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CLINICAL DEVELOPMENT OF A THIRD-GENERATION ONCOLYTIC HSV-1 (G47Δ) FOR MALIGNANT GLIOMA*

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Genetically engineered, conditionally replicating herpes simplex viruses type 1 (HSV-1) are promising therapeutic agents for cancer. We have developed a triple-mutated, third-generation oncolytic HSV-1, G47Δ, by introducing an additional genetic mutation to the viral genome of G207, a second generation HSV-1, used in clinical trials for malignant glioma in the United States. Preclinical studies demonstrated that G47Δ exhibited increased antitumor efficacy in various tumor models while preserving the safety of G207. Prior to the first-in-man clinical trial, G47Δ genome structure analysis, stability tests, and preclinical safety evaluation using HSV-1-susceptible A/J mice were performed. After development and optimization of manufacturing processes, clinical-grade G47Δ was produced at the GMP vector production facility of the University of Tokyo. Quality tests under GLP were completed for clinical products at 4 manufacturing steps. The first clinical trial of G47Δ is designed as an open labeled, single armed, phase I-II study for patients with recurrent glioblastoma. Patients with a single lesion, age 18 or older, and with Karnofsky Performance Scale (KPS) 70% or higher are enrolled. G47Δ is administered stereotactically into multiple sites of the tumor, twice within 14 days. The primary endpoint is to assess the safety of G47Δ, and the secondary endpoint is to assess the efficacy by tumor size and progression-free survival. After 3 years of contact with and review by regulatory authorities, the final governmental approval was obtained in May 2009, and the patient registration began in November 2009. In this paper, we also review the background of the clinical development of G47Δ.

Keywords: Malignant glioma; oncolytic virus; HSV-1; G47Δ; clinical trial.

Introduction

Malignant glioma is the most frequent malignant primary brain tumor. It is highly infiltrative to adjacent brain parenchyma, and radical surgical resection of the tumor is not always feasible due to preservation of cortical functions. Glioblastoma, WHO grade 4, is the most malignant type of glioma. With standard radiation therapy

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and chemotherapy, the median survival is only 14 to 16 months, which remains almost unchanged during the past decades despite recent progresses in radiological imaging, navigation-guided surgery, radiotherapy, and chemotherapy. In addition, there are limited second line therapies upon recurrence. Undoubtedly, there is a high need for a novel, effective therapy for glioblastoma.

The use of genetically engineered oncolytic viruses is a novel therapeutic strategy for cancer. The antitumor activities of viruses have been long known as described in case reports on patients with malignant tumors that regressed after incidental infection to viral diseases (Bluming and Ziegler, 1971; Taqi *et al.*, 1981). In the1960s and 1970s, there were multiple clinical studies using viruses for treatment of cancer (Asada, 1974; Wheelock and Dingle, 1964). However, wild type or naturally attenuated viruses exhibited pathogenicity when high enough amounts were used to cause efficacy. It was then recognized that intentional regulation of viral pathogenicity was important to utilize viruses as antitumor agents. In 1991, Martuza *et al.* reported that *dls*ptk, a mutant HSV-1 that had the thymidine kinase (tk) gene inactivated, could selectively replicate in glioma cells and effectively inhibited the tumor growth when injected into the brain tumors in mice (Martuza *et al.*, 1991). This report demonstrated the concept of conferring replication selectivity to the virus by means of genetic engineering.

When an oncolytic virus infects a tumor cell, the virus replicates, the host tumor cell is destroyed in the course of viral replication, amplified progeny viruses are released and infect surrounding tumor cells, and, in theory, the cycle repeats until all tumor cells are eradicated. On the other hand, oncolytic viruses are designed so that they are unable to infect or replicate in normal cells, so they do not harm normal tissues. Currently, various types of virus have been tested in clinical trials for malignant glioma, including HSV-1, adenovirus (Chiocca *et al.*, 2004), reovirus (Forsyth *et al.*, 2008), Newcastle disease virus (Freeman *et al.*, 2006), and measles virus (Msaouel *et al.*, 2009). Other viruses, *e.g.* vesicular stomatitis virus (Lun *et al.*, 2006), vaccinia virus (Timiryasova *et al.*, 1999), and poliovirus (Goetz and Gromeier, 2010), are being investigated in preclinical studies.

In this report, we provide an updated review on oncolytic HSV-1 therapies that lead to the clinical development of G47Δ.

Oncolytic HSV-1

HSV-1 is an enveloped, double-stranded DNA virus, and known as a pathogen responsible for herpes labialis. Symptoms of initial viral infection are usually mild and subclinical infections are also common; 70 to 80% of the adult population is reported to be seropositive for HSV-1. In rare occasions, it causes severe clinical manifestations, i.e. HSV-1 encephalitis or keratitis. After infection to the skin or the mucosa, the virus may occasionally infect sensory nerves and establish latency in the ganglia. In a latent state, the virus does not replicate but expresses only latency-associated genes, although it can get reactivated and the symptoms may

recur occasionally (Roizman *et al.*, 2007). The reactivation can occur in a subject who is seropositive for HSV-1, indicating that HSV-1 can spread through direct cell-to-cell contact even in the presence of circulating HSV-1 antibodies.

In general, a genetically engineered HSV-1 possesses preferable characteristics as oncolytic virus including the following: (1) it shows little toxicity to normal tissues, and there exist scientific grounds for tumor cell selectivity; (2) the viral genome is stable; (3) it shows no risk of non-specific transmission to the healthy public population; (4) it can efficiently infect a wide range of tumor types and exhibits a potent oncolytic activity; (5) oncolytic activity is minimally affected by circulating antiviral antibodies; (6) inflammatory reactions to the virus are generally mild and repeated administrations are possible; (7) large-scale manufacturing of high-titer, pure virus product is feasible; (8) the final product is stable and can be stored for a decent period; (9) there are antiviral drugs or pooled gamma globulin available to terminate viral replication when undesired events occur; (10) antitumor immune responses will be elicited in the course of oncolytic activities of the virus; (11) the large size of HSV-1 genome (~152 kb) makes it also an attractive vector to express large or multiple foreign genes.

G207 Clinical Trial

G207 is an oncolytic HSV-1 that has double mutations in the viral genome (Mineta *et al.*, 1995). It has deletions in both copies of the *γ34.5* gene, the major determinant of HSV-1 neurovirulence (Chou *et al.*, 1990). *γ34.5*-deficient HSV-1 does not replicate in normal cells, but maintains its ability to replicate within cancer cells. In normal cells, double-stranded RNA-dependent protein kinase (PKR) is activated upon HSV-1 infection, which causes the phosphorylation of the α-subunit of eukaryotic initiation factor 2, resulting in a shutdown of host cell and viral protein synthesis. The product of the *γ34.5* gene (ICP34.5) counteracts to this function, so in *γ34.5*-deficient HSV-1, the protein synthesis is terminated (Cassady *et al.*, 1998; He *et al.*, 1997). Since most types of tumor cells have low PKR activities, *γ34.5*-deficient HSV-1 are still able to replicate in tumor cells (Farassati *et al.*, 2001). G207 also has an insertion of the *E. coli lacZ* gene in the infected-cell protein 6 (*ICP6*) locus, which inactivates ICP6, the large subunit of ribonucleotide reductase. ICP6 is required for viral nucleotide synthesis in normal cells. In cancer cells where cellular enzymes for nucleotide synthesis are abundant, ICP6 deficiency is compensated by the cellular ribonucleotide reductase, allowing ICP6-deficient HSV-1 to replicate (Goldstein and Weller, 1988). ICP6 inactivation also makes the mutant virus more sensitive to antiviral drugs, acyclovir and ganciclovir (Coen *et al.*, 1989).

In preclinical studies using immunocompetent mice, an induction of specific antitumor immunity was observed following tumor cell destruction by G207 (Todo *et al.*, 1999b). In A/J mice bearing bilateral subcutaneous tumors, intratumoral G207 inoculation on one side caused tumor size reduction not only of the inoculated tumors but also of the non-inoculated contralateral tumors. The antitumor

immunity was associated with an elevated cytotoxic T lymphocyte (CTL) activity specific to tumor cells.

After *in vivo* safety evaluation using HSV-1-susceptible mice (Sundaresan *et al.*, 2000) and non-human primates (Hunter *et al.*, 1999; Todo *et al.*, 2000), the G207 phase I clinical trial was performed from 1998 to 2000 at two institutions in the United States (Markert *et al.*, 2000). Twenty-one subjects with recurrent malignant glioma were enrolled, and G207 was administered directly into the tumor via a stereotactic method. The dose started from 1×10^6 plaque-forming units (pfu) per injection and increased up to 3×10^9 pfu, in 7 cohorts with three patients at each dose level. There were no grade 3 or 4 adverse events attributable to G207 and maximal tolerated dose could not be reached. Twenty patients had serial MRI evaluations, and 8 tumors decreased in volume between four days and one month post-inoculation. One glioblastoma patient remained alive 5.5 years after G207 administration.

The result of phase Ib trial has also been published in 2009 (Markert *et al.*, 2009). Six patients with recurrent glioblastoma were enrolled in a single cohort. A catheter was stereotactically placed into the tumor and 1.5×10^8 pfu of G207 (13% of total dose) were injected through the catheter. Two or 5 days later, the tumor was resected together with the catheter, and 1×10^9 pfu of G207 were injected into the tumor bed. Evidence of G207 replication was demonstrated by reverse transcription polymerase chain reaction (RT-PCR) analysis on genes for viral replication. No patients developed encephalitis, and there were no severe adverse events. Although radiological and pathological responses were seen, there were no complete responses or partial responses. Median time to tumor re-progression was 3 months, and median overall survival from G207 administration was 6.6 months.

HSV1716 Clinical Trial

HSV1716 (1716) is an oncolytic HSV-1 with a single gene mutation. Both copies of the *γ34.5* gene are deleted, while its ICP6 gene is intact. Preclinical studies have demonstrated strong cytotoxicity to tumor cells, but there remained some toxicities to the normal brain; half of the mice that had intraventricular injection of 1716 as low as 1×10^3 pfu died (Lasner *et al.*, 1998).

The first phase I clinical trial was initiated in the United Kingdom and the result was published in 2000 (Rampling *et al.*, 2000). Nine patients with recurrent malignant glioma (8 glioblastoma and 1 anaplastic astrocytoma) had stereotactic injections of escalating dose (1×10^3 pfu to 1×10^5 pfu, 3 cohorts, 3 patients in each cohort) of 1716. Dose limiting toxicities were not observed at the highest dose.

In the second phase I study, 12 patients with recurrent malignant glioma (11 glioblastoma and 1 anaplastic astrocytoma) received intratumoral, stereotactic injections of 1716 (1×10^5 pfu) followed by surgical resection of the 1716 injected tumors. Intratumoral viral replication was studied by PCR, and immune reaction

to the virus was assessed by immunohistochemistry. In this study, again, 1716 was shown to be safe at the dose tested (Papanastassiou *et al.*, 2002).

An additional phase I study was performed to evaluate the safety and efficacy of 1716 injected directly to the tumor cavity wall at the time of surgical resection. Twelve patients were treated and there were no severe adverse events attributable to 1716 administration (Harrow *et al.*, 2004). Conventional radiation therapy and chemotherapy were also delivered when indicated, and 3 patients remained in a stable condition one year after 1716 injection.

Recently, a phase III clinical trial of 1716 (Seprehvir, Crusade Laboratories) was approved in Europe. Patients with recurrent glioma patients after surgical resection and radiotherapy will be enrolled.

Construction of G47Δ

In order to further improve the efficacy of oncolytic HSV-1 while maintaining the safety of G207, a new generation oncolytic HSV-1 termed G47Δ was created (Todo *et al.*, 2001). Compared with G207, G47Δ has an additional genetic alteration, i.e., a 312 bp sequence including the *α47* gene and the overlapping *US11* promoter region is further deleted from the G207 genome. The *α47* gene product inhibits transporter associated with antigen presentation (TAP) that translocates peptides across the endoplasmic reticulum of the host cell. Because of TAP inhibition, HSV-1-infected cells have down-regulated levels of MHC class I when the *α47* gene is intact (Hill *et al.*, 1995; Jennings *et al.*, 1985). G47Δ-infected human cells maintained higher levels of MHC class I expression than cells infected with other HSV-1 with intact *α47*. In addition, deletion of the *US11* promoter puts the late gene *US11* under control of the immediate- early *α47* promoter. Expression of the *US11* gene as an immediate-early gene is known to recover the reduced replication capability of *γ34.5*-deficient HSV-1 in tumor cells, without affecting pathogenicity (Mohr and Gluzman, 1996). In athymic mice bearing subcutaneous U87MG human glioma and A/J mice bearing subcutaneous Neuro2a murine neuroblastoma, G47Δ was significantly more efficacious than G207 at inhibiting the tumor growth when inoculated intraneoplastically. Nevertheless, the safety of G47Δ remained unchanged from G207 following injection into the brain of HSV-1-sensitive A/J mice.

G47Δ Clinical Trial

Clinical grade G47Δ was manufactured at the Core Facility for Therapeutic Vectors (CFTV) at the University of Tokyo under current Good Manufacturing Practice (cGMP). A master cell bank was created from WHO Vero cells (Horaud, 1992; Montagnon and Vincent-Falquet, 1998), and comprehensive quality tests were performed in accordance with Guidelines for Biological Materials which has recently been revised (Knezevic *et al.*, 2010). After a process development for manufacturing and purification in a large scale based on the method for G207 (Todo *et al.*, 2000),

clinical lots of G47Δ were obtained. Test samples from unpurified bulk harvest, purified bulk, as well as final vialed product were submitted for extensive quality testing to confirm the absence of any other pathogens, including human viruses as well as bovine and porcine viruses. The entire genome of the virus from the master virus seed stock was sequenced, and the genome stability was confirmed by passaging ten times in Vero cells. The titer of the purified virus in storage was tested at various time points and proved stable at least for 24 hours at 4°C and at room temperature, and for at least 3 years at −75°C. Absence of contamination with wild type or revertant mutant HSV-1 in the final G47Δ product was confirmed by PCR. A/J mice that received intracerebral injections of 2×10^6 pfu of clinical grade G47Δ maintained healthy throughout the observation period while the control mice that received 2×10^3 pfu of wild type HSV-1 strain F all died within 2 weeks.

In 2006, it was decided by the Japanese regulatory authorities that the application for the clinical trial of G47Δ for the treatment of recurrent glioblastoma was to be reviewed according to the Japanese guideline for gene therapy clinical trials. Then, it was the first clinical trial application in Japan using a replication-competent, recombinant virus. The complete application materials were submitted to the government in February 2007, received for review in October 2007, and the final governmental approval was obtained in May 2009. The G47Δ administration and patient care procedures were also reviewed and approved by the government according the Cartagena Law of Japan.

The International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH) is a project that brings together the regulatory authorities and experts from the pharmaceutical industry from Europe, Japan and the United States to discuss scientific and technical aspects of product registration. ICH Considerations on Oncolytic Viruses was revised in September 2009 (http://www.ich.org/). Although this publication became available after the completion of clinical G47Δ production and quality testing, the clinical lots of G47Δ had been designed and tested to meet the current standard requirements.

The G47Δ clinical trial is an open labeled, single armed, phase I-II study at a single institution. Patients with histologically confirmed recurrent glioblastoma that is progressive despite previous or ongoing radiation therapy, age 18 or older, and with Karnofsky Performance Scale (KPS) 70% or higher are enrolled. The primary endpoint is to evaluate the safety of G47Δ, and the secondary endpoint is to access the efficacy by tumor size and progression-free survival. The total dose of the initial dose level is 6×10^8 pfu per subject. A dose of 3×10^8 pfu divided into 2–5 sites within the tumor is administered stereotactically, and within 14 days, the same dose is administered stereotactically to the same coordinates. Three cohorts of 3 subjects each are planed in the dose escalation phase up to 6×10^9 pfu per

Table 1. Summary of the G47Δ clinical protocol.

Target disease	Recurrent glioblastoma
Primary endpoint	Safety (Spectrum and frequency of adverse events)
Secondary endpoint	Efficacy (Change in tumor size on MRI, progression free survival, overall survival)
Design	Open label, single arm, non-randomized, phase I-II study
Key inclusion criteria	— Histologically confirmed recurrent glioblastoma that is progressive despite previous or ongoing radiation therapy — Single enhancing lesion measures 1.0 cm or larger — Karnofsky Performance Scale(KPS) >= 70% — Age >= 18 years
Key exclusion criteria	— Previous history or current diagnosis of other cancer — Multiple intracranial malignant glioma lesions — Subependymal or subarachnoidal dissemination
Number of patients	21 — 3 patients each in dose escalation phase — 12 patients in safety dose
Administration	— Stereotactic intratumoral injection — Total 1 ml/tumor divided into 2–5 sites/tumor — 2 injections, 5 to 14 days apart, to the same coordinates
Dose escalation	— 1st cohort: 6×10^8 pfu (3×10^8 pfu/injection) — 2nd cohort: 2×10^9 pfu (1×10^9 pfu/injection) — 3rd cohort: 6×10^9 pfu (3×10^9 pfu/injection)

subject, and 12 additional subjects will be treated at the highest safe dose (Table 1).

For Improved Efficacy of Oncolytic HSV-1

According to multiple published reports of phase I trials of oncolytic HSV-1, direct inoculation of the viruses to glioma can definitely be performed safely. The questions are, whether the patient can benefit from the treatment, and how to enhance the efficacy. The effect of circulating neutralizing antibody against the virus should be minimal on the efficacy of oncolytic HSV-1, especially when the virus is administered directly into the tumor. Innate immunity and interferon pathways may counteract the oncolytic activity of the virus (Msaouel *et al.*, 2009). In rats, a transient immune suppression by cyclophosphamide was reported to enhance the viral spread within brain tumors when the virus was administered into the carotid artery (Ikeda *et al.*, 1999; Wakimoto *et al.*, 2002). Cells with intact interferon signal pathways counteract with viral infection through activation of the pathway, however, cancer cells are likely defective in interferon pathways and the majority of oncolytic viruses use this defect for preferential replication in tumor cells (Barber, 2004). It should be cautioned that non-specific immune suppression might increase the toxicity of the

virus to normal cells, especially when the patient is already immunocompromised by chemotherapy, radiotherapy, or steroids.

We have previously reported that in immunocompetent mouse tumor models, administration of corticosteroid did not affect the initial tumor growth inhibition by G207 in the inoculated tumors, but resulted in a lower cure rate due to later regrowth (Todo *et al.*, 1999a). G47Δ is designed to elicit antitumor immune responses more efficiently by maintaining the MHC class I expression in G47Δ-infected tumor cells. Results from ongoing G47Δ clinical trial may provide a hint to whether G47Δ effectively elicits antitumor immune responses as intended.

Stereotactic intratumoral injection is the route of administration in the first-in-man G47Δ trial, as the initial step to evaluate safety. Injections into the tumor bed at the time of surgical resection have also been shown to be safe in G207 and HSV1716 studies. Convection-enhanced delivery might be an attractive option (Lun *et al.*, 2010) when the oncolytic virus can retain the activity (titer) during the prolonged period of administration, and still maintain the low toxicity to the surrounding normal brain. Intraarterial or intravenous administration may also be practical, although extensive pharmacokinetics and additional toxicity studies will be necessary. Intelligent drug delivery systems might be helpful for intravascular approaches.

Concluding Remarks

Results from clinical trials of oncolytic HSV-1 therapy for malignant glioma so far have indicated that this novel therapeutic approach can be performed safely. The proof of concept has been demonstrated in humans that oncolytic viruses actually replicate in tumors. Oncolytic HSV-1 therapy is also being tested in patients with other types of cancer, including malignant melanoma and head and neck cancer, in multiple countries. Based on rapid advancement of clinical trials of oncolytic HSV-1 to phases II and III, and also on encouraging results from recent publications, it is without doubt that oncolytic virus therapy would be one of the major treatment modalities for cancer in the future. Use of oncolytic HSV-1 armed with therapeutic transgenes, such as immunostimulatory genes and antiangiogenic genes, may be the step that follows. The results from the G47Δ clinical trial will hopefully contribute to the advancement of this treatment modality.

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Cancer Research

NKX2.2 Suppresses Self-Renewal of Glioma-Initiating Cells

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Abstract

Glioblastoma (GBM) is the most aggressive and destructive form of brain cancer. Animal models that can unravel the mechanisms underlying its progression are needed to develop rational and effective molecular therapeutic approaches. In this study, we report the development of mouse models for spontaneous gliomas representing distinct progressive stages of disease that are governed by defined genetic alterations. Neural stem/progenitor cell (NPC)-specific constitutive Ras activation *in vivo* plus *p53* deficiency led to development of primarily anaplastic astrocytoma (grade III), whereas combined loss of *p53* plus $p16^{Ink4a}/p19^{Arf}$ led to development of GBM (grade IV) at 100% penetrance within 6 weeks. These glioma models showed enhanced stem cell properties (stemness) accompanied by malignant progression. Notably, we determined that, in our models and in human specimens, downregulation of the homeodomain transcription factor *NKX2.2*, which is essential for oligodendroglial differentiation, was correlated with increased tumor malignancy. *NKX2.2* overexpression by GBM-derived glioma-initiating cells (GIC) induced oligodendroglial differentiation and suppressed self-renewal capacity. By contrast, Nkx2.2 downregulation in mouse NPCs accelerated GBM formation. Importantly, the inhibitory effects of NXK2.2 on GIC self-renewal were conserved in human cells. Thus, our mouse models offer pathobiologically significant advantages to investigate the nature of brain tumors, with improved opportunities to develop novel mechanism-based therapeutic approaches. *Cancer Res; 71(3); 1135–45. ©2010 AACR.*

Introduction

Glioblastoma (GBM) is the most common high-grade malignant glioma in humans and is categorized as a WHO grade IV glioma, a highly aggressive, invasive, and destructive brain tumor (1). There are 2 GBM subtypes, primary and secondary, which are distinguished by clinical characteristics. Primary GBM arises *de novo* in the absence of a preexisting low-grade lesion, whereas secondary GBM develops progressively (over 5–10 years) from lower grade gliomas such as anaplastic astrocytoma (AA, grade III). Alterations in several signaling cascades are known to affect gliomagenesis. These pathways include the receptor tyrosine kinase (RTK)/RAS/PI3K pathway (including EGFR, PDGFR, NF1, and PTEN); the p53 pathway (including TP53, CDKN2A/$p14^{ARF}$, and MDM2); and the RB pathway (including RB1, CDKN2A/$p16^{INK4A}$, CDKN2B and CDKN2C; refs. 1, 2).

Several investigators have developed mouse GBM models by genetically engineering glioma mutations. Reilly and colleagues (3) report a mouse model carrying heterozygous *cis*-germline mutations in the gene encoding a Ras GTPase-activating protein, Nf1, an effector of RTK signaling, in combination with *p53* deficiency. These mice develop malignant gliomas, including GBM and AA, with varying penetrance depending on genetic background (3). Mouse models harboring a heterozygous germline or conditional somatic *p53* mutation combined with conditional somatic *Nf1* heterozygosity develop low- to high-grade astrocytomas (4). Tumor formation is accelerated into high-grade astrocytomas similar to primary GBM by additional loss of *Pten* (5). Concomitant central nervous system (CNS)-specific deletion of *p53* and *Pten* generates a high-grade malignant glioma phenotype ranging from grade III to grade IV, with notable clinical, pathologic, and molecular resemblance to human malignant gliomas (6). Furthermore, Alcantara Llaguno and colleagues (7) have shown that inactivation of Nf1 combined with loss of other tumor suppressors (*p53* and *Pten*) in neural stem/progenitor cells (NPC), but not in non-NPCs, was both necessary and sufficient to induce glioma formation, indicating critical roles

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for Ras activation in NPCs on gliomagenesis *in vivo*. Thus, these mouse models have provided critical information regarding molecular mechanisms underlying gliomagenesis. However, in these models, phenotypic variations in malignant progression are observed even in the presence of the same mutations (5, 7). To better understand mechanisms underlying malignant glioma progression, mouse models that reliably control stages of malignant progression are needed.

Although *RAS* mutations are uncommon in human malignant gliomas, the impact of *NF1* inactivation on human and mouse glioma suggested critical roles of RAS activation in gliomagenesis (8). Proliferation of these tumors requires RAS activation and many of these tumors exhibit elevated RAS signaling, which seems to be central to their pathology (9). Consistent with this notion, several mouse GBM models have been generated by inducing constitutive Ras activation (10–12). In this study, we developed mouse models of gliomagenesis by engineering NPCs to express a constitutively active form of K-Ras via the tamoxifen-induced Cre-loxP system. Interestingly, in combination with loss of the tumor suppressors *p53* and $p16^{Ink4a}/p19^{Arf}$, mutant mice developed glioma at 100% penetrance with short latency (within 10 weeks), a clear alteration in malignant progression status. We also observed a correlation between enhanced stem cell properties (stemness) and malignant progression stages, consistent with human samples. Furthermore, we report that Nkx2.2 is a critical factor controlling self-renewal of glioma-initiating cells (GIC), an activity conserved in human GICs. Our mouse brain tumor models could be used to gain important insights into new therapeutic approaches.

Materials and Methods

Mice

$LSL\text{-}K\text{-}Ras^{G12D}$ and $p16^{Ink4a+/-}/p19^{Arf+/-}$ mice were obtained from the Mouse Models of Human Cancers Consortium of NCI-Frederick (13, 14). $p53^{+/-}$ and $Nestin\text{-}CreER^{T2}$ mice were previously described (15, 16). Mice were maintained on a mixed 129SvJ/C57BL/6 background. All animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University and performed in compliance with the University's Guidelines for the Care and Use of Laboratory Animals.

Human brain tumor samples

Tumors from patients with glioma were surgically removed and diagnosed at the Department of Neurosurgery, Kanazawa University and at the Department of Neurosurgery, Kumamoto University. All histologic analyses of NKX2.2 expression levels were performed at low-power magnification, and the entire tissue section was evaluated rather than specific foci or selected high-power fields. NKX2.2 expression levels were scored from negative/weak to positive (>30% of tumor cells), depending on the percentage of $NKX2.2^{+}$ cells in a given tumor. Human GBM patient–derived GICs, termed TGS-01 and TGS-04, were established as described previously (17). All human materials and protocols used in this study were approved by ethics committees of Kanazawa University, Kumamoto University, and the University of Tokyo. Informed consent was obtained from all patients.

Tamoxifen induction

To activate $CreER^{T2}$ *in vivo*, 1 mg tamoxifen (Sigma) in corn oil (Sigma) was administered intraperitoneally to 8-week-old mice once daily for 5 consecutive days. Immunohistochemistry and immunofluorescence analyses were performed on coronal sections of forebrains obtained at 12 weeks of age.

Tissue preparation and histology

Sacrificed mice were perfused with 4% paraformaldehyde (PFA), and brains were dissected and postfixed overnight in 4% PFA at 4°C. Serial sections were prepared at 5 μm for paraffin sections or 10 μm for cryostat sections. Paraffin-embedded mouse brains, mouse gliomas, or human gliomas were deparaffinized and rehydrated prior to immunohistochemical analysis. Tumors were graded according to the WHO grading system for malignant astrocytomas (1).

Antibodies

Immunohistochemistry and immunofluorescence analyses were performed as described (18). Sections were examined using optical, fluorescence, and confocal microscopy (Keyence BZ-9000, Olympus FV1000, and Carl Zeiss Axio Imager A1 microscopes, respectively). Primary antibodies (Abs) recognizing the following proteins were used for immunostaining assays: Nkx2.2 (Developmental Studies Hybridoma Bank; Sigma), Ki-67 (BD), Nestin (BD and Chemicon), Sox2 (Chemicon), CD133 (eBioscience), GFAP (DAKO), Olig2 (Chemicon), βIII-tubulin (Tuj-1, Covance), NG2 (Chemicon), O4 (Chemicon), cleaved caspase-3 (Cell Signaling), HP1γ (Chemicon), γ-H2AX (Upstate), phospho-Akt^{Ser473} (Cell Signaling), CD34 (Chemicon), VEGF (Santa Cruz), PDGFRα (Cell Signaling), myelin basic protein (Mbp; Abcam), and NeuN (Chemicon). Primary Abs were detected using Alexa Fluor-conjugated secondary Abs (Invitrogen), and peroxidase-conjugated secondary Ab (GE Healthcare) plus the DAB Peroxidase Substrate Kit (VECTOR).

Tumor neurospheres

For mouse tumor neurosphere (TNS) formation, mouse glioma cells were isolated from brains of $p53^{-/-}$;NR^{+tamo} or $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice. To assay TNS numbers, dissociated cells (2×10^{3} cells/200 μL) were seeded into 96-well plates and cultured for 7 days in medium containing 20 ng/mL fibroblast growth factor-2 (FGF-2; Peprotech) and 20 ng/mL epidermal growth factor (EGF; Peprotech) as described (18). TNSs derived from TGS-01 and TGS-04 cells were cultured as described (17). Dissociated cells from sphere preparations were transfected with pLXSB-human *NKX2.2* and cultured as adherent monolayers on poly-L-ornithine–coated coverslips, and then selected in blasticidine-S (8 μg/mL) for 4 days. Transfection was performed using Fugene6 transfection reagent (Roche) according to the manufacturer's instructions. The number of spheres of diameter greater than 50 μm was determined using phase-contrast microscopy.

Retrovirus-mediated Nkx2.2 overexpression

cDNA encoding full-length mouse *Nkx2.2* was cloned into the retroviral vector pLXSB (19). Retroviral packaging cells (Phoenix-E) were transiently transfected as above with pLXSB-*Nkx2.2*. Viral titers were estimated by observing increased drug resistance in infected NIH3T3 cells. TNSs derived from $p53^{-/-}$; $p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice were mixed with *Nkx2.2*–expressing viral suspensions and maintained in culture for 7 days, or cultured as adherent monolayers on coverslips with a *Nkx2.2*–expressing virus suspension and then selected in blasticidine-S (8 μg/mL) for 4 days.

Retrovirus-mediated RNA interference

For short hairpin RNA (shRNA)-mediated mRNA knockdown, a retroviral vector (pSM2c) expressing shRNAs under control of the U6 promoter (Open Biosystems) was used. *Nkx2.2* shRNAs were *Nkx2.2* shRNA1 (oligoID: V2HS61850) targeting GGTCAAGATCTGGTTCCAGAA, and *Nkx2.2* shRNA2 (oligoID: V2HS152272) targeting CCAGAACCACCGCTACAAG. Control shRNA was GFP shRNA targeting GCACAAGCTGGAGTACAACTA. NPCs derived from neonatal (P3–5) $p16^{Ink4a-/-}/p19^{Arf-/-}$ mice were cultured as adherent monolayers with a control GFP shRNA or an *Nkx2.2*-shRNA-expressing retrovirus suspension, and then selected with puromycin (2 μg/mL) for 4 days, infected with *EGFRvIII*-expressing retrovirus (pLERNL), and cultured for an additional 7 days. Levels of *Nkx2.2* mRNAs before infection with *EGFRvIII*-expressing retrovirus were determined by quantitative real-time RT-PCR as described in the following section.

Quantitative RT-PCR

RNAs were purified from cultured NPCs using the RNeasy kit (QIAGEN) and reverse-transcribed using the Advantage RT-for-PCR kit (Takara-Clontech). Real-time quantitative PCR was performed using SYBR green Premix EX Taq (Takara) on an Mx3000P real-time PCR system (Aligent Technology). Sense and antisense primers are listed in Supplementary Table S1 online. The following cycle parameters were used: denaturation at 95°C for 10 seconds, and annealing and elongation for 30 seconds at 57°C for *β-actin* and at 60°C for *Nkx2.2*.

Orthotopic transplants

For intracranial injections, TNSs cultured as adherent monolayers were dissociated and resuspended in Hanks Buffered Salt Solution at a concentration of 100,000 viable cells/μL. Female NOD/SCID mice (Charles River) ages 6 to 8 weeks were anesthetized and placed into a stereotactic apparatus equipped with a *z*-axis (Stoelting). A small hole was bored into the skull 0.5 mm anterior and 3.0 mm lateral to bregma using a dental drill. Cell suspensions (2 μL) were injected into the right striatum 3 mm below the surface of the brain using a 10 μL Hamilton syringe with a 26 gauge needle. The scalp was closed using an Autoclip Applier. Animals were monitored daily for neurologic deficits.

Western blotting

Western blotting was performed as described (18). The primary Abs used recognized NKX2.2 (Developmental Studies Hybridoma Bank) or α-tubulin (Sigma).

Statistics

P values were calculated using the unpaired Student's *t* test. Survival curves were plotted using the Kaplan–Meier method, and differences were analyzed using the log-rank test. The significance of the association between NKX2.2 expression and malignancy was determined by the Fisher's exact test (right tail).

Results

Establishment of mouse glioma models that exhibit malignant progression by *in vivo* NPC-specific Ras activation

Because Ras signaling is a major pathway upregulated in gliomagenesis, we asked whether Ras activation in NPCs could induce gliomagenesis *in vivo*. To do so, we established a mouse model in which constitutively activated Ras ($K\text{-}Ras^{G12D}$) could be specifically and temporally induced in NPCs by binding of tamoxifen to a Cre recombinase-modified estrogen receptor ligand-binding domain fusion protein ($Cre\text{-}ER^{T2}$) expressed under control of the *Nestin* promoter/enhancer (13, 15). For these experiments, we designated untreated control $Nestin\text{-}CreER^{T2}$;$LSL\text{-}K\text{-}Ras^{G12D}$ mice as "NR" mice, tamoxifen-treated NR mice as "NR^{+tamo}" mice, $Nestin\text{-}CreER^{T2}$ mice treated with tamoxifen as "$control^{+tamo}$" mice, and NR mice treated with vehicle only as "NR^{-tamo}" mice. First, we treated all 4 groups of mice (at 8 weeks of age) with vehicle or tamoxifen and analyzed their brains at 12 weeks of age. Although histologic analysis revealed that the lateral ventricles were slightly enlarged in NR^{+tamo} mice compared with $control^{+tamo}$ and NR^{-tamo} mice, the gross appearance of the brain in all animals was normal (Supplementary Fig. S1). Further analysis indicated that both expression of the proliferation marker Ki-67 antigen and 5-bromodeoxyuridine incorporation were reduced in NR^{+tamo} subventricular zone (SVZ) compared with $control^{+tamo}$ SVZ (Supplementary Fig. S2A–C). Moreover, immunohistochemical analyses showed no differences between NR^{+tamo} and $control^{+tamo}$ SVZ in numbers of $TUNEL^{+}$ apoptotic cells (Supplementary Fig. S3A) or cleaved $caspase\text{-}3^{+}$ cells (Supplementary Fig. S3B). Although it has been shown that oncogenic signaling induces cellular senescence *in vitro* and *in vivo*, we observed no significant differences between $control^{+tamo}$ and NR^{+tamo} SVZ cells in expression of the senescence-associated markers HP1γ or γ-H2AX (Supplementary Fig. S3C), or in SA-β-gal activity (data not shown; ref. 20). Overall we conclude that enlargement of the lateral ventricles observed in NR^{+tamo} mice was largely because of Ras-induced inhibition of NPC proliferation.

NR^{+tamo} mice did not develop tumors, even after long-term observation (Fig. 1A). Therefore, because inactivation of p53 and $p16^{INK4A}/p14^{ARF}$ is commonly seen in human gliomas, we evaluated the impact of loss of these genes on our NR model mice *in vivo*. First, we crossed our NR model mice with *p53* mutant mice. Following administration of tamoxifen, $p53^{-/-}$; NR^{+tamo} mice began dying of brain tumors approximately 6 weeks later, and 100% of the mice were dead by approximately 10 weeks. Histologic analyses of brain tumors revealed that most (9 of 10) were AA, with only 1 of 10 showing classic features of GBM, such as necrosis, microvascular proliferation,

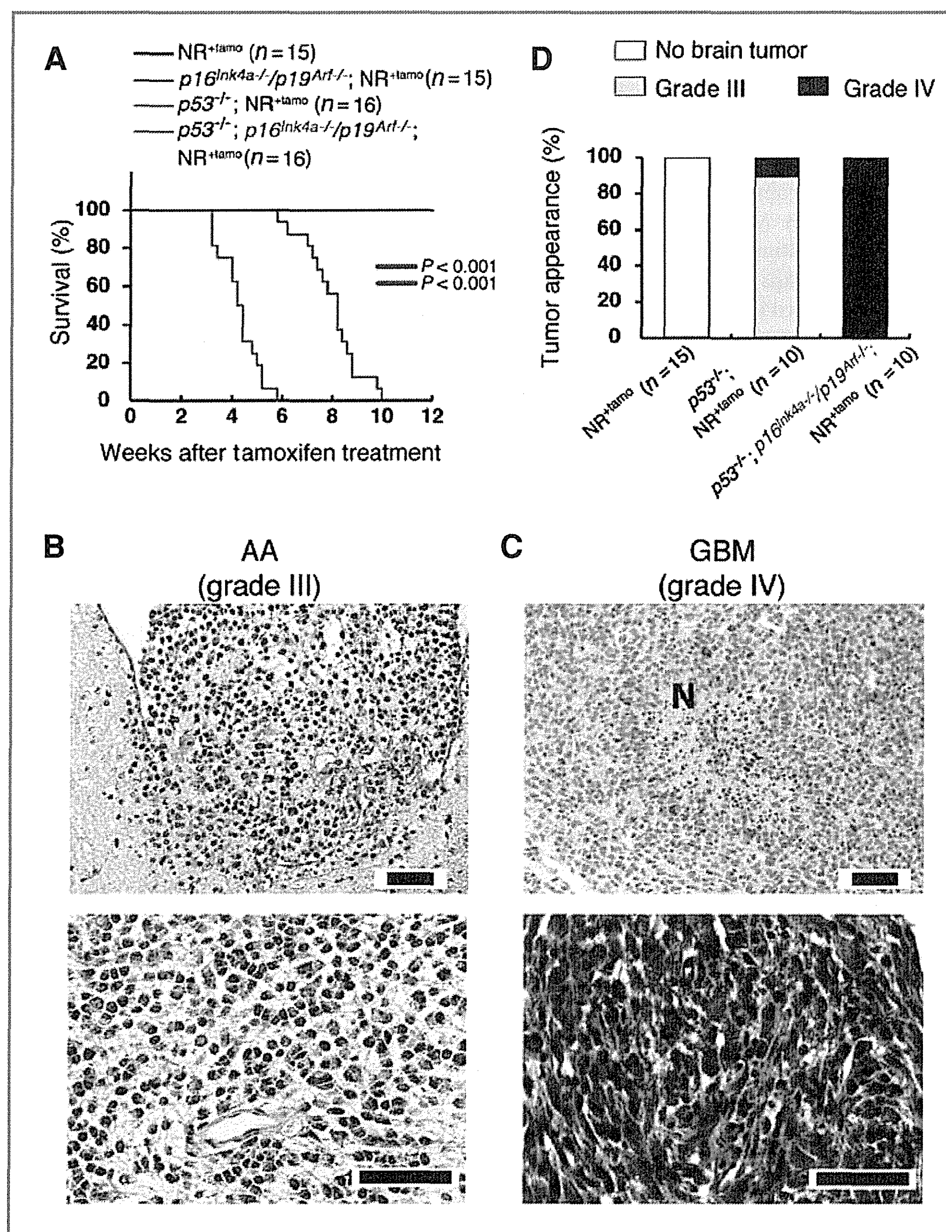


Figure 1. *p53* plus $p16^{Ink4a}/p19^{Arf}$ deficiencies combined with Ras activation drive GBM formation *in vivo*. A, altered survival. Mice of indicated genotypes (8 weeks old) were treated with tamoxifen for 5 days and monitored for tumor development. Kaplan–Meier tumor-free survival curves are shown. B and C, tumor histology. Coronal sections of forebrains of mice in (A) were prepared and stained with H&E. Tumors were graded for severity, as either AA (grade III; B) or GBM (grade IV; C). Scale bars, 50 μm. "N" indicates an area of "palisading" with regional necrosis. Data shown in (B) and (C) are representative of 10 mice/group. D, quantification of (B) and (C). Data shown are the percentage of mice in each group that developed no tumors, or grade III AA, or grade IV GBM.

marked cellular pleomorphism, and highly infiltrative spread to the cerebral cortex (Fig. 1B–D; Supplementary Fig. S4). $p53^{-/-}$;NR^{-tamo} mice did not develop any brain tumors over the period analyzed (data not shown). Next, we crossed our NR model mice with $p16^{Ink4a}/p19^{Arf}$ mutant mice. NR^{+tamo} mice on a $p16^{Ink4a}/p19^{Arf}$ null background also did not develop tumors over the 12-week period analyzed. These findings differ from previous studies showing that cultured NPCs derived from neonatal $p16^{Ink4a-/-}/p19^{Arf-/-}$ mice and infected with retrovirus carrying $K\text{-}Ras^{G12V}$ develop GBMs (10, 21). It was reported that GBM also arises when $p16^{Ink4a-/-}$ or $p19^{Arf-/-}$ mice are engineered to overexpress mutant *K-Ras* gene *in vivo* (10). By contrast to those studies, in our model the mutant *K-Ras* allele is expressed at physiologic levels. Therefore, discrepancies are likely due to differences in the amplitude of oncogenic signaling, the age of the mice used, or the cellular context (transformation *in vitro* or *in vivo*).

To investigate the effect of loss of multiple tumor suppressors in our model, we crossed NR mice with *p53* and $p16^{Ink4a}/p19^{Arf}$ mutant mice. Simultaneous deletion of $p16^{Ink4a}/p19^{Arf}$ plus *p53* significantly shortened the survival time of NR^{+tamo} mice (Fig. 1A). Interestingly, in contrast with $p53^{-/-}$;NR^{+tamo} mice, histologic analyses revealed that 100% of the tumors in $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice were GBMs (Fig. 1D). As shown in Figure 2A and C, GBMs derived from $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice were phenotypically similar to human GBMs. Those mouse tumors showed markedly increased numbers of Ki-67^{+} mitotic cells, and expressed the classic human glioma markers glial fibrillary acidic protein (Gfap) and Nestin, and high levels of vascular endothelial

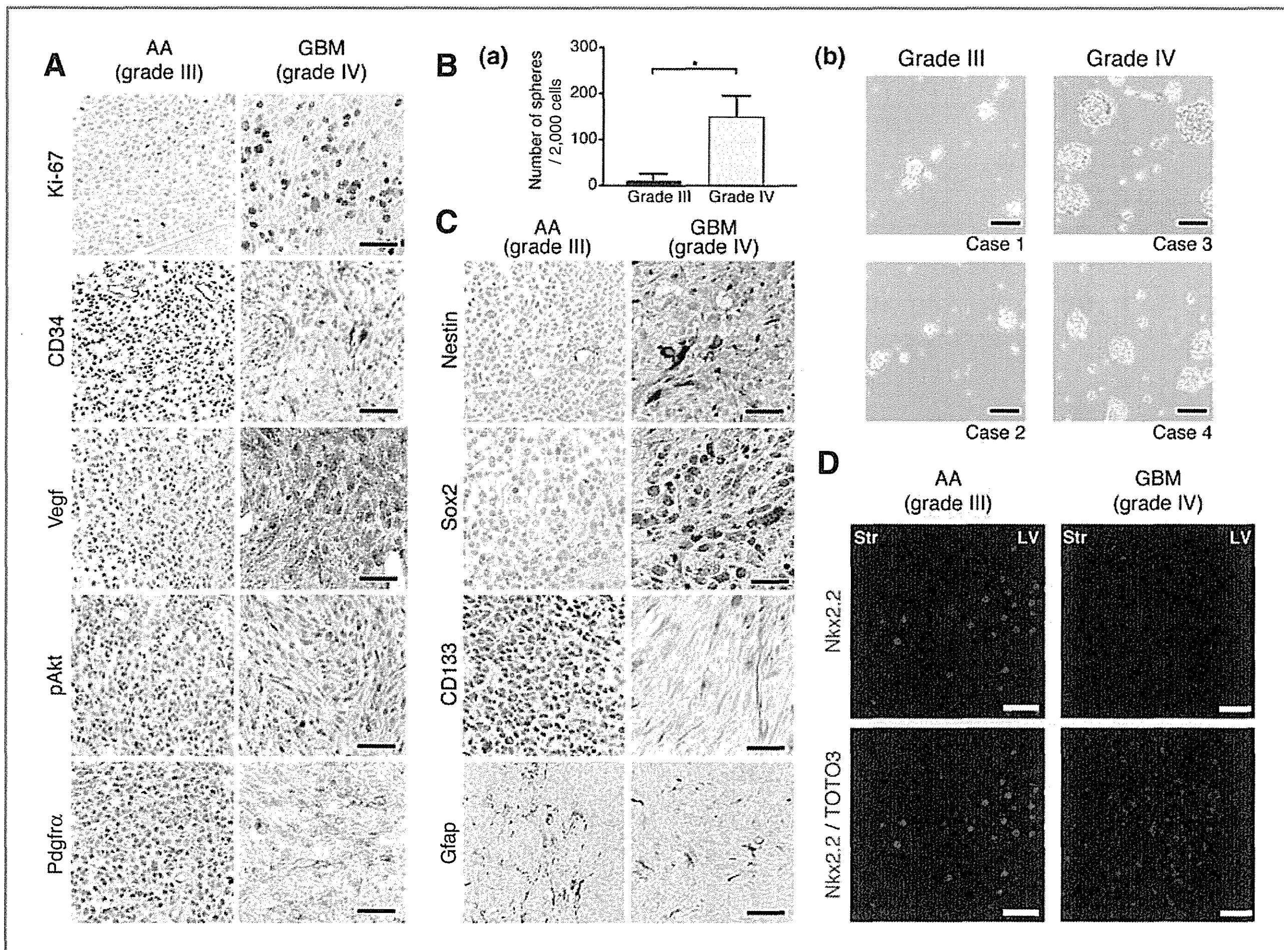


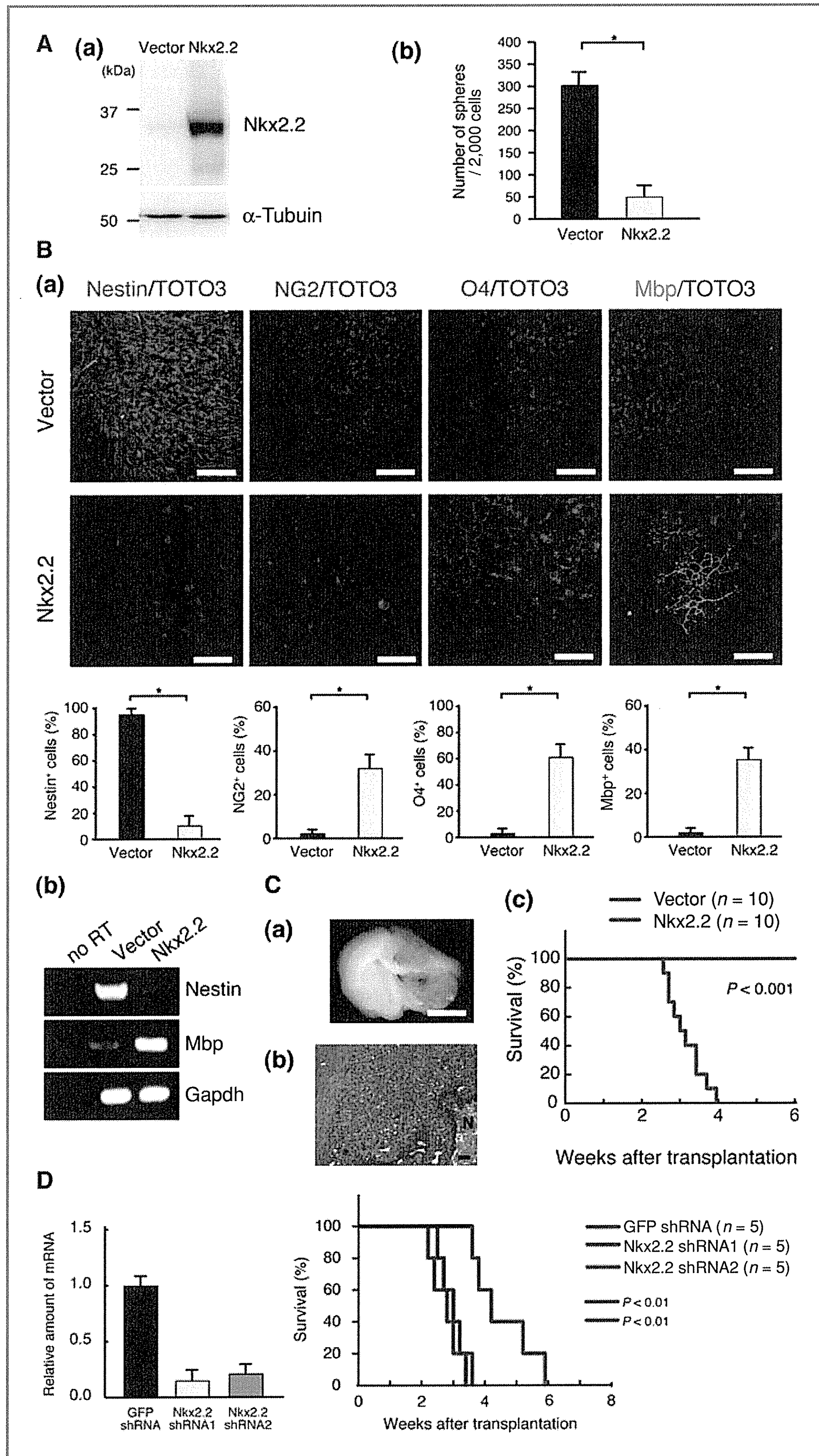
Figure 2. Enhancement of stem cell properties follows malignant glioma progression. A, sections of AAs (grade III) from $p53^{-/-}$;NR^{+tamo} mice and GBMs (grade IV) from $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;NR^{+tamo} mice were immunostained to detect indicated proteins. The resulting staining patterns are highly similar to those seen in human malignant astrocytomas. Scale bars, 100 μm. B, a, increased number of TNSs derived from dissociated mouse GBM cells. b, representative images of the TNSs derived from AAs (grade III, cases 1 and 2) and GBMs (grade IV, cases 3 and 4) are shown. Scale bars, 100 μm. Data shown are the mean number ± SD of TNSs generated per 2,000 cells (n = 5/group). *, $P < 0.001$. C, expression of stem cell and glial differentiation markers. Sections of AA (grade III) from $p53^{-/-}$;NR^{+tamo} mice and GBMs (grade IV) from $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;NR^{+tamo} mice were immunostained to detect indicated proteins. The resulting staining patterns are reminiscent of human malignant gliomas. Scale bars, 100 μm. D, expression of oligodendroglial differentiation markers. Forebrain sections from mice in (A) were stained with anti-Nkx2.2 (red) and TOTO3. Str, striatum; LV, lateral ventricle. Scale bars, 25 μm. A, B, and D, results shown are representative of 5 mice/group and 5 experiments.

growth factor (Vegf) and phosphorylated Akt (p-Akt). Coactivation of multiple RTKs, which is often seen in human primary GBMs, was also evident in our murine GBMs, in which we observed markedly upregulated Pdgfrα expression. By contrast, AA seen in $p53^{-/-}$;NR^{+tamo} mice showed only low levels of Ki-67, Nestin, VEGF, p-Akt, and Pdgfrα (Fig. 2A and C). Microvascular formation in mouse GBMs was confirmed by detection of the endothelial marker CD34 (Fig. 2A).

Enhanced stem cell properties (stemness) accompanies malignant progression in mouse glioma models

Previous reports of human GBMs indicate that several stem cell markers, including Nestin, Sox2, and CD133 are upregulated in these malignancies (22–24). Likewise, we found that Nestin, Sox2, and CD133 were upregulated in GBMs from $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;NR^{+tamo} mice, but not in the AA of $p53^{-/-}$;NR^{+tamo} mice (Fig. 2C). Because it has been shown that population of undifferentiated tumor cells known as tumor stem cells, or tumor-initiating cells, can undergo sphere formation *in vitro* (21), we evaluated the capacity of tumor cells in our model to form TNSs in culture by dissociating the entire tumor. A large number of TNSs was generated from cultures of cells derived from dissociated GBM, whereas AA-derived cells produced very few (Fig. 2B), indicating that mouse GBMs, like human GBMs, contain cells exhibiting stem cell properties (stemness).

Given the established inverse relationship between malignant progression and cellular differentiation, we compared expression levels of several differentiation markers in mouse GBM and AA. Consistent with evidence derived from human studies, tumor cells in either $p53^{-/-}$;NR^{+tamo} or $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;NR^{+tamo} mice did not express markers


A (a) Vector Nkx2.2
(kDa)
37
25
50
Nkx2.2
α-Tubuin
(b)
Number of spheres / 2,000 cells
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B
(a) Nestin/TOTO3 NG2/TOTO3 O4/TOTO3 Mbp/TOTO3
Vector
Nkx2.2
Nestin+ cells (%)
NG2+ cells (%)
O4+ cells (%)
Mbp+ cells (%)
(b)
no RT Vector Nkx2.2
Nestin
Mbp
Gapdh
C
(a)
(b)
(c)
Vector (n = 10)
Nkx2.2 (n = 10)
Survival (%)
P < 0.001
Weeks after transplantation
D
Relative amount of mRNA
GFP shRNA
Nkx2.2 shRNA1
Nkx2.2 shRNA2
Survival (%)
Weeks after transplantation
GFP shRNA (n = 5)
Nkx2.2 shRNA1 (n = 5)
Nkx2.2 shRNA2 (n = 5)
P < 0.01
P < 0.01

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 $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{IArf-/-}$;NR^{+tamo} 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.

Figure 3. 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^{+tamo} 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.