Gliomagenesis arising from Pten and Ink4a/Arf deficient neural progenitor cells is mediated by the p53-Fbxw7/Cdc4 pathway which controls c-Myc

Hong Sug Kim¹, Kevin Woolard¹, Chen Lai, Peter O. Bauer, Dragana Maric, Hua Song, Aiguo Li, Svetlana Kotliarova, Wei Zhang, Howard A Fine*.

Neuro-Oncology Branch, National Cancer Institute, National Institutes of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892, USA

¹ These authors contributed equally to this work

Correspondence: hfine@mail.nih.gov or Howard.Fine@nyumc.org

Anne Murnick Cogan & David H. Cogan Professor of Oncology, NYU Cancer Institute

The New York University (NYU) Langone Medical Center

522 First Avenue, Smilow 1101

New York, NY. 10016

(P) 212-263-9221

(F) 212-263-9210

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common type of primary malignant brain tumor and may arise from a cell with neural stem-like properties. Deregulation of the RB, PI3K and p53 pathways are molecular hallmarks of this disease. Recent work has demonstrated that $p53^{-/-}Pten^{-/-}$ mice form gliomas in a c-Myc dependent manner. To explore the role of the INK4A/ARF locus and Pten deletions in gliomagenesis, we generated $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs and such cells were highly proliferative, self-renewing, relatively refractory to differentiation and induced both low and high grade glioma formation in vivo. In contrast to $p53^{-/-}Pten^{-/-}$ mNSCs, however, $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs do not express appreciable levels of c-Myc in vitro although glioma stem cells (GSCs) derived from these same cells did. Sequencing of $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs-derived tumors revealed spontaneous mutations in $Tp53$ in vivo with subsequent downregulation of $Fbxw7$. Expression of p53 mutants in $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs, or knockdown of Fbxw7, resulted in re-expression of c-Myc with enhanced $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs tumorigenicity. We propose that p53 mutations contribute to gliomagenesis by both allowing the overexpression of c-Myc through downregulation of Fbxw7 and by protecting against c-Myc-induced apoptosis.
INTRODUCTION

Glioblastoma multiforme (GBM) is the most common type of primary malignant brain tumor and one of the most lethal of all cancers and despite advances in neurosurgery, radiotherapy and chemotherapy, still have an overall poor prognosis. Patients with GBM have a median survival of less than 18 months and there are few if any long-term survivors despite aggressive multidisciplinary therapy (1). The majority of cases (> 90%) are primary GBMs, which are genetically characterized by loss of heterozygosity on chromosome 10q (70%), EGFR amplification (36%), p16<sup>INK4A</sup> deletion (31%), and Pten mutation (25%). TP53 mutations are also frequently found in primary GBM and even more frequently in low-grade gliomas that progress to secondary GBMs (2). These glioma-related genetic alterations deregulate growth factor receptor signaling (EGFR, PDGFR, PTEN) disrupting both the cell cycle and normal regulation of apoptosis (INK4A, CDK4, RB, TP53) (3).

The role of several key genes within the RB, p53 and PI3K pathways to gliomagenesis has been investigated using various genetic mouse models. Mice lacking Pten exhibited enlarged, histoarchitecturally abnormal brains resulting from increased cell proliferation, cell size, and reduced cell death in the Pten<sup>−/−</sup> stem/progenitor cell compartment (4, 5, 6). For example, the combination of Ink4a/Arf ablation and expression of constitutive active EGFR in mature astrocytes resulted in the formation of glioma-like lesions following intracranial transplantation in mice (7). Trp53 and Nf1 double mutant mice generated gliomas of various stages ranging from low-grade astrocytoma to GBM (8). Mouse strains with germline or somatic heterozygous mutations of the Trp53, Nf1, and Pten developed high-grade astrocytomomas (9, 10, 11). Despite these important studies, the
precise role and contribution of each of these individual genetic aberrations leading to glioma initiation and progression remains imprecisely understood.

The cancer stem cell hypothesis posits that tumorigenic potential is largely restricted to a subset of self-renewing tumor cells with stem cell-like properties designated as tumor stem cells (TSCs) or tumor-initiating cells (TICs) (12, 13, 14, 15). The relatively recent identification of neural stem/progenitor cells (NSCs) within the brains of adult mammals presents an attractive theoretical and experimental target cells for acquisition of glioma-causing mutations. Consistent with such a possibility, investigation of tumor development in mouse models suggests that the tumors often arise in the subventricular zone (SVZ) where adult NSC reside (9, 10, 11, 16). Despite deletions and mutations in the *Pten* and *Ink4a/Arf* being two of the most frequent findings in human GBMs, combined knockout mouse models have not been established and thus the mechanistic outcome of such combined deletions within the context of a NSC remains unclear. To examine whether mNSCs derived from *Pten*<sup>loxP/loxP</sup>*Ink4a/Arf<sup>-/-</sup> mice facilitate gliomagenesis, we established cells derived from the forebrain of *Pten*<sup>loxP/loxP</sup>*Ink4a/Arf<sup>-/-</sup> embryos. Here, we characterize *Pten<sup>-/-</sup>Ink4a/Arf<sup>-/-</sup> mNSCs and demonstrate that these cells possess increased self-renewal potential and maintain an undifferentiated state compared to WT mNSCs counterparts yet do not overexpress c-Myc <em>in vitro</em>. Despite the lack of c-Myc activity <em>in vitro</em>, *Pten<sup>-/-</sup>*<em>Ink4a/Arf<sup>-/-</sup></em> mNSCs give rise to both low- and high-grade astrocytoma tumors, including GBM, when orthotopically implanted in mice. Finally, we demonstrate that expression of *Fbxw7* in the *Pten<sup>-/-</sup>*<em>Ink4a/Arf<sup>-/-</sup></em> mNSCs is responsible for the post-translational downregulation of c-Myc <em>in vitro</em>. These cells, however, ultimately accumulate c-Myc protein and become tumorigenic GSCs <em>in vivo</em> through spontaneously occurring
mutations in TP53 with subsequent downregulation of Fbxw7. These studies demonstrate that inactivation of p53-Fbxw7 pathway with subsequent derepression of c-Myc expression is crucial for the development of gliomas that originate from Pten−/−Ink4a/Arf−/− neural stem cells.
MATERIALS AND METHODS

Mice

$Pten^{loxP/loxP}$; $Ink4a/Arf^{-/-}$ mice were generated by breeding $Ink4a/Arf^{-/-}$ (FVB.129-
$Cdkn2a^{tm1Rdp}$, NCI-Frederick) (34) with $Pten^{loxP/loxP}$ (C; 129S4-Pten$^{tm1Hwu}$/J, The Jackson
Laboratory) (6). Mice were interbred and maintained on the FvB/C129 hybrid
background in pathogen-free conditions at the National Institution of Health, monitored
for signs of health poor every other day, and euthanized when moribund. $Pten$ and
$Ink4a/Arf$ alleles were examined using genotyping primers. All manipulations were
performed with the Institutional Animal Care and Use Committee (IACUC approval.

Cell Culture

mNSCs were isolated from the forebrain of E14 mouse embryos and GSCs were isolated
from the tumor mass of injected SCID mice brain (Supplementary Fig. S1A). mNSCs and
GSCs were cultured in NBE media consisting of neurobasal media (Invitrogen), N2 and
B27 supplements ($0.5 \times$ each; Invitrogen), and human recombinant bFGF with EGF (25
ng/ml each; R&D Systems). For differentiation studies, cells were cultured in NBE media
with 1% FBS (Invitrogen) in poly-Ornithine coated plates during 7 days.

Histology and Immunohistochemical Staining

Once euthanized, mice were perfused with 4% PFA and brains were dissected, followed
by overnight post-fixation in 4% PFA at 4 °C. 5 µm paraffin serial sections were
prepared and stained with haematoxylin and eosin (Histo Serve, Inc). Tumour grading
was determined on the basis of the WHO grading system. The immunohistochemical
staining was performed using the Dako Cytomation EnVision doublestain system (K1395, Dako) following manufacturer's instructions. Images were captured using a Leica DM1400B microsystem and Leica FW4000 version 1.2.1.

**Intracranial mNSCs and GSCs Injection into SCID Mice**

An intracranial orthotopic model was utilized for evaluation of mNSCs and GSCs tumorigenicity according to an approved animal study proposal by NIH-ACUC. mNSCs and GSCs were resuspended in 2 µl of HBSS and injected stereotactically into the striatum of female SCID mice (6-8 week age) using a stereotactic device (coordinates, 2 mm anterior, 2 mm lateral, 2.5 mm depth from the dura). Following injection, animals were followed up daily for the development of neurological deficits.

**Statistical Analysis**

Student’s t test was used for data analysis and a $p$ value $\leq 0.05$ was considered significant. All values are shown as mean ± standard deviation (SD). Kaplan-Meier survival analysis was performed in Prism 4.0 software.
RESULTS

Generation and characterization of Pten⁻⁻ Ink4a/Arf⁻⁻ mNSCs.

PTEN negatively regulates neural stem cell proliferation by controlling cell cycle and apoptosis whereas Ink4a/Arf deficiency is associated with an increased incidence of spontaneous and carcinogen-induced cancers in mice (6, 17, 18). To evaluate the effects of Pten- and/or Ink4a/Arf- deficiency specifically within mNSCs, we generated PtenloxP/loxPInk4a/Arf⁻⁻ mice by crossing the PtenloxP/loxP and Ink4a/Arf⁻⁻ mouse strains. mNSCs were isolated from the forebrain of E14 embryos and cultured in NBE media (13, 19). PtenloxP/loxPInk4a/Arf⁻⁻ and PtenloxP/loxP mNSCs were transduced with a Cre adenovirus to delete the Pten locus. Ablation of Pten and Ink4a/Arf genes was confirmed by genomic PCR (Fig. 1A and Supplementary Fig.S1). Pten⁻⁻ and Pten⁻⁻ Ink4a/Arf⁻⁻ mNSCs demonstrated increased phosphorylation of AKT and S6 (Fig. 1B). Additionally, Pten⁻⁻ and Pten⁻⁻ Ink4a/Arf⁻⁻ mNSCs exhibited increased proliferative activity (Fig. 1C) and self-renewal capability (Fig. 1D; Supplementary Fig.S1B). By contrast, the Ink4a/Arf⁻⁻ mNSCs displayed proliferation and self-renewal activities similar to WT mNSCs (Supplementary Fig.S1B). These results reveal that Pten deletion expands mNSCs proliferative and self-renewal capacity in vitro in either a WT or Ink4a/Arf⁻⁻ background, but that the Ink4a/Arf deletion alone does not, highlighting the crucial gatekeeper role of Pten in mNSCs.

Pten⁻⁻ Ink4a/Arf⁻⁻ mNSCs generate gliomas in vivo
To examine whether Pten and/or Ink4a/Arf deletion in mNSCs are sufficient to induce glioma formation in vivo, we injected Ink4a/Arf⁻/⁻, Pten⁻/⁻, and Pten⁻/⁻ Ink4a/Arf⁻/⁻ mNSCs (5 X 10⁵ cells per mouse) into the SCID mice. To minimize any potential in vitro artifact, early passages (less than passage 5) mNSCs were injected. As both Pten⁻/⁻ and Pten⁻/⁻ Ink4a/Arf⁻/⁻ mNSCs exhibit similar self-renewal capacity and altered signaling pathways in vitro, we expected that both would be able to induce gliomas (Fig. 1C and 1D). Interestingly, however, only Pten⁻/⁻ Ink4a/Arf⁻/⁻ mNSCs were able to form tumors and did so with 100% penetrance and with a median survival time of 157 days (Fig. 2A). By contrast, Pten⁻/⁻ mNSCs, Ink4a/Arf⁻/⁻ mNSCs and WT mNSCs failed to form tumors in vivo. Pathological examination of the Pten⁻/⁻ Ink4a/Arf⁻/⁻ mNSCs-generated tumors revealed gliomas of varied histologic grades including infiltrating astrocytomas (grade II), anaplastic astrocytomas (grade III) and GBMs (grade IV) (Fig. 2B and Supplementary Table. S1).

Hematoxylin and eosin (H&E) staining of the high-grade tumors demonstrated the salient features of human glioma, including nuclear atypia, vascular proliferation, and necrosis (Fig. 2C). All tumor cells showed high expression of markers associated with human gliomas (GFAP, EGFR and PDGFRA), neural stem cells (Nestin) and proliferation (Ki-67). Consistent with the derivation of these tumors exclusively from Pten⁻/⁻ Ink4a/Arf⁻/⁻ mNSCs, all tumor cells were Pten negative by immunohistochemistry whereas host physiologic astrocytes and vascular cells were Pten positive (Fig. 2D). This pattern indicates that tumors derived from Pten⁻/⁻ Ink4a/Arf⁻/⁻ mNSCs are highly proliferative and maintain features associated with both neural stem cells and glioma. Thus, Pten⁻/⁻ Ink4a/Arf⁻/⁻ mNSCs is capable of developing heterogeneous glioma.
xenografts of varying histologic grades demonstrating key phenotypic features associated with spontaneous human gliomas. In addition, the restriction of tumorigenic capacity to $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs and not to $Pten^{-/-}$ or $Ink4a/Arf^{-/-}$ mNSCs further highlights the necessity of inactivating both rather than any one of these pathways for glioma formation.

**Histologic Grade Influences Heterogeneous GSC Behavior in vitro**

We next sought to determine the tumor forming capacity of GSCs derived from the different low- and high-grade xenograft tumors from the $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs. We isolated the GSCs from mice bearing intracerebral xenograft tumors of grade II, III, and IV, and expanded them in NBE media. Hereafter, cells derived from grades II, III and IV gliomas are designated as “T1”, “T2” and “T3”, respectively. We injected these xenograft derived glioma cells (2.5 X $10^5$ cells per mouse) into the periventricular cortex of adult SCID mice. As expected, GSCs isolated from all tumor grades resulted in secondary tumors in 100 % of mice injected. Interestingly, GSCs isolated from primary xenografts with varying histologic grades induced secondary xenograft gliomas with remarkably different survival dates (Fig. 3A). Kaplan–Meier survival data showed a stepwise progression in malignancy with median survival times of 131, 74.5, and 21 days for T1, T2, and T3 GSCs, respectively. Thus, while T1 GSCs formed slightly more aggressive tumors than the original $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs (131 days versus 157 days), both T2 and T3 were significantly more aggressive than the primary high-grade xenografts they were derived from. All T3- injected mice developed tumors with phenotypic features characteristic of human GBM including marked brain infiltration, large regions of
necrosis, and hemorrhage (Supplementary Fig. S2A). These data suggest that the heterogeneity of histologic grades in xenograft tumors derived from $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs is reflected in biologically relevant and distinct populations of GSCs, with xenograft latency inversely related to histologic grade from the tumor they were derived from. Furthermore, serial GSC orthotopic transplantation selects for increasingly more malignant GSC subpopulations.

Next, we characterized the T1, T2, and T3 GSCs in vitro. To compare the differentiation potential of all GSCs with their parental $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs and WT mNSCs, cells were cultured in FBS media (Fig. 3B). In NBE media, GSCs and mNSCs highly express markers associated with neural stem cells (Nestin and Sox2) and minimally express markers associated with differentiation of astrocyte (GFAP) and neuron (TuJ1). By contrast, culturing cells in the presence of FBS resulted in downregulation of nestin and sox2 along with upregulation of GFAP to a much greater extent in WT mNSCs than in either $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs or the GSCs. Additionally, we analyzed the expression of the CD133 and CD15 surface markers that have been associated with the enrichment for GSC tumorigenic potential (15, 20). When compared to either WT mNSCs, $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs or grade-II derived T1 GSCs, both T2 and T3 GSCs demonstrated an increased CD15-positive (CD15$^+$) population of cells (Fig. 3C), consistent with their accelerated tumorigenic potential. All GSCs and $Pten^{-/-}Ink4a/Arf^{-/-}$ mNSCs expressed barely detectable levels of CD133-positive (CD133$^+$) cells (less than 2%) (Fig. 3D and Supplementary Fig.S2B). In a manner consistent with histologic grade, T3 GSCs exhibited the highest level of proliferative activity whereas T2 and T3 GSCs had significantly greater self-renewal capability than T1 GSCs (Fig. 3E and 3F). These results
demonstrated that GSCs derived from Pten^−/− Ink4a/Arf^−/− mNSCs represent a poorly differentiated, population of cells whose self-renewal and tumorigenic potential increases with the histological grade of the tumor from which they were derived.

c-Myc protein is downregulated in Pten^−/− Ink4a/Arf^−/− mNSCs in vitro but upregulated in xenograft-derived GSCs.

c-Myc is an important transcription factor for the induction and maintenance of iPSCs, ES, neural stem cells and cancer stem cells with its depletion resulting in a loss of self-renewal capacity and the induction of differentiation (16, 21, 22, 23). Furthermore, high c-Myc activity has been shown to be particularly important in glioma biology and was recently demonstrated to be required for p53^−/− Pten^−/− mNSCs mediated gliomagenesis (16). Since Pten^−/− Ink4a/Arf^−/− mNSCs demonstrated increased self-renewal, proliferation, and poor differentiation potential, similar to GSCs and p53^−/− Pten^−/− mNSCs, we investigated whether c-Myc was also increased in Pten^−/− Ink4a/Arf^−/− mNSCs. Surprisingly, Pten^−/− Ink4a/Arf^−/− mNSCs expressed slightly lower levels of c-Myc protein than WT, Pten^−/− and Ink4a/Arf^−/− mNSCs (Supplementary Fig. 3A). More impressively, c-Myc expression level in Pten^−/− Ink4a/Arf^−/− mNSCs was dramatically lower than it was in the GSCs (Fig. 4A). To measure c-Myc activity, we transfected cells with a luciferase c-Myc reporter construct. Consistent with the protein expression, Pten^−/− Ink4a/Arf^−/− mNSCs expressed significantly decreased luciferase activity compared to WT mNSCs (0.28 versus 1). Additionally, GSCs had significantly increased luciferase activity (more than 15 folds) compared to WT mNSCs (Fig. 4B). Consistent with these data, Western blot
analysis demonstrated significantly decreased phospho-S62 c-Myc in \(Pten^{-/-}\) mNSCs and increased phospho-S62 c-Myc in WT mNSCs and especially in GSCs (Supplementary Fig. 3B). Additionally, ubiquitin pulldown assay revealed c-Myc ubiquitination was significantly higher in \(Pten^{-/-}\) mNSCs than in either WT or GSCs whereas c-myc total protein levels became similar in all cell types following treatment with ALLN (Fig. 4C and supplementary Fig. 3C). Nevertheless, despite the significant difference in c-Myc protein expression and activity between \(Pten^{-/-}\) mNSCs, WT mNSCs, and GSCs, there was no significant difference in total c-Myc mRNA expression (Fig. 4D). These data suggested that the difference in c-Myc protein expression and functional activity between \(Pten^{-/-}\) mNSCs, WT mNSC, and GSCs was likely due to post-translational regulation.

**P53-Fbxw7 pathway suppresses c-Myc expression in \(Pten^{-/-}\) mNSCs and is inactivated in gliomas**

We have demonstrated that \(Pten^{-/-}\) mNSCs express decreased levels of c-Myc compared to WT mNSCs through post-translational regulation (Fig. 4 and Supplementary Fig. S3). These data led us to further investigate genes involved in c-Myc regulation. Fbxw7 has been shown to interact with c-Myc and mediate its ubiquitination in a Thr\(^{58}\)-phosphorylation-dependent manner. Fbxw7 is a p53-dependent tumor suppressor and its activation by p53 leads to ubiquitination-mediated suppression of several oncoproteins (24, 25, 26). Hence, we hypothesized that the p53-Fbxw7 pathway might be involved in the suppression of c-Myc expression in \(Pten^{-/-}\) mNSCs \textit{in vitro} and
gliomagenesis in vivo. Endogenous Fbxw7 mRNA expression and protein levels were increased in vitro in Pten\(^{--}\)/Ink4a/Arf\(^{--}\)mNSCs compared to WT mNSCs and decreased in GSCs suggesting an inverse relationship between Fbxw7 and c-Myc protein expression (Fig. 5A and 5B). Knockdown of Fbxw7 resulted in increased c-Myc expression in Pten\(^{--}\)/Ink4a/Arf\(^{--}\)mNSCs and overexpressed Fbxw7 resulted in decreased c-Myc expression in T3 GSCs in vitro (Fig. 5C and 5D). We analyzed several substrates of Fbxw7 and found that c-Jun and Aurora A are increased in GSCs although Cyclin E was not (Supplementary Fig.S4). These data suggest that Fbxw7 suppresses c-Myc in Pten\(^{--}\)/Ink4a/Arf\(^{--}\)mNSCs in vitro, an effect that is relieved in GSCs in vivo.

Since Fbxw7 is a p53 target gene, we next asked whether the decreased Fbxw7 expression in our GSCs correlated with their p53 status. Interestingly, while WT p53 expression is low in Pten\(^{--}\)/Ink4a/Arf\(^{--}\)mNSCs, GSCs expressed much higher levels of the p53 protein (Fig. 6A). Furthermore, Pten\(^{--}\)/Ink4a/Arf\(^{--}\)mNSCs-derived xenograft tumors demonstrated high levels of p53 protein expression within the nuclei of all xenograft tumor cells (Fig. 6B). Since high basal levels of p53 protein are commonly seen when p53 is mutated (27), we sequenced the Tp53 gene in our Pten\(^{--}\)/Ink4a/Arf\(^{--}\)mNSCs generated xenograft tumors. Whereas the p53 sequence was wildtype in Pten\(^{--}\)/Ink4a/Arf\(^{--}\)mNSCs, we identified one of two nonsynonymous point mutations in the p53 gene in each of the xenograft tumors. Eleven tumors had the C132W (C135W in human p53) mutation and three tumors revealed the R270C (R273C in human p53) mutation (Fig. 6C and Supplementary Table S2). Each of these mutations has been previously found in primary human gliomas and both are located in the DNA binding domain of p53 (http://p53.free.fr/Database/p53_cancer/p53_brain.ed by html).
To better understand the relevance of p53 status to the expression of c-Myc, we performed knockdown of p53 or overexpression of either the C132W or the R270C mutation in Pten\(^{-/-}\)Ink4a/Arf\(^{-/-}\)mNSCs. Both p53 knockdown and overexpression of the p53 mutants resulted in decreased Fbxw7 mRNA and protein expression in vitro while increasing c-Myc protein expression with little effect on c-Myc mRNA levels (Fig. 6D and 6E and 6F and 6G). These data suggest that p53 regulates c-Myc protein levels through Fbxw7 and wildtype p53 function needs to be deregulated to allow accumulation of c-Myc which is necessary for gliomagenesis.

**P53-Fbxw7 pathway regulates c-Myc induced apoptosis and gliomagenesis**

It has been previously demonstrated that ectopic expression of c-Myc induces apoptosis in fibroblasts in the absence of growth factors (28), in MEFs (29) and in U87MG glioma cell lines following GSK inhibition (30). We, therefore, hypothesized that elevated c-Myc induces p53-dependent apoptosis in Pten\(^{-/-}\)Ink4a/Arf\(^{-/-}\)mNSCs, an affect that is suppressed in GSCs with p53 mutations. To test this hypothesis, we overexpressed c-Myc or knocked-down Fbxw7 (see Fig. 5C), both of which resulted in elevated c-Myc followed by increased cellular apoptosis as demonstrated by elevation of cleaved caspase 3 (Fig. 7A and 7B and 7D). By contrast, overexpression of the GSC-derived C132W and R270C p53 mutants in Pten\(^{-/-}\)Ink4a/Arf\(^{-/-}\)mNSCs resulted in increased c-Myc but little apoptosis (Fig. 7C and 7D). These data suggest that elevated c-Myc fails to induce p53-dependent apoptosis in GSCs by inactivation of the p53-Fbxw7 pathway.
Finally, to evaluate the effects of Fbxw7 expression on p53 mutant GSCs, we stably overexpressed Fbxw7 in T3 GSCs and injected them (1 X10^5 per mouse) into the periventricular cortex of adult female SCID mice. As can be seen in Figure 7E, mice inoculated with Fbxw7 overexpressing T3 GSCs lived significantly longer than control T3 GSC inoculated animals (Median survival is 39 days for Fbxw7 and 23 days for control) (Fig. 7E). These data demonstrate that the p53-Fbxw7 pathway is a key regulatory mechanism for controlling c-Myc induced apoptosis and gliomagenesis.
DISCUSSION

In this study, we demonstrate that Pten−/−Ink4a/Arf−/− mNSCs exhibit GSC features and induce gliomas in adult SCID mice with 100% penetrance. Like human GSCs, Pten−/−Ink4a/Arf−/−mNSCs display poor differentiation relative to WT mNSCs, possess increased self-renewal and proliferative properties, and are capable of forming a heterogeneous range of glial tumors including both low and high grade astrocytomas. Additionally, we found inactivation of the p53-Fbxw7 pathway during gliomagenesis results in elevated c-Myc expression necessary for gliomagenesis.

High throughput genomic data have demonstrated a wide array of genetic and epigenetic alterations within the genomic landscape of GBM. These alterations can differ significantly from one tumor to another, however, many of these alterations ultimately converge to deregulate three signaling pathways; the PI3K, RB and p53 pathways. Disruption of any single pathway has not proven to be sufficient to generate gliomas in the mouse system. For instance, although Ink4a/Arf null and p53 null mice each are at higher risk of tumor formation, gliomas are rarely seen and Pten null mice are fail to generate gliomas. By contrast to single pathway disruption, perturbations of combinations of pathways have generated gliomagenic phenotypes. For example, mice with mutations in Trp53 and Nf1 as well as mice strains with germ line or somatic heterozygous mutations of Trp53, Nf1, and Pten develop high-grade astrocytomas (8, 9,10, 11). Likewise, Zheng and co-workers demonstrated that p53−/−Pten−/− mNSCs are capable of forming gliomas dependent on c-Myc expression (16). These observations are consistent with previous data showing that although the c-Myc gene is rarely amplified or mutated in gliomas, high c-Myc activity is commonly observed. In human gliomas, c-Myc is
expressed in more than 75% of gliomas and the level of expression correlates with the grade of malignancy (31).

Despite the fact that deletions and mutations in the \textit{Pten} and \textit{Ink4a/Arf} genes are two of the most frequent findings in human GBMs, combined knockout mouse models have not been characterized and thus the mechanistic outcome of such combined deletions within the context of a NSC remains unclear. We had initially hypothesized that the phenotype and tumorigenic properties of \textit{Pten}\textsuperscript{−/−}\textit{Ink4a/Arf}\textsuperscript{−/−} mNSCs would be similar to those of the \textit{p53}\textsuperscript{−/−}\textit{Pten}\textsuperscript{−/−} mNSCs. We had assumed, however, that \textit{Pten}\textsuperscript{−/−}\textit{Ink4a/Arf}\textsuperscript{−/−} mNSCs would generate an even more aggressive phenotype than the \textit{p53}\textsuperscript{−/−}\textit{Pten}\textsuperscript{−/−} mNSCs given the deregulation of all three GBM-associated signaling pathways with the loss of PTEN, deregulation of the RB pathway through loss of p16\textsuperscript{INK4A} and deregulation of p53 through deletion of p14\textsuperscript{ARF}. To our surprise, however, the phenotype of the \textit{Pten}\textsuperscript{−/−}\textit{Ink4a/Arf}\textsuperscript{−/−} mNSCs was different than that of the \textit{p53}\textsuperscript{−/−}\textit{Pten}\textsuperscript{−/−} mNSCs in a couple of important and unexpected ways. First, in contrast to \textit{p53}\textsuperscript{−/−}\textit{Pten}\textsuperscript{−/−} mNSCs, the \textit{Pten}\textsuperscript{−/−}\textit{Ink4a/Arf}\textsuperscript{−/−} mNSCs expresses very little c-Myc protein with correspondingly low c-Myc activity. Despite previous data demonstrating that down regulation of c-Myc inhibits the tumorigenic capacity of \textit{p53}\textsuperscript{−/−}\textit{Pten}\textsuperscript{−/−} mNSCs, \textit{Pten}\textsuperscript{−/−}\textit{Ink4a/Arf}\textsuperscript{−/−} mNSCs still formed gliomas with 100% penetrance. Unlike \textit{Pten}\textsuperscript{−/−}\textit{Ink4a/Arf}\textsuperscript{−/−} mNSCs, however, GSCs cultured from all of the \textit{Pten}\textsuperscript{−/−}\textit{Ink4a/Arf}\textsuperscript{−/−} mNSCs-generated xenografts expressed very high levels of c-Myc.

Since \textit{c-Myc} mRNA levels were not substantially different between WT mNSCs, \textit{Pten}\textsuperscript{−/−}\textit{Ink4a/Arf}\textsuperscript{−/−} mNSCs and GSCs, we reasoned that the difference in c-Myc protein and activity levels between the cells was primarily post-translationally regulated. Our data suggest that the post-translational regulation of c-Myc expression occurs spontaneously...
in vivo through down-regulation of the Fbxw7. Fbxw7 is a p53-dependent tumor suppressor and its activation by p53 leads to ubiquination-mediated suppression of several oncoproteins functioning in cellular growth and division pathways, including c-Myc, Cyclin E, Notch and c-Jun (24, 25). The dependency of Fbxw7 expression on p53 prompted us to look at the status of p53 in our GSCs compared to Pten⁻/⁻Ink4a/Arf⁻/⁻ mNSCs. We found that the c-Myc-dependent glioma formation in vivo required down-regulation of Fbxw7 that occurred as a consequence of spontaneous mutation of p53. Consistent with the importance of p53 regulation of Fbxw7 in gliomagenesis was the fact the C132W and R270C p53 mutations we saw in the glioma xenografts were ones that resulted in defective Fbxw7 expression and up regulation of c-Myc when transfected into Pten⁻/⁻Ink4a/Arf⁻/⁻ mNSCs (Fig. 6 and 7). Furthermore, restoration of Fbxw7 expression in the xenograft GSCs repressed their tumorigenic potential in vivo, confirming the role of Fbxw7 as a tumor suppressor within GSCs (Fig. 7E). Our data are consistent with results from a conditional knockout Fbxw7 mouse model in which inactivation of Fbxw7 resulted in acute T-cell lymphoblastic leukemia/ lymphomas which was accompanied by c-Myc accumulation (32, 33).

In addition to the inhibition of Fbxw7-mediated degradation of c-Myc, p53 mutations likely also contributed the ability of the Pten⁻/⁻Ink4a/Arf⁻/⁻ mNSCs to tolerate high levels of c-Myc activity through inhibition of p53 dependent oncogene-induced stress-mediated apoptosis. Supportive of this idea is the fact that our numerous attempts to establish Pten⁻/⁻Ink4a/Arf⁻/⁻ mNSCs that overexpress c-Myc failed secondary to significant apoptosis that could be inhibited by the C132W and R270C mutant p53 genes. These findings are consistent with our prior work showing that GSK3 inhibition-mediated hyperactivation of
c-Myc activity causes c-Myc induced apoptosis. Thus, it appears that there exists a range of c-Myc activity in gliomas, high enough to support gliomagenesis, but not so high to induce apoptosis. These data suggest that mutation of p53 contributes to gliomagenesis in part both through up regulation of c-Myc activity and through inhibition of c-Myc-mediated apoptosis.

In summary, our PtenuInk4a/Arf−/− mNSCs model supports the hypothesis that genomic alterations commonly occurring in human glioblastoma, and arising within a mammalian neural stem cell compartment, results in malignant transformation and the formation of glioma stem cells. Our characterization of these cells address novel mechanisms in gliomagenesis concerning the regulation of c-Myc accumulation during oncogenic stress, and the importance of the Ptenu, Ink4a/Arf and p53-Fbxw7 pathway in regulating normal neural stem cell and gliomas. These data support efforts to identify and test novel therapeutic approaches targeting the p53-Fbxw7 and c-Myc related pathways for the treatment of gliomas.
SUPPLEMENTAL INFORMATION

Supplemental information includes supplementary Material and methods, 3 figures and 2 tables.

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FIGURE LEGENDS

Figure 1. *In vitro* characterization of neural stem/progenitor cells (mNSCs). (A) *Pten* and *Ink4a/Arf* alleles were confirmed with genomic DNAs by genotyping and subjected to polymerase chain reaction (PCR)-based assay. Note that “W” designates the *Pten* and *Ink4a/Arf* wild-type allele, “L” the conditional (loxP) allele, “D” the inactivated form of conditional allele after Cre-mediated loxP site recombination, and ”KO” the knockout. (B) Western blot analysis of mNSCs with antibodies against Pten, p-AKT, AKT, p-S6, S6, p16 and Actin. (C) and (D) mNSCs proliferation activity examined by cell counting and limiting dilution assay. Histogram shows number of cells (C) and number of wells with spheres (D). Error bars represent SD (performed in triplicates). *Pten*−/− and *Pten*−/−*Ink4a/Arf*−/− mNSCs increase self-renewal and proliferation activity compared to WT and *Ink4a/Arf*−/− mNSCs.

Figure 2. *Pten*−/−*Ink4a/Arf*−/− mNSCs induce transplantable xenograft gliomas. Kaplan-Meier survival curve of mice after intracranial mNSCs injection. (A) *Pten*−/−*Ink4a/Arf*−/− mNSCs injected mice have shorter survival than WT, *Pten*−/− and *Ink4a/Arf*−/− mNSCs injected mice. Kaplan–Meier survival curves of mice injected with *Pten*−/−*Ink4a/Arf*−/− mNSCs show a median survival time of 157 days (log rank test, p<0.0001). (B) Table shows frequency and histologic grade of gliomas versus non-tumors observed in end point of indicated mice from (A). (C) Representative hematoxylin and eosin (H&E) stained brain sections representing the various grade gliomas formed by the *Pten*−/−
Ink4a/Arf<sup>−/−</sup>mNSCs. Grade III or IV astrocytomas with characteristic features of nuclear atypia, mitoses (arrow), vascular proliferation (*), and necrosis (N) are shown. Scale bar = 100 μm (D) Tumors show no PTEN immunoreactivity and express markers associated with human gliomas including GFAP, EGFR, PDGFRA, Nestin, and Ki-67. Scale bar = 100 μm.

**Figure 3. GSCs induce gliomas with different tumorigenic potential.** (A) GSCs taken from different grade glioma xenografts and transplanted back into mice generate tumors with different levels of aggressiveness as demonstrated by differing median survivals of 131 (T1), 74.5 (T2), and 21 (T3) days, respectively (log rank test, p<0.0001). (B). GSCs, WT and Pten<sup>−/−</sup>Ink4a/Arf<sup>−/−</sup>mNSCs were cultured in NBE medium (top) or 1 % FBS differentiation medium (bottom) and immunostained for Nestin, Sox2, Gfap and TuJ1 as indicated. (magnification X20) (C) and (D) Graphs show percentage of CD15 and CD133 positive cells in GSCs, respectively. CD15, but not CD133, is increased in GSCs and correlates with degree of malignancy as assessed by animal survival time. (E) and (F) GSCs proliferative activity examined by cell counting and limiting dilution assays. T3 had the highest proliferative and self-renewal activities, whereas T1 had the lowest. Histogram shows number of cells (E) (* p<0.05 compared to T1) and number of wells with spheres (F) (* p<0.05 and ** p<0.001 compared to T1). Error bars represent SD (performed in triplicates).
Figure 4. c-Myc expression and activity are decreased in Pten$^{-/-}$Ink4a/Arf$^{-/-}$ mNSCs but highly expressed in GSCs. (A) Western blot analysis shows that Pten$^{-/-}$Ink4a/Arf$^{-/-}$ mNSCs express low levels of c-Myc but much higher levels in GSCs compared to WT mNSCs. (B) A pMyc-luciferase assay shows that c-Myc activity is significantly decreased in Pten$^{-/-}$Ink4a/Arf$^{-/-}$mNSCs and increased in GSCs (**p<0.001). Graph indicates relative Luminescence Units (relative pMyc-luciferase to Renilla-luciferase). (C) Western blot analysis shows that ubiquitinated c-Myc is increased in Pten$^{-/-}$Ink4a/Arf$^{-/-}$mNSCs compared to WT mNSCs with and without ALLN treatment. (D) Quantitative RT-PCR shows that c-Myc mRNA in Pten$^{-/-}$Ink4a/Arf$^{-/-}$mNSCs and GSCs is similar to WT. Graph indicates c-Myc mRNA fold change relative to GAPDH mRNA. Error bars represent SD (performed in triplicates).

Figure 5. Fbxw7 is regulated by p53 and controls c-myc levels. (A) Western blot analysis shows that Fbxw7 is increased in Pten$^{-/-}$Ink4a/Arf$^{-/-}$mNSCs and decreased in GSCs. (B) Quantitative RT-PCR analysis for Fbxw7 demonstrates increased Fbxw7 in Pten$^{-/-}$Ink4a/Arf$^{-/-}$mNSCs compared to GSCs and WT mNSCs (** p<0.001 compared to WT and GSCs). Graph indicates Fbxw7 mRNA fold change relative to GAPDH mRNA. Error bars represent SD (performed in triplicates). (C) Knockdown Fbxw7 by ShRNAs shows c-Myc elevation in Pten$^{-/-}$Ink4a/Arf$^{-/-}$mNSCs by western blot analysis. (D) Overexpression of Fbxw7 represents decreased c-Myc in T3 GSCs.
**Figure 6. p53 inactivation induces c-Myc overexpression.** (A) Western blot analysis shows that p53 expression is significantly decreased in *Pten*<sup>−/−</sup>*Ink4a/Arf*<sup>−/−</sup>mNSCs, but highly expressed in GSCs. (B) *Pten*<sup>−/−</sup>*Ink4a/Arf*<sup>−/−</sup>mNSCs-derived tumors showing that p53 is highly expressed in nuclei of tumor cells. Scale bar = 100 μm (B). (C) Sequence traces show the two different mutations of p53 found in gliomas. Condon positions in p53 are shown at the left. * indicated normal (*left*) and mutated (*right*) nucleotides. Both mutations were located in the DNA binding domain. (D) Western blot analysis shows shRNA-mediated knockdown of p53 expression significantly increased c-Myc and decreased Fbxw7 in *Pten*<sup>−/−</sup>*Ink4a/Arf*<sup>−/−</sup>mNSCs. (E) Overexpressed p53 mutants (C132W and R270C) increased c-Myc and decreased Fbxw7 in *Pten*<sup>−/−</sup>*Ink4a/Arf*<sup>−/−</sup>mNSCs. (F) Quantitative RT-PCR shows that both p53 mutants (C132W and R270C) expressed in *Pten*<sup>−/−</sup>*Ink4a/Arf*<sup>−/−</sup>mNSCs significantly decreased *Fbxw7* transcription; R270C > C132W and control (** p<0.001). (G) Quantitative RT-PCR shows that c-Myc mRNA expression is not changed in *Pten*<sup>−/−</sup>*Ink4a/Arf*<sup>−/−</sup>mNSCs in p53 knockdowns and in both p53 overexpressed mutants. Graph indicates *Fbxw7* (F) and c-Myc (G) mRNA fold change relative to *GAPDH* mRNA. Error bars represent SD (performed in triplicates).

**Figure 7. P53-Fbxw7 pathway regulates c-Myc induced apoptosis and gliomagenesis.** (A) and (B) In *Pten*<sup>−/−</sup>*Ink4a/Arf*<sup>−/−</sup>mNSCs, western blot analysis shows that overexpressed c-Myc and ShRNA-mediated Fbxw7 knockdown resulted in increased cleaved caspase 3. (C) Overexpressed p53 mutants (C132W and R270C) in *Pten*<sup>−/−</sup>*Ink4a/Arf*<sup>−/−</sup>mNSCs decreased cleaved caspase 3 compared to control. (D) Apoptosis rates
are quantified by FACS analysis and graph represents apoptosis ratio of (A) and (B) and (C) (** p<0.001 compared to control). (E) Overexpressed Fbxw7 T3 GSCs (2.5 X 10^5 cells per mouse) intracranially injected mice show suppressed tumorigenesis compared to control T3 injected mice. Kaplan–Meier survival curves of mice injected cells show a median survival time of 39 days for Fbxw7 and 23 days for control (log rank test, p<0.05).
Kim et al. Fig 4
Gliomagenesis arising from Pten and Ink4a/Arf deficient neural progenitor cells is mediated by the p53-Fbxw7/Cdc4 pathway which controls c-Myc

Hong Sug Kim, Kevin Woolard, Chen Lai, et al.

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