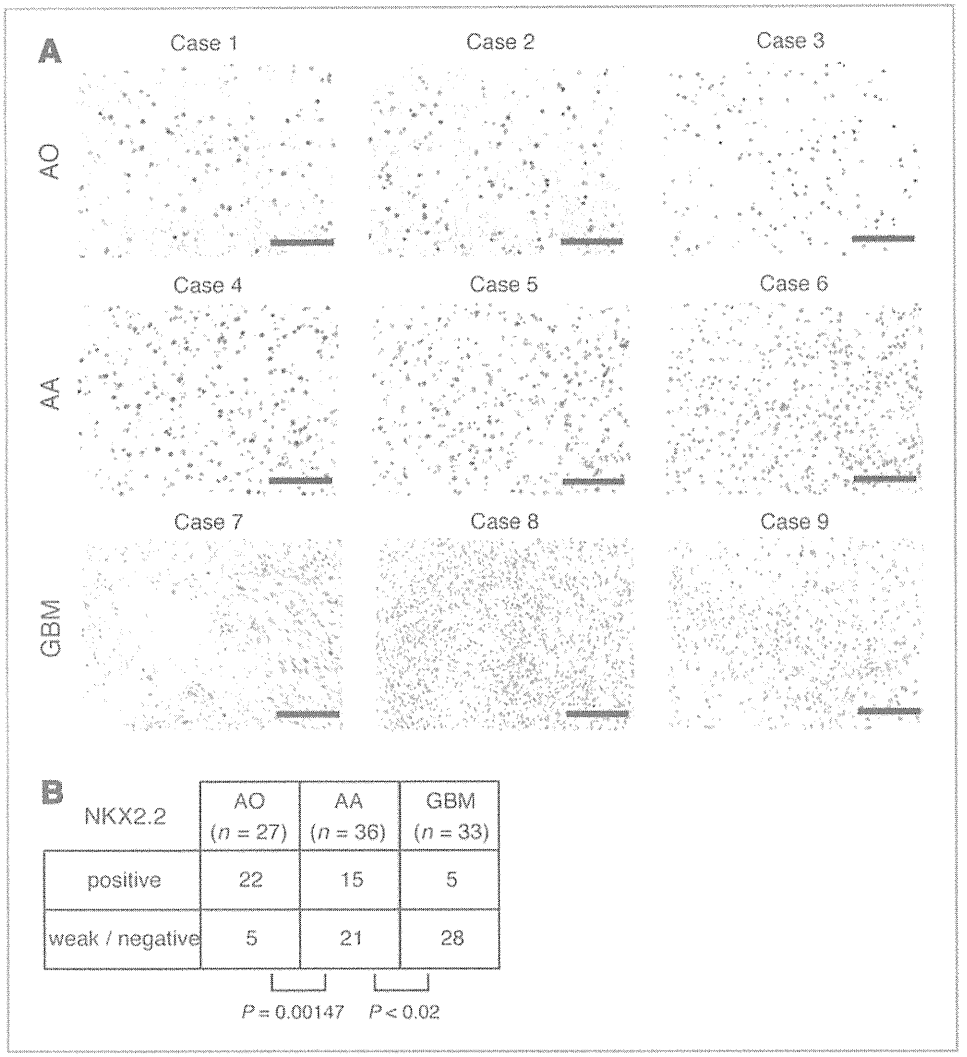

B

NKX2.2	AO (n = 27)	AA (n = 36)	GBM (n = 33)
positive	22	15	5
weak / negative	5	21	28

$P = 0.00147$ $P < 0.02$

Figure 4. Immunohistochemical examination of NKX2.2 expression in human high-grade gliomas. A, sections of 96 human gliomas were stained with anti-NKX2.2 antibody. Three representative samples of 27 AOs (cases 1–3), 36 AAs (cases 4–6), or 33 GBMs (cases 7–9) are shown. Scale bars, 100 μm. B, correlation between downregulated NKX2.2 expression and malignancy. Gliomas in (A) were scored for intensity of NKX2.2 immunostaining. The significance of the association between the level of NKX2.2 protein expression and malignancy grade was calculated, as determined by Fisher's exact test (right tail).

Discussion

Here, we have established mouse models harboring specific mutations that allow us to control stages of malignant glioma progression (AA versus GBM). These models provide significant advantages in comparing characteristics of gliomas of different malignant progression stages. Mutations seen in human cancers may differ from those seen in mouse models. Nonetheless, mouse models are essential and indispensable to fully understand the nature of gliomas. Our models represent powerful tools useful to identify novel factors that control glioma malignant progression.

A stem cell–like gene expression signature (stemness) has been shown in poorly differentiated tumors, based on histologic criteria. Stemness is associated with an unfavorable prognosis in several human cancers, including gliomas (30). Consistent with these data, we observed enhanced stemness characteristics, including upregulation of stem cell markers or sphere formation, in GBM (grade IV) tumors but not AA (grade III) in our mouse models. Although the mechanism is still unclear, several lines of evidence show that genetic loss of *p53* or $p16^{Ink4a}/p19^{Arf}$ enhances stemness. For example, *p53* or $p16^{Ink4a}/p19^{Arf}$ deficiency increases both the kinetics of induced pluripotent stem (iPS) cell reprogramming and the number of emerging iPS cell colonies (31–35). These results indicate that *p53* and $p16^{Ink4a}/p19^{Arf}$ function as barriers to cell reprogramming and acquisition of stemness. Indeed, *p53*/$p16^{Ink4a}/p19^{Arf}$–deficient multipotent hematopoietic progenitors exhibit properties of hematopoietic stem cells that can carry out long-term reconstitution of blood cells (36). Thus, *p53* and $p16^{Ink4a}/p19^{Arf}$ have a central role in limiting expansion of multipotent progenitors. Because differentiation pathways are commonly repressed in tumor cells, the above results plus our findings suggest a mechanism by which incipient neoplastic cells could gain the ability to self-renew, acquire further oncogenic mutations, and become malignant.

Our study reveals a critical role for Nkx2.2 in suppressing glioma development and GIC self-renewal. A direct effect of Nkx2.2 on oligodendroglial differentiation is supported by previous analyses of mouse spinal cord (25). Coexpression of Olig2 and Nkx2.2 in spinal cord (26) or in the ventricular and

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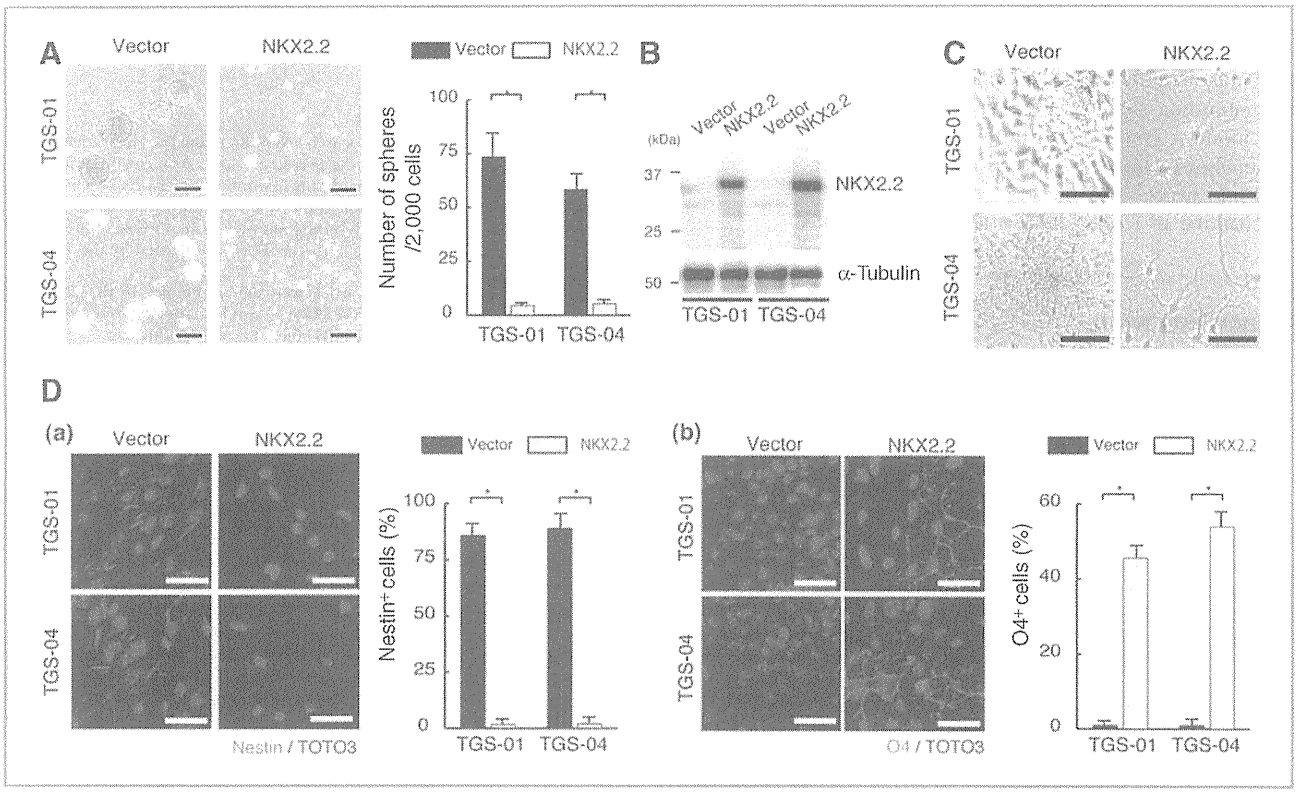

Figure 5. NKX2.2 overexpression inhibits self-renewal of human GICs by induction of oligodendroglial differentiation. A, NKX2.2 overexpression decreases human TNS formation. TGS-01 and TGS-04 cells were cultured as TNSs, transfected with pLXSB (Vector) or pLXSB-*NKX2.2* (NKX2.2), and selected with blasticidine-S for 4 days (left). Scale bars, 100 μm. Right, data shown are the mean number ± SD of TNSs generated per 2,000 cells ($n = 5$/group). *, $P < 0.001$. B, Western blot examination of NKX2.2 protein in representative samples from (A). α-Tubulin: loading control. C and D, decreased Nestin but increased O4 expression. TNSs were cultured on coverslips, transfected with pLXSB (Vector) or pLXSB-*NKX2.2* (NKX2.2), selected with blasticidine-S for 4 days (C, bright-field), and stained with (D, a) anti-Nestin (red) plus TOTO3, or (D, b) anti-O4 (red) plus TOTO3. Data shown represent 5 experiments. Scale bars, 50 μm. Data shown are the mean percentage ± SD of Nestin$^+$ or O4$^+$ cells among TOTO3$^+$ cells ($n = 5$/group). *, $P < 0.001$.

SVZ of the midbrain promotes oligodendrocyte differentiation (37). Olig2 is essential for proliferation and differentiation of oligodendrocyte precursors (38, 39). Olig2-expressing precursors give rise not only to oligodendrocytes but to motor neurons (26, 40), astrocytes, and ependymal cells (41, 42). In contrast to Olig2, Nkx2.2 generally regulates late differentiation and/or maturation, rather than initial specification, of oligodendrocyte precursors (25), although Nkx2.2 does support generation of new oligodendrocytes or remyelination in adults with CNS injury (43, 44). Nkx2.2 cooperates with Olig2 to promote oligodendrocyte maturation to Mbp-positive stages (26, 45). Although several lines of evidence suggest that Olig2 activity represents a mechanistic link between growth of malignant glioma cells and adult NPCs (46, 47), data regarding the role of Nkx2.2 role in gliomagenesis are not available. Our findings suggest that Nkx2.2 functions as a cell fate switch determining whether NPCs receiving oncogenic stimulation develop into benign glial cells or malignant astrocytomas.

Rousseau and colleagues (48) previously reported that NKX2.2 was expressed at higher levels in human AO than in AA and GBMs, suggesting that NKX2.2 is a marker that distinguishes AO from astrocytomas. In addition, it has been reported that NKX2.2 expression is elevated in a proneural subgroup of human GBM (49). In our examination of a larger group of clinically defined samples, we found that NKX2.2 is not only a marker for AO but also an indicator of malignant progression in astrocytomas, suggesting that NKX2.2 expression antagonizes malignant progression of most gliomas. In addition, we showed that impaired oligodendroglial differentiation caused by Nkx2.2 downregulation accelerates GBM formation in a robust murine model of primary gliomagenesis. Our work shows that Nkx2.2 antagonizes glioma initiation and malignant progression induced by activation of oncogenic signaling in NPCs. Finally, we show that forced Nkx2.2 expression in GICs leads to oligodendroglial differentiation and suppression of self-renewal *in vitro*. However, it is unclear whether the inhibitory effects of NKX2.2 on glioma malignancy *in vivo* are mediated by the process of oligodendroglial differentiation, because in both mouse and human samples the mature oligodendrocyte marker, MBP, is not expressed in AA. NKX2.2 may affect malignant progression by an unknown function *in vivo*. Nonetheless, reactivation of NKX2.2 expression in glioma cells suggests a novel therapeutic strategy.

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In summary, our novel mouse glioma models allow us to analyze two grades of glioma rapidly and define molecular mechanisms underlying malignant glioma progression. Thus, understanding signaling driving malignant gliomagenesis in our models could contribute to development of novel approaches to diagnose and/or eradicate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Glioma-initiating Cells Retain Their Tumorigenicity through Integration of the Sox Axis and Oct4*

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Running title: Regulation of glioma-initiating cells by Oct4

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Background: Glioma-initiating cells are underlying causes of development and progression of glioblastoma.
Results: Depletion of Oct4 expression suppresses tumorigenic activity of glioma-initiating cells through down-regulation of Sox2.
Conclusion: Oct4 maintains tumorigenicity of glioma-initiating cells in cooperation with the Sox axis.
Significance: This study uncovers transcriptional network of stemness genes in cancer-initiating cells.

SUMMARY

Although the concept of cancer stem cells or cancer-initiating cells had created a new paradigm for the treatment of malignant tumors, it remains unclear how cancer-initiating cells can be eradicated. We have previously reported that transforming growth factor-β (TGF-β)-Sox4-Sox2 pathway is essential for glioma-initiating cells to retain their stemness, and inhibition of TGF-β signaling may lead to differentiation of glioma-initiating cells (Ikushima et al. (2009) Cell Stem Cell 25, 504-514). Here we demonstrate that Oct4 plays essential roles in retention of the stemness properties of glioma-initiating cells through positive regulation of Sox2 expression. We also show that, in glioma-initiating cells, Oct4 is associated with Sox4 and that Oct4-Sox4 complexes cooperatively activate enhancer activity of the Sox2 gene. In contrast, in fetal neural progenitor cells, Sox2 expression is enhanced by transcriptional complex containing Sox2 protein itself, and this self-reinforcing loop of Sox2 appears to be disrupted in glioma-initiating cells, suggesting that Sox2 expression in glioma-initiating cells is differently regulated from that in neural progenitor cells. Our findings reveal differences between glioma-initiating cells and fetal neural progenitor cells, and may open the way to depriving glioma-initiating cells of tumorigenic activity without affecting normal tissues.

Glioblastoma, also known as grade IV astrocytoma, is the most aggressive form of malignant glioma and is one of the most

malignant human cancers, with an estimated median survival of only around 1 year (1, 2). Despite past huge efforts, this statistic has not markedly improved over the past decades.

Cancer stem cells or cancer-initiating cells are tumor cells characterized by their ability to induce tumorigenesis and to self-renew (3). Similar to other types of tumor cells, glioma-initiating cells (or glioma stem cells) have been isolated from human glioblastoma tissues (4, 5). Following their identification, glioma-initiating cells have been intensively investigated, and have been found to exhibit strong resistance to chemotherapy and radiotherapy (6, 7). It has been suggested that the failure to cure glioblastoma may be due to existing therapeutic strategies that affect only the tumor bulk and not glioma-initiating cells (8). These findings indicate the need for an innovative therapeutic strategy enabling functional eradication of glioma-initiating cells.

Although it has yet to be fully determined how the stemness of glioma-initiating cells is maintained, a few signaling pathways, including Hedgehog (9), bone morphogenetic protein (BMP) 4 (10), and transforming growth factor (TGF)-β (11-13), have been implicated to contribute to maintenance of the stemness properties of these cells. Although the transcriptional machinery required is under investigation, we have recently reported crucial roles for the Sox axis. Sox4 interacts with the Sox2 enhancer region to induce Sox2 expression, and this "Sox4-Sox2" axis maintains stemness properties of glioma-initiating cells under the control of TGF-β signaling (11).

The POU class 5 transcription factor Oct4 (also known as Pou5f1) is essential for establishing and maintaining the pluripotent state of embryonic stem cells (14, 15). Deletion of Oct4 from embryonic stem cells results in trophoblast differentiation (16). Introduction of Oct4 together with Sox2, Klf4, and c-Myc into human or mouse adult fibroblasts results in the generation of induced pluripotent stem (iPS) cells (17, 18). In addition, Oct4 has been detected in high-grade glioma and specific types of testicular germ cell tumors (19-21). However, the role of Oct4 in cancer stem cells has yet to be fully determined.

Here, we report that Oct4 is a factor of crucial importance for the maintenance of tumorigenic activity of glioma-initiating cells. We have previously reported that in contrast to Sox4 and Sox2, the expression of Oct4 is not regulated by TGF-β signaling in glioma-initiating cells (11). However, inhibition of Oct4 expression in glioma-initiating cells resulted in suppression of sphere formation *in vitro* and tumor formation *in vivo*. Oct4 knockdown also potentiated sensitivity to conventional chemotherapy. We also demonstrated that Oct4 interacted with Sox4 and cooperatively activated the Sox2 enhancer region to maintain stemness properties of glioma-initiating cells. Notably, Sox2 expression in glioma-initiating cells was induced by the Oct4-Sox4 complex acting on the Sox2 enhancer region to maintain stemness properties, while that in fetal neural progenitor cells was regulated by transcriptional complex containing Sox2 protein itself through a self-reinforcing regulatory loop. These findings indicate that Oct4 plays a role in the tumorigenic activity of glioblastoma and suggest that the stemness properties of glioma-initiating cells are regulated by mechanisms different from those of neural progenitor cells.

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 of the University of Tokyo Hospital. 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). Characteristics of the glioma-initiating cells were evaluated in our previous study (11). Normal human fetal neural progenitor cells were obtained from Lonza, and cultured in maintenance medium (NPMM; Lonza). U373MG cells were maintained in DMEM containing 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, sodium pyruvate (1 mM), and non-essential amino acids (0.1 mM). The antibodies used were as follows: anti-Musashi (Chemicon); anti-Nestin (Chemicon); anti-glial fibrillary acidic protein (GFAP; Dako); anti-Tuj1 (Covance); anti-Oct4 (Santa Cruz); anti-Sox2 (R&D); anti-Sox4 (Santa Cruz); anti-α-tubulin (Sigma-Aldrich).

Sphere-forming assay- Glioma-initiating cells were cultured in non-tissue-culture-treated flasks (BD Biosciences) with vented caps (BD Biosciences) for 7 days. Floating spheres in five fields per sample were counted under a microscope (magnification; x40).

Limiting dilution assay- Sphere cells were dissociated and plated in 96-well plates in 200 μl serum-free medium. After 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.

RNA interference- siRNAs (see Supplemental Table 1 for sequences) were purchased from Invitrogen and introduced into cells using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions.

Immunostaining- Glioma-initiating cells were seeded on poly-L-ornithine (Sigma)- and fibronectin (Sigma)-coated slide glasses and cultured for 7 days with the indicated siRNA in serum-free medium. Cells were fixed with 3.7% paraformaldehyde, permeabilized with PBS containing 0.3% Triton X-100, and incubated with the 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).

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 (22). All samples were run in triplicate in each experiment. The primers used are listed in Supplemental Table 1. Values were normalized to that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

ChIP (chromatin immunoprecipitation) and ChIP Re-IP (ChIP re-immunoprecipitation)- ChIP was performed as described previously (11). PCR primers are listed in Supplemental Table 1. For ChIP Re-IP assays, protein-DNA complexes were eluted from

immunoprecipitation by incubation with 10 mM DTT at 37 °C for 30 min and diluted 1:50 in buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2mM EDTA, 1% Triton X-100), followed by Re-IP with secondary antibodies.

Cell viability assay- Quantitation of cell viability was performed using a colorimetric assay for mitochondrial dehydrogenase activity (WST-8; Nacalai Tesque) after treatment with temozolomide (LKT Laboratories).

Luciferase assay- The Sox2 enhancer region (+3553 ~ +4290) was cloned into a pGL4 vector (Promega) with a minimal promoter and luciferase assay was performed as described previously (22). Values were normalized to Renilla Luciferase activity under the control of thymidine kinase promoter.

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.

RESULTS

Oct4 is an essential factor for retention of stemness of glioma-initiating cells in vitro.

The transcriptional network essential for maintenance of glioma-initiating cells has not been fully determined. We used glioma-initiating cells obtained from two patients with glioblastoma, termed TGS-01 and TGS-04, and cultured in serum-free medium to study this network. The glioma-initiating capacities of these cells were characterized in our previous studies (11).

Oct4 is known to be one of the most crucial self-renewal genes and to play pivotal roles in maintaining stemness of embryonic stem cells and neural stem cells. We have demonstrated that expression of Oct4 is not affected by TGF-β signaling in glioma-initiating cells (11). To study the role of Oct4 in glioma-initiating cells, we first examined the effects of Oct4 knockdown on their biological properties. After Oct4 expression was knocked down (Supplemental Fig. 1), glioma-initiating cells exhibited marked reduction of sphere-forming ability in serial sphere-forming assay (Fig. 1A), suggesting that Oct4 is required for self-renewal of glioma-initiating cells. In limiting dilution assay, TGS-01 or TGS-04 with Oct4 small interfering RNA (siRNA) also showed less capacity for self-renewal than control cells (Fig. 1B). Similar results were obtained with the use of glioma-initiating cells, TGS-02, TGS-03, and TGS-05, derived from other patients with glioblastoma (Supplemental Fig. 2). We also examined the effects of Oct4 knockdown on proliferation and apoptosis of glioma-initiating cells. Treatment of siRNA against Oct4 did not significantly induce apoptosis, but reduced proliferation, of TGF-01 and TGS-04 cells (Supplemental Fig. 3)

Glioma-initiating cells have been reported to express neural precursor cell markers, but to only minimally express neural or glial differentiation markers (11). To examine the expression of these marker proteins in each type of cell, spheres in serum-free medium were disaggregated and seeded on poly-L-ornithine- and fibronectin-coated slide glasses. Knockdown of Oct4 expression by siRNA 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) (Fig. 1C). These results indicate that Oct4 is required for maintenance of the stemness properties of glioma-initiating cells *in vitro*.

Knockdown of Oct4 expression decreases tumorigenicity of glioma-initiating cells in vivo.

To study the role of Oct4 in the tumorigenic activity of glioma-initiating cells *in vivo*, we next examined the effects of Oct4 knockdown on intracranial growth of glioma-initiating cells. We treated dissociated glioma-initiating cells with siRNA against Oct4. Cells from the newly formed glioma spheres were orthotopically inoculated into the brains of immunocompromised mice. The growth of glioma-initiating cells was inhibited by pretreatment with siRNA against Oct4, and the mice inoculated with the pretreated glioma-initiating cells survived significantly longer than those inoculated with control cells (Fig. 2). We also examined tumor formation in the brain 30 days after transplantation. Whereas mice with control glioma-initiating cells displayed large tumors in the brain, those with the pretreated glioma-initiating cells exhibited no evidence of tumor on histopathologic examination (Fig. 2). These results suggest that Oct4 is essential for the maintenance of tumorigenicity of glioma-initiating cells, and that loss of tumorigenicity by Oct4 knockdown is an irreversible process.

Knockdown of Oct4 expression in glioma-initiating cells affects sensitivity to chemotherapy.

As suggested by the cancer stem cell model, the resistance of glioma-initiating cells to conventional chemotherapy may be a major cause of the low cure rate for glioblastoma (3). Although oral administration of temozolomide, a new alkylating agent, has shown efficacy in patients with glioblastoma, it was found that glioma-initiating cells were resistant to temozolomide-induced cell death, causing tumors to recur (7). We examined the effects of Oct4 knockdown on the sensitivity of glioma-initiating cells to temozolomide-induced cell death. Control TGS-01 and TGS-04 cells exhibited low sensitivities to temozolomide treatment (Fig. 3; Supplemental Fig. 4). In contrast, treatment with increasing concentrations of temozolomide suppressed the viability of TGS-01 and TGS-04 cells pretreated with Oct4 siRNA in dose-dependent fashion. These results suggest that Oct4 is involved in acquisition of drug-resistance properties of glioblastoma.

Oct4 directly induces Sox2 expression to maintain stemness properties of glioma-initiating cells.

We next studied the molecular mechanisms that underlie the putative pathological roles of Oct4 in glioma-initiating cells. Oct4 regulates stemness properties of embryonic and neural stem cells via several mechanisms (23). Among them, Sox2, which has recently been reported to be a critical regulator of the stemness of glioma-initiating cells (11, 24), is known to be one of the major downstream target genes of Oct4 in embryonic stem cells (25). We therefore examined whether Sox2 acts downstream of Oct4 in glioma-initiating cells. Oct4 knockdown in glioma-initiating cells resulted in down-regulation of Sox2 expression (Fig. 4A), indicating that Oct4 positively regulates Sox2 expression in glioma-initiating cells. To examine whether this regulation is directly mediated at the transcriptional level, we performed a ChIP assay using an antibody to Oct4. It has been

demonstrated that the enhancer element located in the 3' flanking region of the Sox2 gene is important for the regulation of Sox2 expression in embryonic stem cells (25, 26). Recruitment of Oct4 to the Sox2 enhancer element was observed in glioma-initiating cells (Fig. 4B). In contrast, Oct4 was only minimally associated with the Sox2 enhancer element in matched "differentiated" cells that were derived from the same patient but were cultured in media containing 10% fetal bovine serum to induce differentiation. These results appear to be due to the lower levels of expression of Oct4 in the "differentiated" cells compared with the "sphere" cells (Fig. 4C).

These findings together indicate that Oct4 induces Sox2 expression in glioma-initiating cells through direct binding to the Sox2 enhancer region.

Oct4 induces Sox2 expression cooperatively with Sox4 and activates the Sox4-Sox2 cascade in glioma-initiating cells.

In our previous study, another transcription factor, Sox4, was shown to associate with the Sox2 enhancer region and maintain the stemness and tumorigenicity of glioma-initiating cells (11). In addition, consensus sequences of Sox proteins and Oct4 exist proximally in the Sox2 enhancer region (Fig. 5A). These findings prompted us to study the interaction of the Sox axis and Oct4 in the maintenance of stemness properties of glioma-initiating cells. First, we examined the interaction between Sox4 and Oct4. As shown in Fig. 5B, endogenous Oct4 physically interacted with endogenous Sox4 in glioma-initiating cells. Moreover, ChIP Re-IP experiments demonstrated that Sox4 and Oct4 exist in the same transcription complex on the Sox2 enhancer region (Fig. 5C).

We next studied the effects of the Oct4-Sox4 complex on Sox2 expression in glioma-initiating cells. When Oct4 and Sox4 were both knocked down, Sox2 expression was more strongly down-regulated than by separate knockdown of Oct4 or Sox4 (Fig. 6A). Suppression of Sox2 enhancer activity by knockdown of Oct4 or Sox4 was also confirmed in luciferase assay using TGS-01 and TGS-04 cells (Fig. 6B). Moreover, the enhancer activity was synergistically activated by Oct4 and Sox4 overexpression in glioblastoma cell line U373MG (Fig. 6B), in which the levels of expression of Oct4 and Sox4 were significantly lower than in glioma-initiating cells (data not shown).

To confirm a direct association of Oct4 and Sox4 with the Sox2 enhancer region, we generated luciferase constructs with mutated Oct4 and/or Sox4 binding elements in the Sox2 enhancer region (Fig. 6C). Mutation of one of the two elements led to a reduction of enhancer activity compared with the wild-type enhancer. When both binding elements were mutated, the enhancer activity was more strongly reduced. These results indicate that both Oct4 and Sox4 directly interact with the Sox2 enhancer region and synergistically induce Sox2 expression.

Transcription factor complexes on the Sox2 enhancer region in glioma-initiating cells are distinct from those in neural progenitor cells.

As demonstrated here using glioma-initiating cells, Sox2 expression is also induced by Oct4 through interaction of Oct4 with the Sox2 enhancer region in embryonic stem cells. Furthermore, in embryonic stem cells, Sox2 is associated with Oct4 and the Oct4-Sox2 complex cooperatively activates the Sox2 enhancer region to form a positive regulatory loop (25, 26). To determine whether this regulatory loop exists in neural progenitor cells

and glioma-initiating cells, we examined recruitment of these transcription factors to the Sox2 enhancer region in ChIP assay (Fig. 7). Anti-Oct4 antibody enriched the DNA fragments of Sox2 enhancer region equally well in fetal neural progenitor cells and glioma-initiating cells. In addition, anti-Sox2 antibody immunoprecipitated this region in neural progenitor cells. However, strong enrichment of the same region by anti-Sox2 antibody was not observed in glioma-initiating cells. These results indicate that transcription factor complex on the Sox2 enhancer region does not contain Sox2 in glioma-initiating cells, and that Sox2 expression in glioma-initiating cells is regulated by mechanisms different from those in fetal neural progenitor cells.

We next examined recruitment of Sox4 to the Sox2 enhancer region in neural progenitor cells and glioma-initiating cells. In contrast to the experiment using anti-Sox2 antibody, anti-Sox4 antibody immunoprecipitated the DNA fragments of the Sox2 enhancer region in glioma-initiating cells, while the enrichment observed in fetal neural progenitor cells was much weaker. These findings together indicate that Sox2 expression in glioma-initiating cells is potentiated by the Oct4-Sox4 complex acting on the Sox2 enhancer region to maintain tumorigenic activity, while that in neural progenitor cells may be promoted by transcriptional complex containing Sox2 protein itself through a positive regulatory loop.

DISCUSSION

Although the origin of glioma stem cells (or glioma-initiating cells) is controversial (27), several studies have suggested that glioma-initiating cells share characteristics with neural or glial stem/progenitor cells (28, 29). Glioma stem cells express neural stem cell markers, including Nestin, Musashi, and Prominin-1 (CD133). Like normal neural stem cells, glioma stem cells are located in specific niches surrounding the tumor vasculature. A recent study has shown that the perivascular niches control self-renewal of glioma stem cells through endothelial cell-derived factors (30). However, in terms of transcription factor complexes, the similarities and differences between glioma stem cells and neural stem cells have not been clearly determined.

Here we have shown that Oct4 expression is required for the maintenance of the self-renewal capacity of glioma-initiating cells. In addition, transient suppression of Oct4 by siRNA abolished the induction of Sox2 by TGF-β (Ikushima H. and Miyazono K. unpublished observation) and decreased the tumorigenic activity of glioma-initiating cells (Fig. 2), suggesting that impairment of stemness properties via Oct4 knockdown may be an irreversible process. We also demonstrated that Oct4 knockdown increases sensitivity to a chemotherapeutic alkylating agent, temozolomide.

Oct4 is essential for establishing and maintaining the pluripotent state of stem cells (14, 15). Moreover, Oct4 is one of the key factors in the generation of iPS cells (17, 18). However, the role of Oct4 in the development and progression of malignant tumors has not been fully determined. Our findings indicate that Oct4 is an essential factor for glioma-initiating cells and play roles similar to those in embryonic stem cells.

One of the most intensively investigated topics in current cancer research is the identification of specific therapeutic compounds that can effectively eliminate cancer-initiating cells. Recent studies have identified factors

essential for retention of cancer-initiating cells, including several growth factor signaling pathways such as Wnt, Hedgehog, Notch, PI3K-mTOR, TGF-β and LIF (9, 31-36). While new therapeutic targets have been intensively sought based on findings related to these pathways, one problem is that almost all of these signaling pathways are also indispensable for normal stem cells. Inhibitors of these signaling pathways may affect the characteristics of normal stem cells and impair maintenance of normal tissues. Thus, from a clinical standpoint, it is important to identify factors not only essential for the maintenance of cancer-initiating cells but also different from those present in normal stem cells.

Here, we have demonstrated that Oct4-Sox4 complex activates the enhancer region of Sox2 genes to sustain stemness properties of glioma-initiating cells. Oct4 and Sox2 are also important for the maintenance of normal stem cells, and Oct4-Sox2 complex activates the Sox2 enhancer region to form a positive regulatory loop. However, in glioma-initiating cells, Sox2 is not predominantly present in the transcription factor complex on the Sox2 enhancer region. Instead, Sox4 forms a transcriptional complex with Oct4 in glioma-initiating cells to activate the enhancer region of Sox2, a gene essential for the maintenance of tumorigenicity of glioma-initiating cells. These findings suggest that Sox2 expression in glioma-initiating cells can be potentiated via up-regulation of Sox4, while Sox2 expression in neural progenitor cells is regulated by a self-reinforcing regulatory loop and is relatively self-contained (Fig. 7, Supplemental Fig. 5). In other words, the positive regulatory loop of Sox2 is not active in glioma-initiating cells, and alternatively, Oct4 acts with Sox4 to enhance Sox2 expression. We also confirmed that, in neural progenitor cells, Sox2 is only weakly induced by TGF-β stimulation (Supplemental Fig. 6), whereas this cytokine activates the Sox4-Sox2 cascade in glioma-initiating cells (11). Loss of the regulatory loop of Sox2 expression may thus cause glioma-initiating cells to become susceptible to exogenous stimuli. However, we should bear in mind that our glioma-initiating cells were obtained from adult tumors, whereas neural progenitor cells were from a fetus. Further studies in neural progenitor cells from adults may be important to elucidate the differences between glioma-initiating cells and normal neural progenitor cells.

We examined combined effects of siRNAs against Sox4 and Oct4 in a limiting dilution assay, but failed to observe any significant synergistic effects (Supplemental Fig. 7). It may be because a defect of either factor in the Sox4-Oct4 complex results in significant inactivation of the Sox2 enhancer and/or because a single effect of siSox4 or siOct4 is strong enough to reduce sphere-forming ability of glioma-initiating cells.

It remains to be determined why the common Oct4-binding sequence and Sox-binding elements are differently regulated in neural progenitor cells and glioma-initiating cells. On differentiation of erythroid precursors into mature erythrocytes, GATA-binding protein 2 (GATA2) on some promoter regions is replaced by GATA1 (37). This process is termed the "GATA switch", and is an essential step in the maturation of erythrocytes and the expression of α-globin. One of the crucial mediators of this switching is Friend of GATA1 (FOG-1, also known as Zfpm1), a multi-zinc-finger protein critical for the development of erythrocytes and megakaryocytes (38, 39), and GATA-FOG interaction is believed to be required for "GATA switch" (40). Like the "GATA switch", the

Sox-binding element on Sox2 enhancer region in glioma-initiating cells is differently regulated from that in neural progenitor cells, although the mechanism responsible for this remains to be determined.

Although Sox4 plays a crucial role in the retention of tumorigenicity of glioma-initiating cells through up-regulation of Sox2 expression (11), $Sox4^{-/-}$ mice exhibit no neurological defects (41). This finding suggests that the mechanism of action of Sox4 in glioma-initiating cells is distinct from that in neural stem/precursor cells. Because the self-renewal and proliferation of normal stem cells are likely strictly regulated, perhaps by genetic or epigenetic programs, the uncontrolled expansion of cancer-initiating cells may result from deregulation of such strict programs. In support of this conclusion, we found that the self-regulatory loop of Sox2 expression observed in neural progenitor cells was disrupted in glioma-initiating cells. This finding may enable the determination of a novel molecular target and eventually yield a therapeutic approach to eradication of glioblastoma without affecting the normal brain.

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FOOTNOTES

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The abbreviations used are:

BMP bone morphogenetic protein
TGF-β transforming growth factor-β
GFAP glial fibrillary acidic protein
Tuj1 βIII-tubulin
ChIP chromatin immunoprecipitation
GAPDH glyceraldehyde-3-phosphate dehydrogenase
HPRT1 hypoxanthine phosphoribosyltransferase 1

FIGURE LEGENDS

FIGURE 1. Oct4 is essential for retention of stemness of glioma-initiating cells. (A) TGS-01 and TGS-04 cells were dissociated into single cell populations, transfected with control (NC) or Oct4 siRNA duplex, and cultured for 7 days (1st). After the seven-day culture, spheres were dissociated into single cell populations and equal numbers of cells were cultured for another 7 days (2nd). Values are the number of glioma spheres formed (means ± SEM of five fields). *: $p<0.001$. Scale bars: 100 μm. (B) Knockdown of Oct4 expression by siRNA in TGS-01 and TGS-04 cells resulted in decrease of self-renewal capacity in limiting dilution assay. (C) 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 control (NC) or Oct4 siRNA duplex for 7 days. Quantification of Nestin-, Musashi-, Tuj1-, or GFAP-positive cells is shown in the right graphs. *: $p<0.01$. Scale bars; 50 μm.

FIGURE 2. Development of brain tumors after intracerebral transplantation of 5 x 10^4 TGS-01 cells pretreated with control (NC) or Oct4 siRNA duplex for 7 days. Survival of mice (n = 7 mice for each condition) was evaluated by Kaplan-Meier analysis (left graph). *P* values were calculated by the log-rank test. Right panels show the results of histological examination of the samples dissected at 30 days after intracerebral transplantation. Tissue sections were stained with hematoxylin-eosin. Scale bars: 50 μm. Experiments were repeated twice with essentially similar results.

FIGURE 3. Knockdown of Oct4 expression enhances sensitivity to chemotherapy in glioma-initiating cells. TGS-01 and TGS-04 cells with control (NC) or Oct4 siRNA duplex were seeded in 96-well plates and treated with temozolomide (0, 10, 30, 100, 300, 1000 μM) for 72 h. Cell viability was assessed by WST-8 assay. *: $p<0.01$ (siNC vs siOct4#1 and siNC vs siOct4#2).

FIGURE 4. Oct4 is associated with the Sox2 enhancer region to up-regulate expression levels of Sox2 in glioma-initiating cells. (A) Effects of Oct4 knockdown on expression of Sox2. Amounts of Sox2 protein were determined after treatment with control (NC) or Oct4 siRNA #1 duplex for 24 h. α-tubulin was used as a loading control. (B) Association of Oct4 with the Sox2 enhancer region. ChIP analysis was performed using TGS-01 cells ("Sphere") and matched "Differentiated" cells. Eluted DNAs were subjected to conventional RT-PCR. The first intron of hypoxanthine phosphoribosyltransferase (HPRT) 1 was used as a negative control. Input: 1%. (C) Levels of expression of Oct4 protein in "Sphere" cells and "Differentiated" cells. α-tubulin was used as a loading control.

FIGURE 5. Oct4 physically interacts with Sox4 on Sox2 enhancer region. (A) The sequences of Oct4-binding element and Sox-binding element in the Sox2 enhancer region. (B) Physical interaction of endogenous Oct4 with endogenous Sox4 in TGS-01 cells. Cell lysates were subjected to immunoprecipitation with anti-Oct4 antibody followed by immunoblotting with anti-Sox4 (left), or with anti-Sox4 antibody followed by immunoblotting with anti-Oct4 (right). Asterisk: nonspecific band. (C) Recruitment of the Oct4-Sox4 complex to the Sox2 enhancer region. Soluble chromatin was prepared from TGS-01 cells and ChIP analysis was performed using anti-Sox4 and anti-Oct4 antibodies. Subsequently, ChIP Re-IP of protein-DNA complex eluted from the first immunoprecipitation was performed. Eluted DNAs were subjected to conventional RT-PCR.

FIGURE 6. Oct4 acts in concert with Sox4 to potentiate Sox2 enhancer activity. (A) Effects of Oct4 and/or Sox4 knockdown on expression of Sox2. Amount of Sox2 protein was determined after treatment with indicated siRNA duplex for 24 h. (B) Roles of Oct4 and Sox4 in activation of the Sox2 enhancer region. Effects of Oct4 and/or Sox4 knockdown on Sox2 enhancer activity were examined in TGS-01 and TGS-04 cells (left graphs). Effects of Oct4 and/or Sox4 overexpression on Sox2 enhancer activity were examined in U373MG cells (right graph). Error bars represent SEM. *: $p<0.001$. (C) TGS-01 or TGS-04 cells were transfected with luciferase constructs containing wild-type or mutated Sox2 enhancer region. The cells were collected 24 h after transfection and luciferase activity was examined. *: $p<0.001$. The right panel indicates the sequence of the Sox2 enhancer region and

corresponding mutations (underlined) used in this study.

FIGURE 7. The partner of Oct4 on Sox2 enhancer region in glioma-initiating cells is distinct from that in neural progenitor cells. Soluble chromatin was prepared from glioma-initiating cells (TGS-01 and TGS-04) and neural progenitor cells (NPC). ChIP analysis was performed using anti-Oct4, anti-Sox2, and anti-Sox4 antibodies. 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.

Figure 1