Dedifferentiation of Neurons and Astrocytes by Oncogenes Can Induce Gliomas in Mice

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Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive malignant primary brain tumor in humans. Here, we show that gliomas can originate from differentiated cells in the central nervous system (CNS), including cortical neurons. Transduction by oncogenic lentiviral vectors of neural stem cells (NSCs), astrocytes, or even mature neurons in the brain of mice can give rise to malignant gliomas. All the tumors, irrespective of the site of injection (initiating population), share common features of high expression of stem or progenitor markers and low expression of differentiation markers. Microarray analysis revealed that tumors of astrocytic and neuronal origin match the mesenchymal GBM subtype. We propose that most differentiated cells in the CNS upon defined genetic alterations undergo dedifferentiation to generate a NSC or progenitor state to initiate and maintain the tumor progression, as well as to give rise to the heterogeneous populations observed in malignant gliomas.

Despite progress in genetic analysis and classification of gliomas based on pathology and genomics, the prognosis for patients with brain tumors continues to be very poor (1). One of the reasons for the lack of clinical advances of GBMs for decades has been the insufficient understanding of the underlying mechanisms of progression and recurrence of gliomagenesis.

We have recently used Cre-inducible lentiviral vectors to generate a novel mouse glioma model (2). Here we have expanded the utility of our lentiviral system by generating a new construct that carries two shRNAs: one targeting neurofibromatosis type I gene (NF1: mutated in 18% of GBMs) and the other one targeting p53 (mutated in over 35% of GBMs) (Fig. 1, A and B). It has previously been shown that combined loss of both Nf1 and p53 results in high-grade glioma formation (3, 4). Loss of NF1 leads to increased Ras mitogenic

signaling and augments cell proliferation, while loss of functional p53 induces genomic instability, two important events relevant for tumorigenesis that were part of our rationale for using H-RasV12 and inactivation of p53 in the initial pTomo-lentivector (2).

As shown in Fig. 1C and fig. S1, stereotaxic injection of oncogenic lentivector containing either shNF1-shp53 or H-RasV12-shp53 in the hippocampus of GFAP-Cre mice gives rise to gliomas with similar histological and morphological characteristics. Glial cells (5, 6), oligodendrocyte precursor cells (OPCs) (7, 8) and NSCs (4, 9) have been suggested to be good candidates for the cell of origin of gliomas. Here we show that neurons can also be the target of transformation and generate malignant gliomas. Injections of shNF1-shp53 virus in the cortex of Synapsin I-Cre transgenic mice (SynI-Cre; 8–16 weeks old), which express Cre specifically in neurons but not in glial cells (10), showed the formation of gliomas (Fig. 2A). Since the shRNAs targeting either NF1 or p53 genes are not regulated by Cre (see Fig. 1A), the tumors that we obtained were a mixture of GFP+/RFP- or GFP+RFP+ (due to leakiness from IRES; see arrows in Fig. 2B). Only tumor cells that are GFP+/RFP- are considered to be of neuronal origin, because they are expressing Cre to delete RFP. We extended these results by transducing H-RasV12-p53 vector in the cortex of Syn1-Cre mice. Neurons transduced with this oncogenic vector expressed only GFP, because expression of Ras is regulated by Cre (fig. S2) (10).

Analysis of brain sections five days after the injection of the lentivirus revealed GFP+/RFP- expression specifically in NeuN+ and Tuj1+ cells (see representative images in Fig. 2C and quantification of staining in table S1) and the same specificity was observed when SynI-Cre mice were crossed with a LacZ reporter line (fig. S3A), both results showing that Cre is specifically expressed in terminally differentiated neurons (10). To provide further evidence that mature neurons can be transformed by these oncogene/tumor suppressor genes as observed in vivo, we isolated primary cortical neurons from SynI-Cre mice and transduced them in vitro with shNF1-shp53 virus. The isolated neurons were Map2 +ve (a marker of mature neurons), GFAP −ve, doublecortin (DCX) negative (a neuronal progenitor marker) and Ki67 −ve (marker for cell proliferation) (figs. S4A and S5). The transduced neurons were transplanted into NOD-SCID mice, and the resulting tumors (fig. S4B) exhibited the same histopathology features as those observed with the direct in vivo stereotaxic transductions. Interestingly, these tumors also expressed high levels of progenitor markers Nestin and Sox2 (fig. S4C).

To determine the frequency of the tumor initiating cells in tumors obtained by shNF1-shp53 injections in the cortex of SynI-Cre mice, we dissociated the tumors in single cell suspension and sorted them in two different populations: GFP+/RFP- and GFP+/RFP+ cells. Following limiting dilution analysis we transplanted these cells back into new mice and obtained in both cases and with similar frequencies high grade gliomas (fig. S4D). In culture these cells present all the characteristics of tumor initiating cells (TIC) (fig. S6).

We also used a second transgenic model, CamK2a-Cre mice (11) to target mature neurons with our oncogenic lenti-vectors (figs. S3 and S7). Even though both SynI-Cre and CamK2a-Cre mice were injected using the same virus titer (1×10^9 IU), the later developed tumors with a much longer latency. These results can be explained by the different subtypes of neurons that are targeted using CamK2a-Cre mice, mostly excitatory neurons, which may be either more refractory to transformation, in addition to being less numerous in the cortex (11).

In the central nervous system (CNS) of adult mice, constitutive neurogenesis is observed mainly in two regions - the subventricular zone and the subgranular zone (SGZ) of the hippocampus (1, 12). Glial-lineage progenitors have been found in areas outside the classic

neurogenic regions, including the optic nerve, striatum, hypothalamus, and subcortical white matter, but in these areas the majority of cells expressing GFAP are mostly differentiated astrocytes (13). For the next series of injections we elected to use the oncogenic vector (H-RasV12-shp53) where the oncogene is Cre-inducible (limitation with shNF1-shp53 vector). Furthermore at the gross histological and transcriptional level, the two oncogenic vectors exhibit similar characteristics (Fig. 1 and see later). All transduced GFAP-Cre mice developed tumors when injected either in the hippocampus (HP), subventricular zone, striatum or cortex (CTX) (table S2). When injecting Nestin-Cre mice, tumors were obtained only when the virus was injected in the hippocampus (SGZ) and subventricular zone but not in the cortex or striatum, since Nestin expressing neural progenitor/stem cells are infrequent in these locations (4). Similar results were obtained when Sox-2-Cre mice were injected in the same locations (table S2). Taking advantage of the GFP reporter in our oncogenic vector, we decided to follow the kinetics of expression of some of these markers during tumor development. Five days after injection of the vector in the cortex of 8 weeks old GFAP-Cre mice (Fig. 3A, approximately 60 cells are infected), GFP+/RFP- cells are negative for NeuN, Ki67, Nestin, Olig2, and Rip markers but positive for GFAP, suggesting that glial cells were transduced. Using high-resolution large-scale mosaic images (14) we analyzed the expression of GFAP and Nestin markers at 2, 4 and 8 weeks post-injection of the oncogenic vector either in the cortex (Fig. 3B) or in the hippocampus (fig. S8, A and B) of GFAP-Cre mice. Already at the early stages of cell proliferation (tumor initiation), most of the GFP+ cells tend to loose GFAP expression, and as tumor growth progresses the GFAP expression is progressively diminished (Fig. 3B). On the other hand, Nestin expression that was hardly detectable in the transduced cells, 5 days post-injection (Fig. 3A), increased significantly as tumors developed (Fig. 3B). Most of the cells that express Nestin by 8 weeks post-injection also express Sox2, another progenitor marker (fig. S8C). Using qRT-PCR, we observe an increase of more progenitor/stem cell markers (15) as the tumor progresses and the mice succumb to the disease, and diminished expression of differentiation state markers (16) (fig. S9). We suggest that the mature/differentiated cell initially infected, in the process of transformation, acquires the capacity to dedifferentiate to a cell that has the attributes of a neuroprogenitor cell, which can then not only maintain its pluripotency, but also give rise to the heterogeneous cell populations observed in malignant gliomas (2).

To further confirm this hypothesis and support our in vivo observations, we established primary cortical astrocytes cultures from GFAP-Cre P11 (postnatal day 11) mice. These cells were characterized by immunofluorescent staining and expressed only bona fide markers of astrocytes (s-100β, GFAP+ve, Fig. 4A panels ii–iv) and are Nestin and Sox-2 −ve (Fig. 4A, panel v). Early passage GFAP+ primary astrocytes (that expressed Cre as shown in Fig. 4A, panel vi, and are Ki67 −ve as shown in fig. S5) were transduced in vitro with either shNF1-shp53 or H-RasV12-shp53 virus (fig. S10A, see expression of GFP; efficiency of transduction was usually more than 90%) and transplanted into the brains of NOD-SCID mice (3×10^5 cells per injection). All the transplanted mice developed tumors with similar clinical and histopathological features (Fig. 4B) that were observed in the tumors obtained in the lenti-induced model (Fig. 1). Confocal microscopy analysis of these tumors revealed that the vast majority of the tumor cells (GFP+) express progenitor markers Nestin and Sox2 (fig. S10B) and a few cells express Tuj1 (neuronal marker) (see arrows in Fig. 4B). These findings further support the idea that terminally differentiated astrocytes can give rise to tumors that are composed of some differentiated neurons, thus providing evidence that these astrocytes underwent dedifferentiation (or possibly transdifferentiation) during tumorigenesis. To mimic this dedifferentiation process in vitro, we took both the shNF1-shp53 or H-RasV12-shp53 infected cortical astrocytes that were generated in media containing serum (under this condition the cells maintained their astrocyte identity, see Fig. 4C, panel i) and transferred them to stem cell media devoid of serum and supplemented with

FGF-2. Under these new conditions, the cells started to change morphology, from more elongated shape to contracted cytoplasm and in a time period of approximately one week, in the center of each aggregate of cells a small neurosphere like structure formed and detached from the dish (Fig. 4C, panels ii–iv). These neurospheres when collected from the supernatant, treated with low grade trypsin (TrypLE) and cultured back in new dishes, continue to proliferate. These neurospheres show expression of Nestin and Sox2 (Fig. 4D). Under the same tissue culture conditions, uninfected primary cortical astrocytes never changed their morphology and continue to retain the immunofluorescent markers of fully differentiated astrocytes (fig. S11, A and B). Primary astrocytes infected with a lentivirus containing only the shRNA targeting p53 or expressing only H-RasV12 (fig. S11, C and D) remained astrocytes. Our data support the notion that the cooperation between p53 deficiency and the receptor tyrosine kinase (RTK) signaling pathway (through RAS activation) are both required for the dedifferentiation of astrocytes during tumorigenesis. Similarly, transduced mature neurons in vitro when switched to NSC media supplemented with FGF-2 followed the same dedifferentiation changes as observed in the experiments described above using astrocytes and also form neurosphere-like structures (fig. S12).

We performed qRT-PCR analysis to show that the dedifferentiated GFP+ NSCs acquired the expression of NSC-specific genes and largely lost the astrocyte-specific genes (fig. S13). These GFP+ NSCs also expressed CD133 a marker previously associated with brain cancer stem cells (17) (fig. S14A). More interestingly these cells also expressed CD15, better known as SSEA1, a glycoprotein usually expressed in embryonic stem cells and commonly used for iPS cell characterization (18) (fig. S14A). This was not the only common feature that we found our dedifferentiated GFP+ NSCs share with reprogramming of normal iPS cells; the GFP+ NSCs also express the transcription factors Sox2, c-myc and Nanog and have an open, more relaxed chromatin structure (fig. S14, B and C).

Finally, the human relevance of our findings was supported by mi-croarray analysis which revealed that tumors derived from GFAP-Cre mice injected either in the cortex (CTX), hippocampus (HP) or subventricular zone (SVZ), as well as tumors derived from SynI-Cre mice injected in the CTX exhibit a very strong Mesenchymal molecular subtype signature, while most of the Nestin Cre derived tumors display a Neural subclass (fig. S15, A and B, and database S1). These results support our observations that both astrocytic and neuronal derived tumors not only have overlapping histopathological features but also share the same molecular signature. Our mouse model of GBM resembles the mesenchymal subtype, which is of high interest to the field, since patients having this classification exhibit worse survival and are more resistant to treatment compared to the other groups (19–21). The mesenchymal subtype is reminiscent of a transition that has been linked to dedifferentiated and transdifferentiated tumors (22), while the neural subtype is most similar to normal brain tissue, and its signature reflects a tendency to differentiation phenotype (23). Finally, the TCGA study has revealed that both Mesenchymal and Neural subtypes harbor deletions/ mutations in the NF1 and p53 genes (23) further supporting our findings that H-RasV12-shp53 tumors are representatives of the shNF1-shp53 tumors. Similar results were obtained when our microarray data was compared to the molecular signature identified by Phillips HS *et al.* (20) (fig. S15C).

We have shown that both NSCs residing either in the SVZ or hippocampus (e.g., in Nestin-Cre and Sox2-Cre injected mice) as well as mature neurons and astrocytes can be targets of genetic alterations that can lead to gliomagenesis. Although we have not formally shown using our lentiviral vectors system, it has already been reported that oligodendrocytes progenitor cells can also act as cell of origin for glioma (7, 8). We propose that the genetically altered differentiated cell acquires the capacity to dedifferentiate to a more progenitor (stem cell) state, and that tumor progression probably requires a permissive

microenvironment composed of cell types and molecular signals that can sustain both differentiation of tumor cells as well as maintenance of tumor stem-like cells. Our results offer an explanation of recurrence of gliomas following treatment, because any tumor cell that is not eradicated can continue to proliferate and induce tumor formation, thereby perpetuating the cycle of continuous cell replication to form malignant gliomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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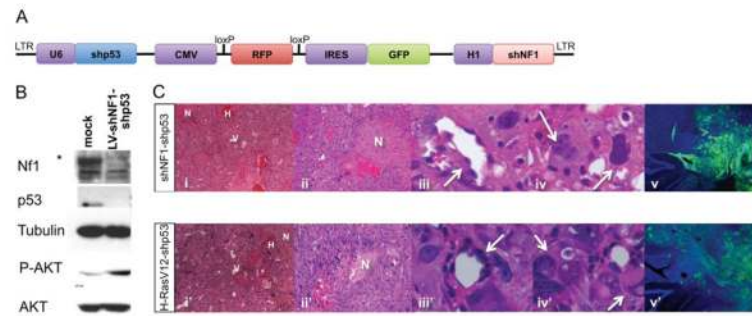
Fig. 1.
Glioblastomas induced by a single lentiviral vector. (**A**) Schematic representation of the lentivector. In the shNF1-shp53 vector, the hairpin targeting NF1 was cloned under the H1 promoter at the 3′ UTR and the hairpin targeting p53 was cloned 5′ to the CMV promoter and under the U6 promoter. (**B**) Western Blot (WB) showing silencing of both NF1 and p53. NF1 loss resulted in increased phosphorylation of Akt (P-Akt); total Akt expression was used as a control. MEF/Ikk2$^{-/-}$ cells were infected with the indicated lentivectors. The cell lysate was collected and analyzed by WB. Tubulin detection was used as a loading control. (**C**) Hematoxylin and eosin (H&E) histology and immunofluorescence of sections from either shNF1-shp53 or H-RasV12-shp53 induced glioblastomas in GFAP-Cre mice (hippocampus injected). Panels i and i′ show images of the tumors (x20) where increased cellularity, vascularity (V), hemorrhage (H) and necrotic areas (N) can be observed. The rest of the panels show all the classical GBM features: necrotic areas (N; panels ii and ii′; x40), perivascular infiltration (indicated by arrows in panels iii and iii′; x40 plus 2 electrical zoom) and multinucleated giant cells (se arrows in panels iv and iv′; x40 plus 2 electrical zoom). Panels v and v′ show by immunofluorescence staining the infiltrative characteristic of the tumor, crossing the midline and migrating to the other hemisphere (blue = DAPI, green = GFP; x5).
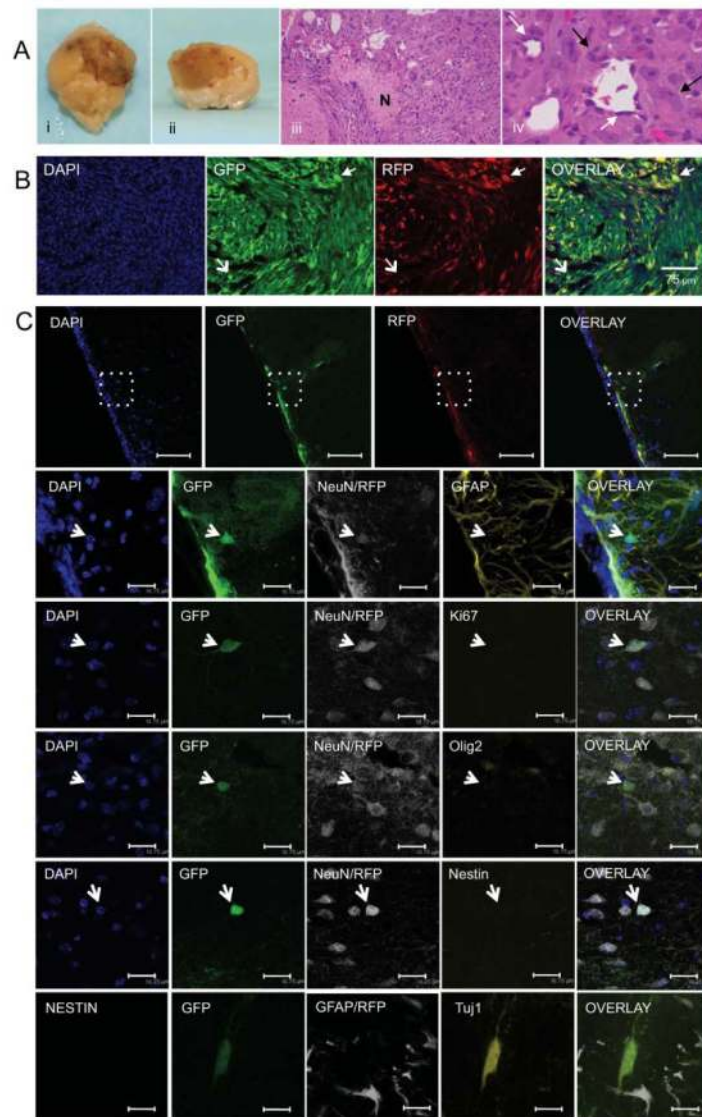
Fig. 2.
Induction of gliomas by shNF1-shp53 lentiviral transduction of neurons. (**A**) Photographs (panels i–ii) showing the massive lesion in the brain and H&E staining of shNF1-shp53 induced tumors in the cortex of SynI-Cre mice (iii, magnification, x40; iv, magnification x40 plus two electrical zoom, showing perivascular infiltration (white arrows) and multinucleated giant cell (black arrows). (**B**) Confocal images showing the presence of both GFP+/RFP− (open arrowhead) and GFP+/RFP+ cells (filled arrowhead) in the tumor. (**C**) Confocal images showing oncogene/tumor suppressor expression specifically in NeuN+ and Tuj1+ cells. Five days after injection of the virus in the cortex of SynI-Cre transgenic mice, brains were collected and fixed. Sections of the brain were stained with the indicated antibodies. The upper panels show representative confocal images of GFP/RFP before antibodies staining (Scale bar = 75 μm). The lower panels show a representative (see arrows) co-labeling using the antibodies/markers indicated in each panel. When possible DAPI staining was also assessed (in blue). Scale bars = 18.75 μm. N = necrosis.
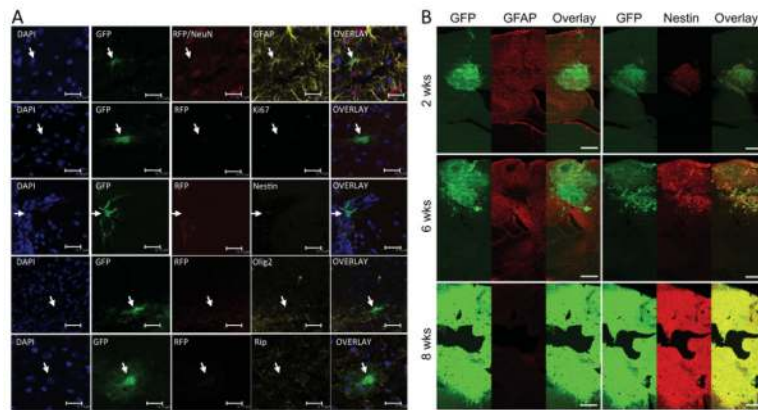
Fig. 3.
Tumorigenesis in the cortex. (**A**) Confocal microscopy analysis of brain sections 5 days after injection of H-RasV12-shp53 virus in the cortex of GFAP-Cre mouse. Panels show representative (see arrows) co-labeling with the markers indicated in each of the images. Scale bars = 18.75 µm. (**B**) Maximum intensity projections of large-scale mosaic volumes. Immunolabeling for GFAP and Nestin in tumors at 2, 6, and 8 weeks following injection of lentivirus in the cortex. GFAP staining intensity is seen to decrease with tumor progression, while Nestin labeling increases. Scale bars = 500 microns.
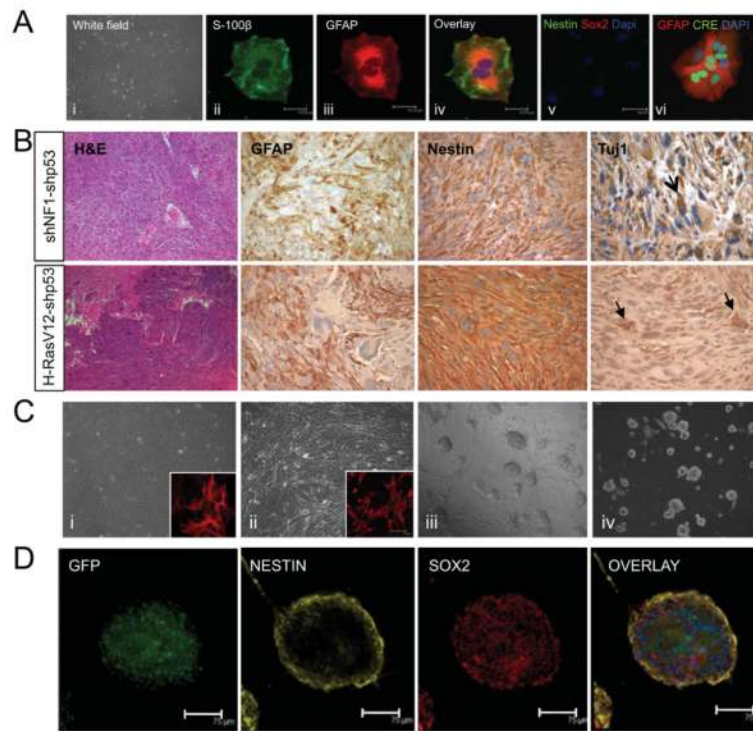
Fig. 4.
Mature astrocytes transduced with either shNF1-shp53 or H-RasV12-shp53 virus dedifferentiate to a neural progenitor/stem cell like state. (**A**) Morphology and staining of cortical astrocytes obtained from GFAP-Cre mice. Confocal microscopy analysis shows double staining for S-100β and GFAP (panels ii–iv), lack of expression of stem/progenitor makers Nestin and Sox2 (panel v) and expression of Cre in all GFAP+ astrocytes (panel vi: Red = GFAP, Green = Cre, Blue = DAPI). (**B**) Tumors derived from either shNF1-shp53 or H-RasV12-shp53 transduced astrocytes orthotopically transplanted into the hippocampus of NOD-SCID mice; H&E histology and immunohistochemistry. Arrows point to representative Tuj1 +ve cells. (**C**) i) astrocytes before transduction maintained in media plus serum express GFAP (inset red = GFAP, scale bar = 75 μm). ii) transduced astrocytes in media plus serum (inset red = GFAP), iii and iv) transduced astrocytes transferred to serum free media supplemented with FGF-2. Light microscopy magnification x20. (**D**) Confocal microscopy analysis of the neurospheres described in (C) panel iv (green = GFP (vector), yellow = Nestin, red = Sox2; scale bar = 75 mu;m).