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 (AA, 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 as well as 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 (GICs) induced oligodendroglial differentiation and suppressed self-renewal capacity. By contrast, Nkx2.2 down-regulation 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.
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 two GBM subtypes, primary and secondary, which are distinguished by clinical characteristics. Primary GBM arises de novo in the absence of a pre-existing 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/ARF and MDM2); and the RB pathway (including RB1, CDKN2a/p16INK4A, CDKN2B and CDKN2C) (1, 2).

Several investigators have developed mouse GBM models by genetically engineering glioma mutations. Reilly et al. report a mouse model carrying heterozygous cis-germline mutations in the gene encoding a Ras GTPase-activating protein (RasGAP), Nf1, an effector of receptor tyrosine kinase signaling, in combination with Trp53 (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 IV, with notable clinical, pathological and molecular resemblance to human malignant gliomas (6). Furthermore, Alcantara Llaguno et al. have shown that inactivation of Nf1 combined with loss of other tumor suppressors (p53 and Pten) in neural stem/precursor cells (NPCs), but not in non-NPCs, was both necessary and sufficient to induce glioma formation (7), indicating critical roles 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 appears 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/p19, 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 (GICs), 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

$LSL-K-Ras^{G12D}$ and $p16^{hkap1a+/p19^{trf+/-}}$ mice were obtained from the Mouse Models of Human Cancers Consortium (MMHCC) of NCI-Frederick (13, 14). $p53^{+/−}$ and Nestin-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 histological 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<sup>T2</sup> *in vivo*, 1 mg tamoxifen (Sigma) in corn oil (Sigma) was administered intraperitoneally (i.p.) 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 post-fixed 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
Sections were examined using optical, fluorescence, and confocal microscopy (Keyence BZ-9000, Olympus FV1000, and 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; 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-AktSer473 (Cell Signaling), CD34 (Chemicon), VEGF (Santa Cruz), PDGFRα (Cell Signaling), Mbp (Abcam), and NeuN (Chemicon). Primary Abs were detected using Alexa Fluor-conjugated secondary Abs (Invitrogen), and peroxidase-conjugated secondary Ab (Amersham Biosciences) plus the DAB Peroxidase Substrate Kit (VECTOR).

Tumor neurospheres (TNSs)

For mouse TNS formation, mouse glioma cells were isolated from brains of p53<sup>-/-</sup>;NR<sup>+tam</sup> or p53<sup>-/-</sup>;p16<sup>ink4a/-/p19Arf/-</sup>;NR<sup>+tam</sup> mice, respectively. To assay TNS numbers, dissociated cells (2x10<sup>3</sup> cells/200 µl) were seeded into 96-well plates and cultured for 7 days in medium containing 20 ng/ml fibroblast growth factor-2 (FGF2; 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 blasticidin-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 >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. TNS derived from $p53^{-/-}$p16$^{inkda/-}/p19^{Arf/-};NR^{tumo}$ mice were mixed with pLXSB-Nkx2.2-expressing viral suspensions and maintained in culture for 7 days, or cultured as adherent monolayers on coverslips with a pLXSB-Nkx2.2-expressing virus suspension and then selected in blasticidin-S (8 μg/ml) for 4 days.

**Retrovirus-mediated RNA interference**

For 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 GGTCAGATCTGTCCAGAA, and
Nkx2.2 shRNA2 (oligoID: V2HS152272) targeting CCAGAACCACCGCTACAAG. Control shRNA was GFP shRNA targeting GCACAAGCTGGAGTACAACTA. NPCs derived from neonatal (P3-5) p16<sup>Ink4a-/−/p19<sup>Arf-/−</sup> 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 below.

**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 (Stratagene). Sense and antisense primers are listed in Supplementary Table S1 online. The following cycle parameters were used: denaturation at 95°C for 10 sec, and annealing and elongation for 30 sec 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) aged 6–8 weeks were anesthetized and placed into a stereotactic apparatus equipped with a Z axis (Stoelting). A small hole was bored into the skull 0.5mm anterior and 3.0mm lateral to bregma using a dental drill. Cell suspensions (2 μl) were injected into the right striatum 3mm below the surface of the brain using a 10 ml Hamilton syringe with a 26 gauge needle. The scalp was closed using an Autoclip Applier. Animals were monitored daily for neurological 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-Ras<sup>G12D</sup>) 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-ER<sup>T2</sup>) expressed under control of the Nestin promoter/enhancer (13, 15). For these experiments, we designated untreated control Nestin-CreERT<sup>T2</sup>; LSL-K-Ras<sup>G12D</sup> mice as “NR” mice, tamoxifen-treated NR mice as “NR+tamo” mice, Nestin-CreERT<sup>T2</sup> mice treated with tamoxifen as “control+tamo” mice, and NR mice treated with vehicle only as “NR-tamo” mice. First, we treated all four groups of mice (at 8 weeks of age) with vehicle or tamoxifen and analyzed their brains at 12 weeks of age. Although histological analysis revealed that the lateral ventricles were slightly enlarged in NR+tamo mice compared to 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 (BrdU) incorporation were reduced in NR+tamo SVZ compared to control+tamo SVZ (Supplementary Fig. S2A-C). Moreover,
immunohistochemical analyses showed no differences between NR\textsuperscript{tamo} and control\textsuperscript{tamo}
SVZ in numbers of TUNEL\textsuperscript{+} apoptotic cells (Supplementary Fig. S3A) or cleaved
caspase-3\textsuperscript{+} cells (Supplementary Fig. S3B). Although it has been shown that oncogenic
signaling induces cellular senescence \textit{in vitro} and \textit{in vivo}, we observed no significant
differences between control\textsuperscript{tamo} and NR\textsuperscript{tamo} SVZ cells in expression of the
senescence-associated markers HP1\textsuperscript{γ} or γH2AX (Supplementary Fig. S3C), or in SA-β-gal
activity (data not shown) (20). Overall we conclude that enlargement of the lateral
ventricles observed in NR\textsuperscript{tamo} mice was largely due to Ras-induced inhibition of NPC
proliferation.

NR\textsuperscript{tamo} mice did not develop tumors, even after long-term observation (Fig. 1A).
Therefore, because inactivation of p53 and p16\textsuperscript{Ink4a}/p19\textsuperscript{Arf} is commonly seen in human
gliomas, we evaluated the impact of loss of these genes on our NR model mice \textit{in vivo}.
First, we crossed our NR model mice with p53 mutant mice. Following administration of
tamoxifen, p53\textsuperscript{−/−};NR\textsuperscript{tamo} mice began dying of brain tumors approximately 6 weeks later,
and 100\% of the mice were dead by approximately 10 weeks. Histological analyses of
brain tumors revealed that most (9/10) were AA, with only 1/10 showing classical features
of GBM, such as necrosis, microvascular proliferation, marked cellular pleomorphism, and
highly infiltrative spread to the cerebral cortex (Fig. 1B-D and Supplementary Fig. S4).

p53\textsuperscript{−/−};NR\textsuperscript{tamo} mice did not develop any brain tumors over the period analyzed (data not
shown). Next, we crossed our NR model mice with p16\textsuperscript{Ink4a}*/p19\textsuperscript{Arf}* mutant mice. NR\textsuperscript{tamoxifen} mice on a p16\textsuperscript{Ink4a}/p19\textsuperscript{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\textsuperscript{Ink4a}/p19\textsuperscript{Arf}-/ mice and infected with retrovirus carrying K-Ras\textsuperscript{G12V} develop GBMs (10, 21). It was reported that GBM also arises when p16\textsuperscript{Ink4a}/ or p19\textsuperscript{Arf}-/ mice are engineered to overexpress mutant K-Ras gene \textit{in vivo} (10). By contrast to those studies, in our model the mutant K-Ras allele is expressed at physiological 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 \textit{in vitro} or \textit{in vivo}).

To investigate the effect of loss of multiple tumor suppressors in our model, we crossed NR mice with p53 and p16\textsuperscript{Ink4a}/p19\textsuperscript{Arf} mutant mice. Simultaneous deletion of p16\textsuperscript{Ink4a}/p19\textsuperscript{Arf} plus p53 significantly shortened the survival time of NR\textsuperscript{tamoxifen} mice (Fig. 1A). Interestingly, in contrast with p53-/;NR\textsuperscript{tamoxifen} mice, histological analyses revealed that 100% of the tumors in p53-; p16\textsuperscript{Ink4a}/p19\textsuperscript{Arf}-/;NR\textsuperscript{tamoxifen} mice were GBMs (Fig. 1D). As shown in Fig. 2A and 2C, GBMs derived from p53-; p16\textsuperscript{Ink4a}/p19\textsuperscript{Arf}-/;NR\textsuperscript{tamoxifen} mice were phenotypically similar to human GBMs. Those mouse tumors showed markedly increased numbers of Ki-67* mitotic cells, and expressed the classical human glioma markers GFAP and Nestin, as well as high levels of VEGF and phosphorylated Akt (p-Akt). Co-activation
of multiple receptor tyrosine kinases, which is often seen in human primary GBMs, was also evident in our murine GBMs, where we observed markedly upregulated Pdgfrα expression. By contrast, AA seen in p53<sup>-/-;NR<sup>tamo mice showed only low levels of Ki-67, Nestin, VEGF, p-Akt and PDGFRα (Fig. 2A, 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<sup>-/-;p16<sup>ink4a/p19<sup>Arf-/-;NR<sup>tamo mice, but not in the AA of p53<sup>-/-;NR<sup>tamo mice (Fig. 2C). Since it has been demonstrated 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 tumor neurospheres (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 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 $p53^{+/+};Ink4a/Arf^{+/+};NR^{-tamo}$ mice and examined TNS formation. Nkx2.2 overexpression
markedly reduced both the numbers of TNSs formed and their expression of Nestin (Fig. 3A, B). Notably, cells within these TNSs showed increased expression of the oligodendrocyte markers, NG2, O4 and MBP. These data indicate that Nkx2.2 can induce oligodendroglial differentiation of GICs, decreasing their self-renewal capacity (Figure 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 (10/10) 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<sup>Ink4a</sup>/p19<sup>Arf</sup>-/- NPCs expressing EGFRvIII plus Nkx2.2 shRNA, mouse survival was significantly shorter than that of recipients that received p16<sup>Ink4a</sup>/p19<sup>Arf</sup>-/- NPCs expressing EGFRvIII plus control shRNA (Fig. 3D). Histological 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 oligodendrogial 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 (AOs). 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, that 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 (Fig. 5D).

To examine whether NKX2.2 is directly regulated by TGFβ or BMP signal, which is involved in maintenance of GICs (17, 28, 29), we examined the effects of the TGFβ inhibitor (SB431542, 1 μM) and BMP4 (100 nM) 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.
Discussion

Here, we have established mouse models harboring specific mutations that allow us to control stages of malignant glioma progression (AA vs. 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 demonstrated in poorly differentiated tumors, based on histological 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 demonstrate that genetic loss of p53 or p16INK4a/p19ARF enhances stemness. For example, p53 or p16INK4a/p19ARF 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 p16INK4a/p19ARF function as barriers to cell reprogramming and acquisition of stemness. Indeed, p53/p16INK4a/p19ARF-deficient multipotent hematopoietic progenitors exhibit properties of hematopoietic stem cells, which can carry out long-term...
reconstitution of blood cells (36). Thus, p53 and p16\textsuperscript{Ink4a/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). Co-expression of Olig2 and Nkx2.2 in spinal cord (26) or in the ventricular and subventricular zones 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 myelin basic protein-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 neural stem cells (46, 47), data regarding the role of Nkx2.2 role in
gliomagenesis is 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 et al. previously reported that NKX2.2 was expressed at higher levels in human oligodendrogliomas than in AA and GBMs (48), suggesting that NKX2.2 is a marker that distinguishes oligodendrogliomas 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 oligodendroglioma 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 demonstrates 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, since 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, re-activation of Nkx2.2 expression in glioma cells suggests a novel therapeutic strategy.

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.
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Figure Legends.

**Figure 1. p53 plus p16<sup> Ink4a</sup>/p19<sup>Arf</sup> 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,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.

**Figure 2. Enhancement of stem cell properties follows malignant glioma progression.**

(A) Sections of AAs (grade III) from p53<sup>−/−</sup>;NR<sup>+</sup>tamo mice and GBMs (grade IV) from p53<sup>−/−</sup>; p16<sup>Ink4a</sup>/p19<sup>Arf</sup><sup>−/−</sup>;NR<sup>+</sup>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, Case1,2) and GBMs (Grade IV, Case3,4) are shown. Scale bars, 100 μm. Data shown are the mean number ± s.d. 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<sup>−/−</sup>;NR<sup>+</sup>tamo mice and GBMs (grade IV) from p53<sup>−/−</sup>; p16<sup>Ink4a</sup>/p19<sup>Arf</sup><sup>−/−</sup>;NR<sup>+</sup>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. For A, B and D, results shown are representative of 5 mice/group and 5 experiments.

**Figure 3. Critical roles of Nkx2.2 in mouse gliomagenesis.**

(A) Nkx2.2 overexpression decreases TNS formation. Primary mouse glioma cells isolated from p53<sup>−/−</sup>; p16<sup>Ink4a</sup>/p19<sup>Arf</sup><sup>−/−</sup>;NR<sup>+</sup>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 ± s.d. of TNSs generated per 2,000 cells (n=5/group). *P<0.001. (B) Decreased Nestin but increased NG2,
O4 and MBP expression. TNSs were cultured on coverslips, infected with Vector- or Nkx2.2-expressing retrovirus, selected with blasticidin-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 ± s.d. 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 p16Ink4a/p19Arf⁻/⁻ 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 ± s.d. of Nkx2.2 mRNA relative to β-actin (n=3/group). (right) Decreased mouse survival. SVZ cells derived from neonatal p16Ink4a/p19Arf⁻/⁻ 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.

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).

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 blasticidin-S for 4 days (left). Scale bars, 100 µm. (right) Data shown are the mean number ± s.d. 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, 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 ± s.d. of Nestin⁺ or O4⁺ cells among TOTO3⁺ cells (n=5/group). *P<0.001.
NKX2.2 suppresses self renewal of glioma-initiating cells

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