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Sox11 Prevents Tumorigenesis of Glioma-Initiating Cells by Inducing Neuronal Differentiation

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Abstract

Recent findings have shown that malignant tumors contain cancer-initiating cells (CIC), which self-renew and are tumorigenic. However, CICs have not been characterized properly due to lack of specific markers. We recently established a mouse glioma cell line, NSCL61, by overexpressing an oncogenic *HRas*^{L61} in p53-deficient neural stem cells. Using limiting dilution assays, we show that only 2 of 24 NSCL61 clones retained their tumorigenicity *in vivo*, although the others also expressed oncogenic *HRas*^{L61} and could proliferate in culture. A comparison of the gene expression profiles of tumorigenic and nontumorigenic clones showed that the tumorigenic clones had lost Sox11 expression. We show that overexpression of *sox11* prevented tumorigenesis of NSCL61s by inducing their neuronal differentiation accompanied with decreased levels of *plagl1*. We also show that overexpression of *plagl1* abolished neuronal commitment of nontumorigenic cells and induced them to become tumorigenic. Moreover, we show that human glioma-initiating cells lost *sox11* expression, and overexpression of *sox11* prevented their tumorigenesis *in vivo*. Together with the clinical evidence showing that downregulation of *sox11* mRNA correlates with a significant decrease in survival, these findings suggest that Sox11 prevents gliomagenesis by blocking the expression of oncogenic *plagl1*. [Cancer Res 2009;69(20):7953–9]

Introduction

Cancer-initiating cells (CIC; also known as cancer stem cells) are capable of indefinite self-renewal and generate amplifying cancer cells, which make up the majority of cells in a tumor (1–4). CIC-enriched populations can be obtained from cancers and cancer cell lines using the common features of tissue-specific stem cells, including cell surface antigens such as CD133, side population, floating sphere formation assays, or a combination of these features (2–4). However, with other lines of evidence showing that CD133$^-$ cells and non–side population cells from tumors and cancer cell lines can form tumors when transplanted *in vivo* (5–7), it remains uncertain whether existing isolation methods can identify bona fide CICs.

The overexpression of oncogenes can induce hematopoietic stem/precursor cells to transform into leukemic stem cell–like cells in culture, which can cause leukemias when small numbers are transplanted *in vivo* (8, 9). This suggests the existence of CIC-like cells in such induced cancer models. Using a similar approach, we recently established an induced mouse glioma cell line, NSCL61, by transforming *p53*-deficient neural stem cells (NSC) with oncogenic *HRas*^{L61}; it has been shown that p53 is the most frequently mutated tumor suppressor gene in human glioblastoma multiforme, one of the most malignant brain tumors (10–14). Increased activation of the Ras signaling pathway is also found in human gliomas, in ~90% of glioblastoma multiforme cases (13–15). NSCL61s self-renew, express NSC markers including Nestin and Sox2, and form transplantable glioblastoma multiforme showing hypercellularity, pleiomorphism, multinuclear giant cells, mitosis, and necrosis within 2 months, even when as few as 10 cells are transplanted *in vivo* (Supplementary Fig. S1), suggesting that NSCL61 is highly enriched in glioma-initiating cell (GIC)–like cells. Given that NSCL61s contain both tumorigenic cells (GIC-like cells) and non-tumorigenic cells (non-GIC), it should be possible to isolate, characterize, and use GIC-like cells to identify novel therapeutic targets for glioblastoma multiforme by comparing the gene expression profiles of GIC-like cells with that of non-GICs. To examine this possibility, we performed limiting dilution assays, established 24 NSCL61 sublines, and found that only 2 sublines retained their tumorigenicity *in vivo*.

Here, we show that mouse GIC–like cells and human GICs (hGIC) from malignant glioma lost *sox11* expression, and overexpression of *sox11* in these cells blocked tumorigenesis by inducing their neuronal differentiation accompanied with decreased levels of *plagl1*. We also show that overexpression of *plagl1* abolished neuronal commitment of non-GICs and induced them to become malignant. Thus, these data reveal that Sox11 prevents gliomagenesis by blocking the expression of oncogenic *plagl1*.

Materials and Methods

Animals, cells, and chemicals. Mice were obtained from the Laboratory for Animal Resources and Genetic Engineering at the RIKEN Center for Developmental Biology and from Charles River Japan. All mouse experimental protocols were approved by the RIKEN Center for Developmental Biology Animal Care and Use Committee. Mouse NSCL61s and hGICs were cultured in DMEM/F-12 (Life Technologies) supplemented with chemicals, basic fibroblast growth factor (10 ng/mL), and epidermal growth factor (10 ng/mL) as described previously (16). Chemicals and growth factors were purchased from Sigma and Peprotech, respectively, except where indicated.

Vector construction. Full-length mouse and human *sox11* were amplified from mouse NSC cDNA and human fetal brain cDNA libraries

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

T. Hide and T. Takezaki contributed equally to this work.

Accession numbers for microarray data: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17062 and http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17076. InvivoGen's small interfering RNA Wizard (http://www.sirnawizard.com/).

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(Clontech) using reverse transcription-PCR (RT-PCR) and Phusion polymerase (Finnzymes) according to the manufacturer's instructions and were cloned into a pDrive vector (Qiagen). The nucleotide sequences were verified using the BigDye Terminator Kit version 3.1 (Applied Biosystems) and an ABI sequencer model 3130xl (Applied Biosystems). Human or mouse *sox11* cDNA was inserted into the pcDNA3.1-hyg vector (Invitrogen), resulting in pcDNA3.1-hyg-hsox11 and pcDNA3.1-hyg-msox11, respectively. To construct the FLAG-tagged Sox11 expression vector, mouse *sox11* cDNA was inserted into p3xFLAG-CMV10 vector (Sigma; FLAG-cont), resulting in p3xFLAG-sox11 (FLAG-Sox11).

Mouse *plagl1* cDNA was cloned as mentioned above and inserted into the pcDNA3.1-hyg vector (Invitrogen), resulting in pcDNA3.1-hyg-mplagl1.

To knockdown mouse *sox11* and *plagl1*, their hairpin sequences were generated using InvivoGen's small interfering RNA (siRNA) Wizard and inserted into psiRNA-h7SKhygro G1 expression vector (InvivoGen), resulting psiRNA-h7SKhygro-msox11sh and psiRNA-h7SKhygro-mplagl1sh, respectively. The siRNA target sequence for mouse *sox11* was 5′-GCTGACTACCCCGACTACAAG-3′. The siRNA target sequence for mouse *plagl1* was 5′-GCCCCATAGGATAACCTGTCT-3′. The siRNA target sequence for *EGFP* was 5′-GCAAGCTGACCCTGAAGTTCA-3′.

To construct the series of mouse *plagl1*-promoter-firefly luciferase expression vectors, mouse *plagl1* 5′ genomic DNA was amplified with Takara PrimeSTAR GXL DNA polymerase (Takara) and cloned into a pCR-XL-TOPO vector (Invitrogen) according to the manufacturer's instructions. After verifying the nucleotide sequence, mouse *plagl1* genomic DNA fragments were inserted into the pGL3-basic vector (Promega), producing pGL3-plagl1p-full (full), pGL3-plagl1p-R1 (R1), or pGL3-plagl1p-R2 (R2). The following oligonucleotide DNA primers were synthesized: for full *plagl1* 5′ genomic DNA (−5,241 to −1), the 5′ primer was 5′-TACGCGTCTTCTTTCTGCCCACCCCCAT-3′ and the 3′ primer was 5′-AGTCGACGGCCTTTGGTTCTCACACTTTC-3′. For *R1* (−2,241 to −1,160), the 5′ primer was 5′-TACGCGTGGGTTGGTTGAGAGGTAGCATA-3′ and the 3′ primer was 5′-AGTCGACGGGTGTTCTGCTGAAACACAGG-3′. For *R2* (−1,160 to −1), the 5′ primer was 5′-TACGCGTCCTGTGTTTCAGCAGAACACCC-3′ and the 3′ primer was 5′-AGTCGACGGCCTTTGGTTCTCACACTTTC-3′. The translation start site is considered as position +1.

Transfection. Transfections of NSCL61 cells and hGICs were done using the Nucleofector system according to the supplier's instructions (Amaxa). In brief, 2×10^6 cells were suspended in the Mouse NSC Nucleofector Solution (100 μL) with 10 μg vectors and then transfected using the Nucleofector Device. Transfected cells were selected in optimized medium with hygromycin (0.3 mg/mL; Wako Chemical) for 10 days. All the experiments were conducted using mixed stable hygromycin-resistant clones (>50 clones).

Immunostaining. Immunostaining was carried out as described previously (17). The following antibodies were used to detect antigens: rat anti-Nestin [1:1,000 (BD Pharmingen) and 1:200 (Chemicon) for human cells], mouse anti–microtubule-associated protein 2 (MAP2; a+b; 1:200; Abcam), rat anti–green fluorescent protein (1:1,000; Nacalai Tesque), and rabbit anti-human Sox11 (1:50; Sigma). The antibodies were detected with goat anti-rat IgG-A488 (1:400; Molecular Probes), goat anti-rabbit IgG-Cy3 (1:400; Jackson ImmunoResearch), and goat anti-mouse IgG-A488 (1:400; Molecular Probes). All nuclei were counterstained with Hoechst 33342 (1 μg/mL).

Proliferation assay. Two thousand cells were cultured in 100 μL culture medium in each well of a 96-well plate. To examine cell proliferation, a MTT assay was done as follows: Ten microliters of MTT (5 mg/mL; Nacalai Tesque) were added to each well on days 0, 2, 3, and 4 *in vitro*. The cells were then incubated for 3 h, the medium was replaced with 100 μL DMSO, and the cells were dissociated. Cell proliferation was quantified using a microplate reader (Bio-Rad) with the absorption spectrum set at 570 nm.

Soft-agar assay. Soft-agar assays were done to examine whether cells could proliferate anchorage-independently. *Sox11*-overexpressing NSCL61 cells, hGICs, and their parental cells were suspended in 0.3% top agar made with the optimized medium and layered onto 0.6% bottom agar made with the same medium. After the top agar had polymerized, culture medium was added and the cells were cultured for 20 days with medium changes every 3 days.

Human brain tumors. Ten glioblastoma multiformes, two anaplastic astrocytoma, two anaplastic oligodendroglioma, two hGICs, hGIC1 and hGIC2, and two normal brains were used following the research guidelines of RIKEN Center for Developmental Biology and Kumamoto University Graduate School of Medical Science. The detailed characterization of hGICs will be reported elsewhere.[3] Poly(A)$^+$ RNA was prepared using a QuickPrep mRNA Purification kit (GE Healthcare), and the cDNA was synthesized using a Transcription First-Strand cDNA Synthesis Kit (Roche).

Paraffin-embedded tumors were prepared into 6-μm-thick sections. For Sox11 staining, the antigen was retrieved by HistoVT One according to the supplier's instructions (Nacalai Tesque). Endogenous peroxidase activity was inactivated by applying 2% H_2O_2 for 15 min at room temperature. The sections were then pretreated with 5% skim milk in PBS-Tween 20 for 30 min at room temperature, immunostained with an anti-Sox11 antibody (1:50) for 2 h at room temperature, and visualized using the Vectastain elite ABC kit (Vector Laboratories) and diaminobenzidine (Vector Laboratories).

Intracranial cell transplantation into the brain of nude mice. The detailed characterization of NSCL61s and hGICs will be reported elsewhere. *Sox11*-overexpressing GICs and their parental cells were suspended in 5 μL culture medium and injected into the brain of 5- to 8-week-old female nude mice that had been anesthetized with 10% pentobarbital. The stereotactic coordinates of the injection site were 2 mm forward from lambda, 2 mm lateral from the sagittal suture, and 5 mm deep.

Luciferase assay. Luciferase assays were carried out as described previously (16). In brief, GIC-like cells were transfected with 1 μg of different types of *plagl1* promoters containing firefly luciferase expression vectors and 0.04 μg of the internal control vector pEF-Rluc (gift from S. Nagata and K. Shimozaki, Osaka University) using the Superfect Transfection Reagent according to the supplier's instructions (Qiagen). After 24 h, the activities of the two luciferases were measured using the Dual-Luciferase Reporter Assay System according to the supplier's instructions (Promega).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays were carried out as described previously (16). In short, mouse GIC–like cells were transfected with FLAG-cont or FLAG-Sox11 vectors using the Nucleofector device. On the following day, transfected cells were fixed and sonicated. The cell extracts were harvested by centrifugation and immunoprecipitated with anti-FLAG M2 antibody (1:100 dilution) and protein G-Sepharose (GE Healthcare; 50 μL of 50% suspension). The precipitated DNA fragments were purified by phenol/chloroform extraction and used for PCR with primer sets for R1 and R2. PCR conditions were 30 s at 94°C, 30 s at 60°C, and 75 s at 72°C. Cycle parameters were 50 cycles for both R1 and R2.

Statistical analysis. The survival data were analyzed for significance by Kaplan-Meier methods using GraphPad Prism version 4 software (P values were calculated with the log-rank test).

Materials and some other methods were described in Supplementary Data.

Results

Induced glioma cells, NSCL61s, contain both GIC-like cells and non-GICs. To test whether these sublines retained their tumorigenicity, we injected 1,000 cells of each clone into the brains of nude mice (n = 4) and found that only two sublines, 6B and 8B, formed glioblastoma multiformes and killed the mice (Fig. 1*A* and *B*), although all clones could be expanded in culture and expressed the oncogenic $HRas^{L61}$ (Fig. 1*C*). In culture, we noticed that the tumorigenic sublines (GIC-like cells) proliferated at a slower rate than the nontumorigenic ones (non-GICs), although reasons for this are unknown; this was confirmed by the MTT assay (Fig. 1*C*). We also immunolabeled both types of sublines for the NSC markers Nestin and Sox2, the glial markers A2B5 and glial fibrillary acidic protein, and the neuronal marker MAP2. As shown in Fig. 1*D*,

[3] T. Takezaki and T. Kondo, unpublished data.

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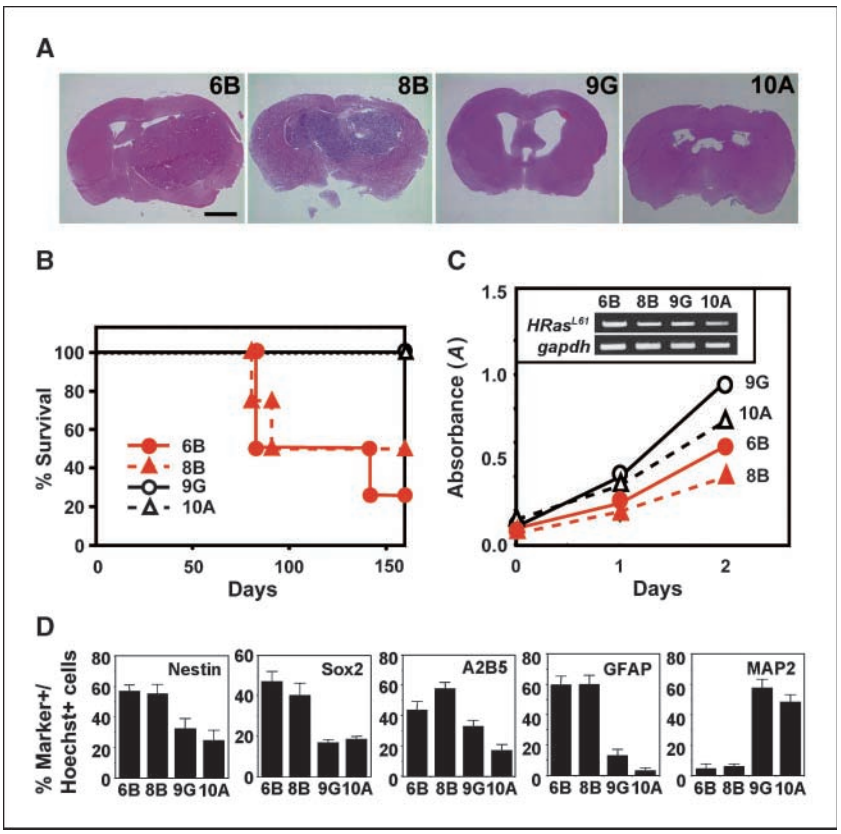

Figure 1. Characterization of NSCL61 sublines. *A,* brain histology injected with NSCL61 sublines 6B, 8B, 9G, and 10A. *Bar,* 2 mm. *B,* survival curves for mice injected with 6B, 8B, 9G, and 10A. Non-GIC sublines, 9G and 10A, are used as negative controls. *C,* proliferation of NSCL61 sublines was determined by the MTT assay. Mean ± SD of three cultures. *Inset,* oncogenic $HRas^{L61}$ expression in NSCL61 sublines was examined by RT-PCR. Expression of *gapdh* was an internal control. *D,* proportion of marker-positive cells per Hoechst 33342–positive cells in NSCL61 sublines. Mean ± SD of three cultures.

GIC-like cells 6B and 8B predominantly expressed the NSC markers and glial markers but not MAP2. In contrast, non-GICs 9G and 10A lost both their NSC and glial markers but expressed MAP2. Moreover, we found that all six 6B subclones and five of six 8B subclones, which were further established by limiting dilution assays, retained their tumorigenic ability *in vivo* (data not shown). Together, these data suggest that GIC-like cells consist of a homogenous cell population that retains characteristics of NSCs and glial cells, whereas the non–mouse GICs are committed to a neuronal fate.

hGICs and mouse GIC–like cells lose *sox11* expression. We next analyzed the gene expression differences between GIC-like cells and non-GICs by DNA microarray analysis and found that 547 genes were upregulated in the GIC-like cells and 402 genes were upregulated in the non-GICs (Supplementary Fig. S2*A*). Using RT-PCR, we confirmed the expression patterns of several genes, including *sox11* and *map2*, which appeared at least twice in the top 30 probe sets for genes upregulated in either GIC-like cells or non-GICs (Supplementary Fig. S2*B*). To identify potential candidate genes involved in GICs, we established two hGIC lines, hGIC1 and hGIC2, from primary anaplastic oligodendroglioma and glioblastoma multiforme, respectively, as shown previously (18–20). Both hGIC lines formed transplantable malignant gliomas in the brains of nude mice even when as few as 10 cells were transplanted. In addition, all of the 10 hGIC sublines, which were established by limiting dilution assays, retained their tumorigenic ability *in vivo*, suggesting that hGIC lines are homogeneous.[3] We confirmed that both hGIC lines lost *sox11* expression, whereas expression in those other candidate genes was not found (Supplementary Fig. S2*B*), leading us to study the functions of Sox11 in our system. In addition, many lines of evidence suggest that GICs are negative for Sox11: first, Sox11 is expressed in the committed neuronal precursor cells but not in NSCs (21–24). Second, overexpression of *sox11* induces the expression of the neuronal markers MAP2 and βIII tubulin in proliferating neural precursors (23). This is consistent with our finding that the non-GICs expressed *sox11* and *map2* mRNA (Supplementary Fig. S2*B*) and also the Sox11 and MAP2 protein (Fig. 1*D*; Supplementary Fig. S2*C*). Finally, the overexpression of a dominant-negative form of Sox11 blocks neuronal differentiation (23). Taken together, these findings suggest that Sox11 inhibits tumorigenicity in GIC-like cells by inducing their neuronal differentiation.

Although it has been shown that Sox11 can be used as a potential diagnostic marker for gliomagenesis, as Sox11 is seen upregulated in malignant glioma, and *sox11* expression is undetectable in the normal adult brain (23–26), the National Cancer Institute Repository for Molecular Brain Neoplasia Data database (27) revealed that downregulation of *sox11* mRNA correlated with a significant decrease in survival of glioma patients (Supplementary Fig. S3). These results are not contradictory because primary glioblastoma multiformes likely contain a large number of non-GICs and a small population of GICs, which are involved in the recurrence (18, 19). Indeed, we found that human primary glioblastoma multiformes (n = 11) express *sox11* mRNA and Sox11 protein, whereas their recurrent ones lose Sox11 expression and increase Nestin expression (Fig. 2*A* and *B*). We also found that the transplanted hGICs were negative for Sox11, although a small number of cells were labeled for Sox11 (Fig. 2*C, arrowheads*). Together, these data suggest that bona fide GICs would be Sox11-negative.

Overexpression of sox11 inhibits tumorigenesis of GICs by inducing their neuronal differentiation. We next addressed whether overexpression of *sox11* could inhibit tumorigenesis of GICs. We noticed that *sox11* overexpression prevented proliferation

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of NSCL61s and hGICs, as measured in a MTT assay (Fig. 3*A*), inhibited Nestin expression (Supplementary Fig. S4*A*), and increased MAP2 expression (Supplementary Fig. S4*B*), consistent with past reports of the involvement of Sox11 in neuronal differentiation (22–25). To examine the tumorigenicity of *sox11*-overexpressing NSCL61s (NSCL61s-S11) and hGICs (hGICs-S11), we first performed colony formation assays in soft agar. As shown in Fig. 3*B*, the *sox11*-overexpressing cells lost their ability to form colonies. One hundred NSCL61s-S11 or hGICs-S11 were then transplanted into the brains of nude mice. All of the mice (n = 5 in each experiment) that received control NSCL61s or hGICs developed glioblastoma multiformes and died within 45 days, whereas a significant number of mice (4 of 5, 4 of 5, and 5 of 5) that received *sox11*-overexpressing cells (NSCL61s-Sox11, hGIC1s-Sox11, and hGIC2s-Sox11, respectively) survived >60 days (Fig. 3*C*).

We further examined whether Sox11 negatively regulates gliomagenesis using a specific short hairpin RNA for *sox11* (Supplementary Fig. S5*A*). Knockdown of Sox11 not only blocked *map2* expression (Supplementary Fig. S5*B*) but also induced non-GICs 10A to become tumorigenic (Supplementary Fig. S5*C*). Together, these findings revealed that Sox11 negatively regulates tumorigenesis of GICs by inducing their neuronal differentiation.

Sox11 negatively regulates the expression of *plagl1* that is involved in tumorigenesis of GIC-like cells. To identify targets of Sox11 in the tumorigenic cells, we analyzed gene expression differences between NSCL61s and NSCL61s-S11 using DNA microarray analysis (Supplementary Fig. S6*A*). We selected several genes, whose expression was significantly affected by *sox11* overexpression, and confirmed their expression by RT-PCR (Fig. 4*A*; Supplementary Fig. S6*B*). Among the potential candidate genes, we focused on *plagl1* for the following reasons: first, *plagl1* is shown to be expressed in neural stem/progenitor cells in developing neuroepithelial cells and decreases on differentiation (28). Second, *plagl1* is expressed in malignant human gliomas as well as hGICs (Fig. 4*B*), although it is thought to be a tumor suppressor candidate (29). Third, Plagl1 regulates several imprinted genes, including *Igf2, H19*, and *Dlk1*, all of which are involved in tumorigenesis as well as early development (30). Fourth, using the Repository for Molecular Brain Neoplasia Data database, we found that glioma patients with down-regulated *plagl1* mRNA show increased survival rates compared with patients with intermediate levels of *plagl1* expression (data not shown). Thus, these data suggest that Plagl1 plays an important role in GICs.

To test whether Sox11 directly regulates *plagl1* expression in GIC-like cells, we constructed a variety of luciferase-reporter vectors that contain the 5′ promoter region of the mouse *plagl1* gene (full) or two deleted forms (R1 and R2) of the promoter region (Fig. 4*C, left*), which contain potential Sox11 binding sites (Supplementary Fig. S7). As shown in Fig. 4*C*, we observed luciferase activity when the full or R1 reporter vectors were transfected into 6B GIC-like cells, whereas no activity was induced in cells transfected with the control or R2 reporter vectors. When Sox11 was overexpressed in 6B GIC-like cells, it blocked the luciferase activity induced by either the full or R1 reporter vectors. We also observed similar results using another GIC-like line, 8B (data not shown). Moreover, we found that some Sox11 binds to the R1 enhancer, but not R2, in GIC-like cells using chromatin immunoprecipitation (Fig. 4*D*). Together, these findings suggest that some Sox11 protein binds to the R1 enhancer in *plagl1* gene and represses its expression in GIC-like cells.

We then addressed whether Plagl1 is involved in the tumorigenicity of GIC-like cells. When the *plagl1* gene was overexpressed in non-GIC 10A cells, it inhibited MAP2 expression and induced Nestin expression in the cells (Fig. 5*A*). In addition, when 1,000 *plagl1*-expressing non-GIC 10A cells were injected into the brains of nude

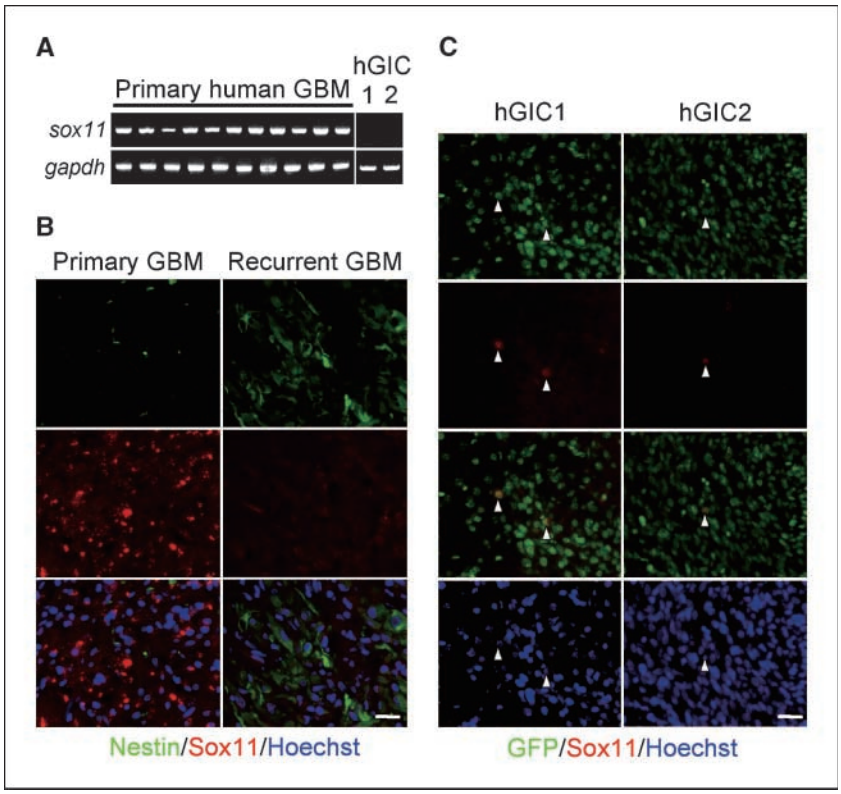

Figure 2. Recurrent glioblastoma multiformes and xenografted tumors are Sox11 negative. *A, sox11* expression in human primary glioblastoma multiformes (*GBM*) and hGICs was examined by RT-PCR. Expression of *gapdh* was an internal control. *B,* specimens of primary (*left*) and recurrent (*right*) glioblastoma multiformes were immunostained for Sox11 (*red*) and Nestin (*green*). *C,* xenografted tumors generated by hGICs were immunostained for Sox11 (*red*) and green fluorescent protein (*GFP*; *green*). All nuclei were counterstained with Hoechst 33342 (*blue*). *Bar,* 100 μm (*B* and *C*).

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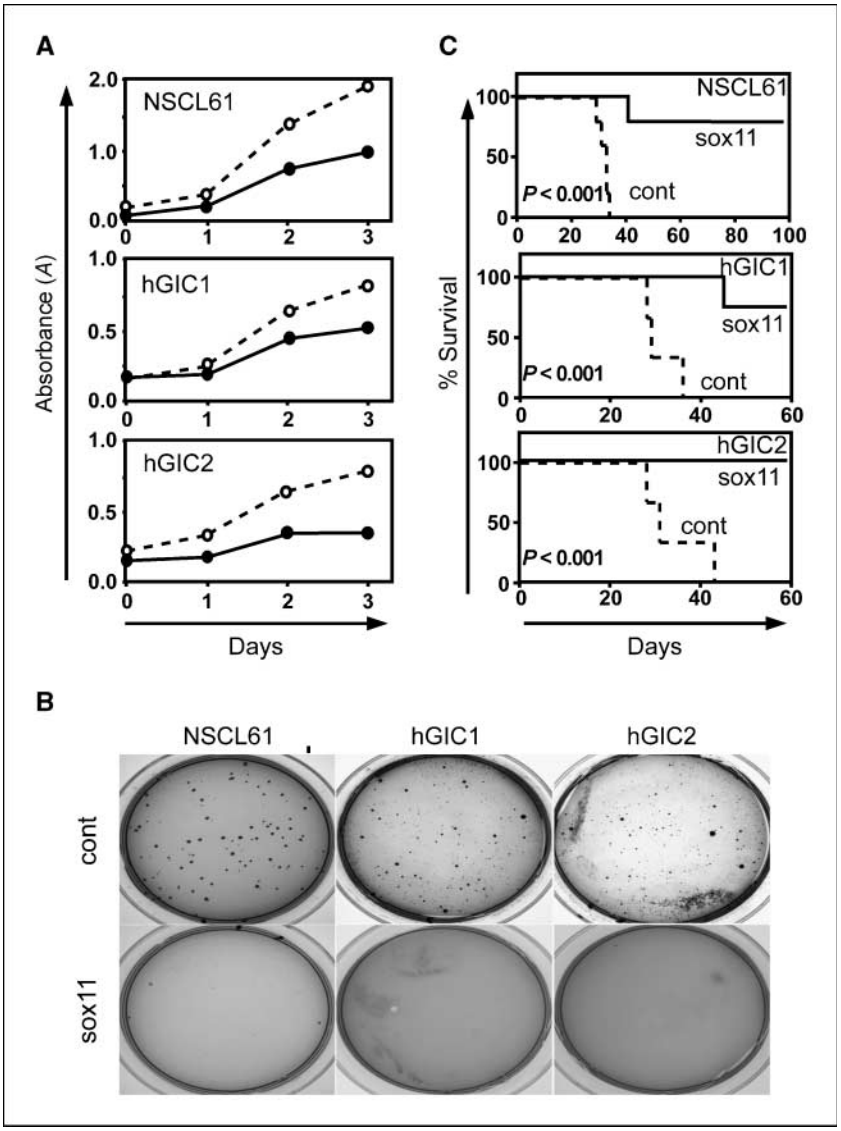

Figure 3. Overexpression of *sox11* abolishes the tumorigenicity of NSCL61s and hGICs. *A,* proliferation of control vector-transfected cells (*dotted line*) and *sox11*-overexpressing cells (*solid line*) was determined by MTT assay. Mean ± SD of three cultures. *B,* colony formation ability of control vector-transfected cells (*cont*) and *sox11*-overexpressing cells was examined in soft agar. *C,* survival curves for mice injected with control vector-transfected cells (*dotted line*) and *sox11*-expressing cells (*solid line*).

mice (n = 3), all of the mice developed brain tumors and died (Fig. 5*B*). To further evaluate the role of Plagl1 in tumorigenesis, we constructed *plagl1*-specific short hairpin RNA expression vector (Supplementary Fig. S8), overexpressed the vector in NSCL61s, and then injected 1,000 of those cells into the brains of nude mice. Knockdown of Plagl1 did not affect either Nestin expression in NSCL61s or their proliferation (data not shown). However, all mice (n = 4) that received *plagl1* short hairpin RNA–expressing NSCL61s survived >40 days, although all mice (n = 4) that received control short hairpin RNA–expressing NSCL61s died within 20 days (Fig. 5*C*). Collectively, these results suggest that Plagl1 plays an important role in tumorigenesis of GICs.

Discussion

We showed here that an induced glioma cell line, NSCL61, contains GIC-like cells and non-GICs, both of which are *p53*-deficient and express oncogenic $HRas^{L61}$. This heterogeneity might be generated by the insertion sites of the exogenous $HRas^{L61}$. Alternatively, because cultured NSCs are likely a heterogeneous cell population, although all of the cells are positive for conventional NSC markers including Sox2, Musashi1, and Nestin, the overexpression of oncogenic $HRas^{L61}$ might transform some NSC sublines, possible cells-of-origin of glioma, which have high tumorigenic potential into GIC-like cells but not others. In either case, however, the differences between GIC-like cells and non-GICs can be useful to characterize GICs and to find therapeutic targets.

We found that GIC-like cells and hGICs lost *sox11* expression, and recurrent human glioblastoma multiformes and xenografted tumors were largely negative for Sox11, whereas non-GICs and primary human glioblastoma multiformes express Sox11. We showed that overexpression of *sox11* inhibited tumorigenesis of GICs by inducing their neuronal differentiation and increased their sensitivity to anticancer drugs etoposide and Taxol,[4] whereas knockdown of *sox11* induced non-GICs to become malignant. Moreover, clinical evidences from the Repository for Molecular Brain Neoplasia Data database also indicated that downregulation of *sox11* mRNA correlated with a poor survival of glioma patients. It was also

[4] Y. Nakatani and T. Kondo, unpublished data.

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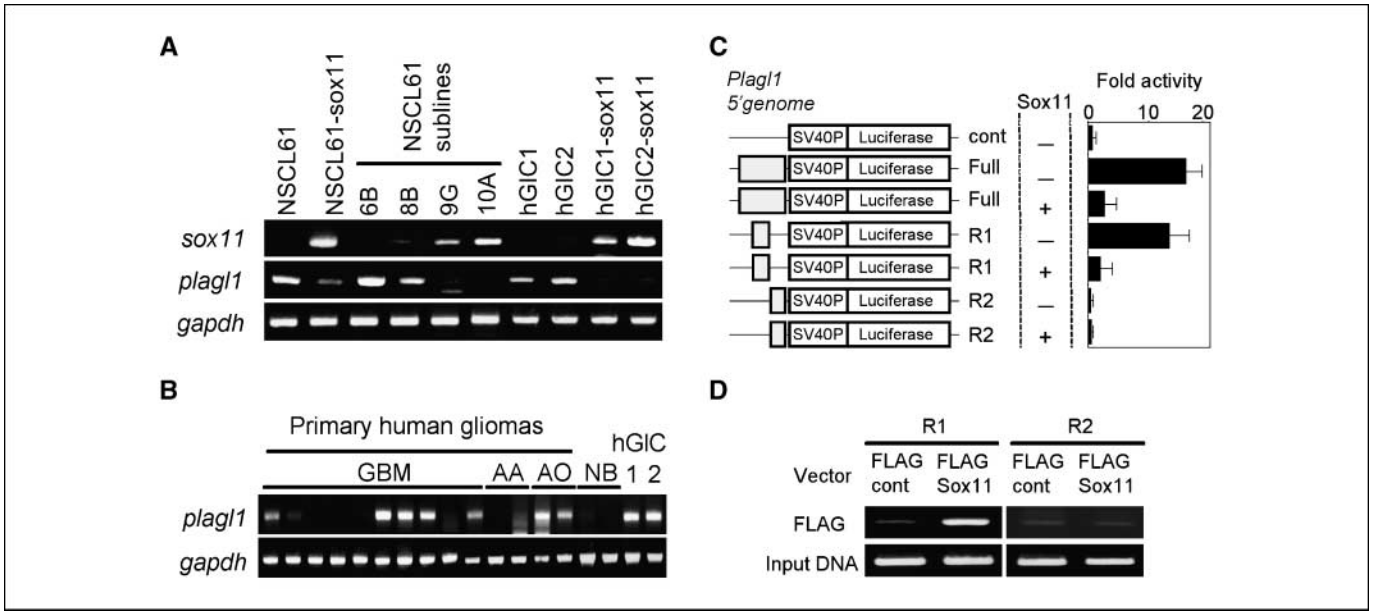

Figure 4. Sox11 inhibits the expression of *plagl1* in GICs. *A, plagl1* and *sox11* expression in NSCL61s, NSCL61 sublines (6B, 8B, 9G, and 10A), hGICs, and their *sox11*-expressing cells were examined by RT-PCR. *B, plagl1* expression in primary human gliomas was examined by RT-PCR. *AA,* anaplastic astrocytoma; *AO,* anaplastic oligodendroglioma; *NB,* normal brain. *C,* firefly luciferase activity in 6B cells transfected with a combination of vectors (shown), and pEF-Rluc was analyzed and normalized to sea pansy luciferase activity. Mean ± SD ($n = 3$). *D,* 6B cells were transfected with either FLAG-cont or FLAG-Sox11. Chromatin immunoprecipitation analysis was done as described in Materials and Methods. Amplified R1 and R2 sequences from input DNA are shown in the bottom (input DNA). Expression of *gapdh* was an internal control.

evident that Sox11 is expressed in neuronal precursors and immature neurons but not in either NSCs or mature neurons (24). Together, these data suggest that bona fide GICs, which retain NSC characteristics, should be negative for Sox11. We summarize these in a model shown in Fig. 5*D*.

Plagl1 was originally shown to regulate both cell cycle arrest and apoptosis; however, its functions appear to depend on the cell context: Plagl1 expression was detected in many brain areas with a high cellular proliferation activity, including the ventricular and subventricular zones where there are few apoptotic cells (28, 31, 32). Although overexpression of *plagl1* transformed non-GIC-like cells into GICs, knocking down *plagl1* inhibited tumorigenesis of NSCL61s. Moreover, Plagl1 has been shown to regulate the expression of several imprinted genes, including *Igf2,*

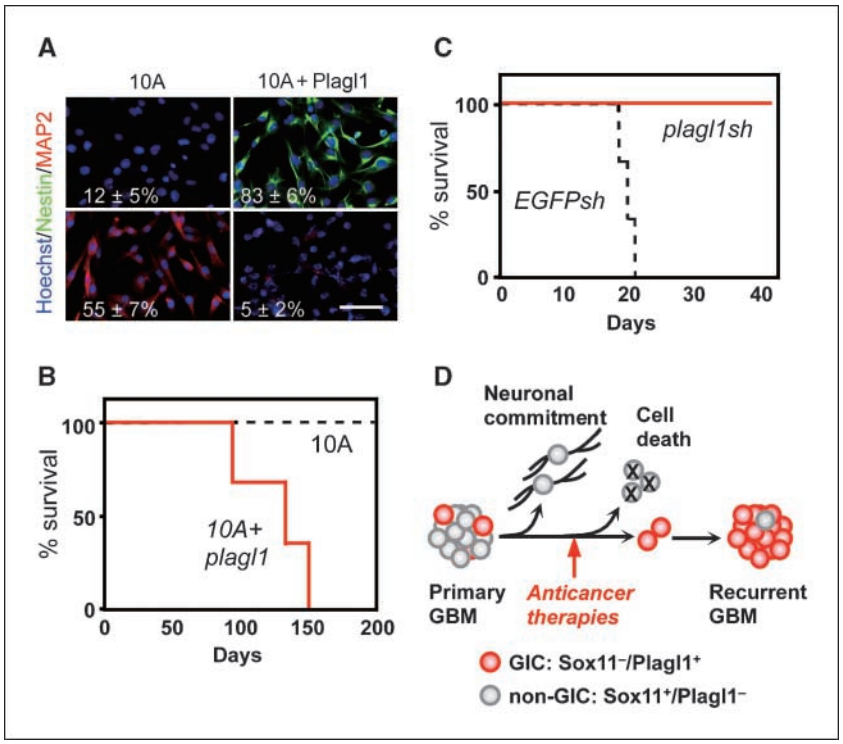

Figure 5. Plagl1 is involved in the tumorigenesis of NSCL61s. *A,* 10A cells were transfected with either control vector (*left*) or *plagl1* expression vector (*right*) and immunolabeled for Nestin (*green*) or MAP2 (*red*). Nuclei were counterstained with Hoechst 33342 (*blue*). *Bar,* 50 μm. *B,* survival curves for mice injected with 10A and *plagl1*-expressing 10A cells. *C,* survival curves for mice injected with *EGFPsh*-expressing NSCL61s and *plagl1sh*-expressing NSCL61s. *D,* model of tumorigenesis by sox11^{-}plagl1^{+} GICs.

H19, and *Dlk1*, which are involved in tumorigenesis and embryonic growth (30). Collectively, these findings suggest that Plagl1 plays an important role in the tumorigenesis of GIC-like cells. The next step will be to determine the functions of Plagl1 in GICs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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