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Innate neural stem cell heterogeneity determines the patterning of glioma formation in children

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Summary

The concept that gliomas comprise a heterogeneous group of diseases distinguished by their developmental origin raises the intriguing possibility that neural stem cells (NSCs) from different germinal zones have differential capacities to respond to glioma-causing genetic changes. We demonstrate that NSCs of subventricular zone of lateral ventricle are molecularly and functionally distinct from those of the third ventricle. Consistent with a unique origin for pediatric low-grade glioma, third ventricle, but not lateral ventricle, NSCs hyperproliferate in response to mutations characteristic of childhood glioma. Finally, we demonstrate that pediatric optic gliomas in *Nf1* genetically-engineered mice arise from the third ventricle. Collectively, these observations establish the importance of innate brain region NSC heterogeneity in the patterning of gliomagenesis in children and adults.

Keywords

optic glioma; astrocytoma; ventricular zone; stem cell niche; neurofibromatosis type 1; subventricular zone; third ventricle

Introduction

The importance of the cell of origin in tumorigenesis and clinical behavior of brain tumors (Singh et al., 2004; Taylor et al., 2005) has been strengthened by the observation that histologically-identical brain tumors are composed of molecularly-distinct subtypes that reflect their progenitor cell of origin (Gibson et al., 2010; Johnson et al., 2010; Kalamarides et al., 2011; Sharma et al., 2007). These findings suggest that brain tumors with distinct cellular origins are unique diseases with different growth control regulatory networks, genetic changes, and responses to therapy. Consistent with this, we have shown that mouse NSCs from the brainstem, but not the neocortex, exhibit increased proliferation and gliogenesis following inactivation of the neurofibromatosis-1 (NF1) tumor suppressor gene (Lee et al., 2010). This differential sensitivity to *Nf1* loss closely parallels the propensity for pilocytic astrocytomas (PAs) in children with NF1 to form within the optic pathway and brainstem but rarely in the cortex (Guillamo et al., 2003). A similar geographic pattern of

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Accession number of microarray data. Human PA (GSE5675) and mouse NSC (GSE37832) microarray data were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>).

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gliomagenesis is observed for sporadic pediatric PAs harboring *KIAA1549:BRAF* fusions (Jacob et al., 2009), which predominantly form in the cerebellum.

Within the brain, there are several germinal zones potentially germane to brain tumorigenesis, including lv-SVZ, TVZ, and the fourth ventricle (Quinones-Hinojosa et al., 2006; Weiss et al., 1996; Xu et al., 2005). While the lv-SVZ is often considered to be the likely stem cell compartment for cerebral hemisphere glioma formation in mice following the introduction of genetic alterations observed in high-grade human adult gliomas (Alcantara Llaguno et al., 2009; Jacques et al., 2010; Wang et al., 2009), other populations, including NG2⁺ cells (Assanah et al., 2006; Masui et al., 2010) and oligodendrocyte precursors (Liu et al., 2011; Sugiarto et al., 2011) can serve as potential cells of origin for malignant glioma. However, the origin of optic glioma, the second most common low-grade pediatric gliomas, remains unresolved. Based on the proximity of the optic nerve/chiasm to the TVZ and that optic nerve oligodendrocyte precursors can originate from the TVZ (Ono et al., 1997), we hypothesized that TVZ may be the progenitor compartment for these pediatric brain tumors.

Results

NSCs from the lv-SVZ and TVZ are molecularly-distinct populations

We obtain several lines of evidence supporting that TVZ is a true stem cell niche. First, cells lining the TVZ in the embryonic day 15.5 (E15.5) mouse brain express NSC markers Sox2, nestin and BLBP, and exhibit robust proliferation (Ki67 immunostaining) (Figure 1A). Second, TVZ NSCs can generate Sox2⁺, nestin⁺ and BLBP⁺ neurospheres and undergo self-renewal, similar to their lv-SVZ counterparts (Figure 1B). Third, dissociated single cells from TVZ neurospheres differentiate into neurons, astrocytes and oligodendrocytes *in vitro* (Figure 1C).

We then employed gene expression profiling to demonstrate that TVZ NSCs and lv-SVZ NSCs are molecularly distinct populations. Initially, E17.5 TVZ and lv-SVZ NSCs from three females were used for the profiling; however, one outlier lv-SVZ NSC sample, based on principal component analysis (PCA), was eliminated from the following analyses (Figure S1A). Using hierarchical clustering methods, lv-SVZ and TVZ NSCs were easily separable (Figure 2A and S1A). The differential expression of several genes were validated by quantitative RT-PCR (qRT-PCR) (Figure 2B) and by *in situ* hybridization (Allen Brain Atlas at <http://www.brain-map.org/>): *Chl1* and *Slit2* expression was higher in the hypothalamus/TVZ region compared to the anterior forebrain/lv-SVZ region, whereas *Dcx* and *Cntn1* expression was higher in the anterior forebrain/lv-SVZ region relative to the hypothalamus/TVZ region (Figure S1B). A subset of these genes was also similarly differentially expressed in older mice (Figure S1B and C). Together, these data demonstrate that TVZ and lv-SVZ contain molecularly-distinct NSC populations.

lv-SVZ and TVZ NSCs exhibit unique cell-autonomous responses to glioma-causing genetic mutations

To determine whether TVZ and lv-SVZ NSCs exhibit different responses to glioma-associated genetic events, we measured NSC proliferation in response to *KIAA1549:BRAF* expression, a representative pediatric glioma-causing genetic change (Jones et al., 2008), *PTEN* loss, a representative adult glioma-causing genetic change (Pollack et al., 2006), and *p53* loss, which occurs in both adult and pediatric gliomas (Hayes et al., 1999; Kim et al., 2010). The *KIAA1549:BRAF* mutation is found in 62% of hypothalamus/optic pathway PAs, but is uncommon in histologically-identical tumors of the cerebral hemispheres (14%) (Jacob et al., 2009). *p53* inactivation increased proliferation and decreased apoptosis of both

lv-SVZ and TVZ NSCs (Figure 2C, **top** and Figure S1D). *Pten* inactivation increased proliferation of lv-SVZ, but not in TVZ, NSCs, whereas *KIAA1549:BRAF* overexpression increased proliferation of TVZ, but not in lv-SVZ, NSCs, with no effect in apoptosis (Figures 2C and **S1D**). Decreased apoptosis was observed in both NSC populations following *Pten* loss, while *KIAA1549:BRAF* overexpression resulted in no change. These differential responses do not reflect a failure to activate AKT following *Pten* loss in TVZ NSCs or MEK following *KIAA1549:BRAF* expression in lv-SVZ NSCs (Figure S1E).

Mouse *Nf1* optic gliomas arise from TVZ

To identify the ventricular zone of origin for optic glioma, we chose NF1 as a model experimental system because gliomas predominate in the optic pathway of children with this syndrome (Guillermo et al., 2003). Similar to human NF1-associated gliomas, optic gliomas form in the prechiasmatic and chiasmatic regions of *Nf1*^{+/-} mice following complete *Nf1* inactivation in glial progenitors (Figure 3A) (Bajenaru et al., 2003). These gliomas could arise from NSCs in the lv-SVZ, TVZ (Figure 3B), optic nerve, or retina.

We first excluded the retina and optic nerve as cell of origin of these gliomas because true NSCs capable of self-renewal and multi-lineage differentiation could not be generated from either E17.5 or postnatal day (PN) 1 retina cells or the optic nerve (Figure S2A, Cicero et al., 2009; Lee et al., 2010). Additionally, Cre transgene expression (LacZ⁺ cells) was not detected in the retina or optic nerve until after PN2 or E17.5, respectively (Figure S2B). We then show that *Nf1*^{+/-} NSCs from the TVZ, but not the lv-SVZ, exhibit increased proliferation relative to wild-type NSCs, with no effect on apoptosis *in vitro* (Figure 3C and **S2C**). To provide *in vivo* support for these *in vitro* observations, we inactivated *Nf1* in BLBP⁺ NSCs beginning at E9.5 and found that the numbers of Olig2⁺ glial progenitors (Figure 3D) and GFAP⁺ astrocytes (Figure 3E) were increased in the TVZ, but not in the lv-SVZ, of PN8 *Nf1*^{BLBP} mice compared to control littermates *in vivo*.

To examine whether human hypothalamic/optic gliomas recapitulate the gene expression pattern of TVZ (Sharma et al., 2007), PCA and hierarchical clustering revealed that hypothalamic/optic pathway gliomas were separated from their supratentorial counterparts (Figure S2D). While some of differentially-expressed genes were not represented on the human Affymetrix Gene Chip, we found that one differentially-expressed TVZ transcript (*Slit2*) was significantly higher (3.7 fold; p=0.001) in hypothalamic/optic PAs compared to supratentorial PAs. Two other TVZ-overexpressed transcripts (*Gmnn* and *Nr2f2*) and two lv-SVZ-overexpressed transcripts (*Cntn1* and *ApoE*) also exhibited increased expression in hypothalamic/optic gliomas and supratentorial gliomas, respectively, although not reaching statistical significance, likely due to the small sample size (p=0.08–0.1) (Figure S2D).

Finally, we sought to define a developmental window when optic glioma formation is favored by virtue of the selective proliferative activity of the TVZ and lv-SVZ using three different GFAP-Cre driver lines with distinct patterns of Cre-mediated *Nf1* inactivation *in vivo* (Figures S3A and 4A). The *GFAP-Cre:IRES-LacZ* strain used to generate *Nf1*^{+/-GFAP} mouse optic gliomas has detectable LacZ expression in the lv-SVZ and TVZ beginning at E15.5 (Figure 4B). The GFAP-Cre* strain (Zhuo et al., 2001) initiates Cre expression in the anterior part of forebrain by E13.5 (Figure 4A and 4C) and in the hypothalamus, which includes the TVZ, by E16.5 (Figure 4C). *Nf1*^{+/-GFAP*} mice also develop optic glioma (Zhu et al., 2005) (Figure 4E). Analysis of the TVZ and lv-SVZ in these mice reveals that nestin⁺ and Ki67⁺ progenitor cells reside in both germinal zones at E15.5 (Figure 4B).

To distinguish between these two germinal zones, we employed the GFAP-Cre^{ER} strain, which was similar to the first GFAP-Cre strain, but expressed a tamoxifen-regulatable Cre (Cre^{ER}; Chow et al., 2008). Recombination and inactivation of the *Nf1* gene was verified by

recombination PCR (Figure 4D), while Cre activity in the brain, optic chiasm, and optic nerve following tamoxifen injection was demonstrated using ROSA-GREEN reporter mice (Figure 4D). Since lv-SVZ contained nestin⁺ Ki67⁺ cells at PN8–14 whereas these proliferating progenitors disappeared after PN2 in TVZ (Figure 4B, S3B and S3C), we inactivated *Nf1* either during the first postnatal week of life (PN 1–3) or at 2 weeks of age when only the lv-SVZ harbors significant numbers of proliferating (Ki67⁺) progenitor (nestin⁺) cells (Figure S3B and S3C). *Nf1* loss at these times did not result in glioma formation at 3 months of age (Figure 4E). As an internal control for the fidelity of the GFAP-Cre^{ER} strain for inducing optic glioma, we treated >20 litters of pregnant females with tamoxifen at E16.5 (50 µg/g i.p.). The vast majority of pregnant dams did not deliver viable mice; however, the one embryonically-treated pup that survived to 3 months of age developed an optic glioma (Figure S3D) with increased numbers of Ki67⁺ cells and increased numbers of GFAP⁺ astrocytes (Figure S3E). Taken together, these data establish that optic gliomas arise from neural stem/progenitor cells in the proliferative TVZ during embryogenesis rather than from astrocytes at later postnatal stages.

Discussion

Our finding that NSCs from two different germinal zones are molecularly-distinct stem cell populations is consistent with previous reports examining mouse embryonic spinal cord and brain NSCs as well as human neural progenitor cells from the developing cortex and ventral 8 midbrain (Johnson et al., 2010; Kelly et al., 2009; Kim et al., 2009; Taylor et al., 2005). In each case, the unique genetic signature reflects the regional identity of the progenitors. Importantly, we show that the heterogeneity revealed at the molecular level translates into unique functional responses to glioma-causing genetic changes seen in children and adults. While the precise etiologies for these innate differences are unknown, they likely reflect transcriptional networks and signaling set-points unique to these brain regions. For example, we have previously shown that the expression of the mTOR component rictor underlies the ability of *Nf1*-deficient NSCs to increase their proliferation and glial differentiation (Lee et al., 2010), whereas basal cAMP levels in specific brain regions partly dictate the spatial pattern of gliomagenesis in NF1 (Warrington et al., 2010).

We also provide several lines of converging evidence that optic gliomas likely originate from stem/progenitor cells residing in the TVZ. While both TVZ and lv-SVZ germinal zones could provide cells of origin for these tumors, only TVZ, but not lv-SVZ, NSCs exhibit increased proliferation and gliogenesis following *Nf1* inactivation. In addition, optic gliomas do not form in mouse strains following postnatal *Nf1* inactivation when only lv-SVZ NSCs are proliferating. These latter experiments also demonstrate that *Nf1* inactivation in GFAP-expressing astrocytes in young mice does not result in optic gliomagenesis. One report employing immunohistochemical and gene expression analysis similarly suggested that human optic gliomas might derive from third ventricle glial progenitors (Tchoghandjian et al., 2009). This result parallels the developmental origins of another optic nerve glial cell population in which oligodendrocyte precursor cells generated in the floor of the TVZ differentiate and migrate into the optic nerve in response to signaling molecules from retinal ganglion axons (Gao and Miller, 2006; Ono et al., 1997).

While our mouse experimental data argue that optic gliomas in children with NF1 arise from the TVZ, it is possible that we have modeled only one type of human optic glioma, and that other subtypes of optic glioma originate from different progenitor cells akin to other CNS cancers (Gibson et al., 2010; Johnson et al., 2010). Additional potential progenitors could be NG2⁺ oligodendrocyte precursor cells recently implicated in malignant gliomagenesis (Assanah et al., 2006; Liu et al., 2011; Masui et al., 2010; Sugiarto et al., 2011). However, *Nf1* inactivation in NG2⁺ cells of *Nf1*^{+/-} mice, similar to the *Nf1* mouse models described

here, is not sufficient for glioma formation (Solga A, manuscript in preparation). Future studies aimed at subdividing these common pediatric tumors into molecularly-distinct diseases will facilitate the development of brain tumor therapies targeted to the specific growth regulatory pathways that drive cell growth and differentiation in these distinct cancer-initiating cell populations.

Experimental Procedures

Mice

All strains were generated (Supplemental Experimental Procedures), maintained on a C57BL/6 background and used under an approved Animal Studies Committee protocol at Washington University.

NSC isolation and analysis

Iv-SVZ and TVZ NSCs from *Nf1*^{flox/flox}, *p53*^{flox/flox} and *Pten*^{flox/flox} PN1 mouse pups were infected with adenovirus containing LacZ or Cre, and protein loss confirmed by Western blotting (Lee et al., 2010). NSCs expressing *KIAA1549:BRAF* were generated following retrovirus infection (Peter Collins, University of Cambridge) and verified by RT-PCR (Supplemental Experimental Procedures). pBABE-puro retrovirus was used as control. NSC proliferation and multi-lineage differentiation assays were performed as described previously (Lee et al., 2010).

Immunohistochemistry and immunocytochemistry

Tissues and cells were prepared as previously reported (Hegedus et al., 2007) prior to staining with appropriate antibodies (Supplemental Experimental Procedures).

Microarray analysis

RNA from three independent litters of E17.5 C57BL/6 Iv-SVZ and TVZ NSCs were subjected to microarray profiling (Supplemental Experimental Procedures), and differentially-expressed probe sets ($p < 0.05$; fold change > 3 -fold increase or decrease) prioritized for validation.

qRT-PCR

mRNA expression was determined by qRT-PCR using NSCs from independently-generated litters as described previously (Yeh et al., 2009) (Supplemental Experimental Procedures).

X-gal staining

Six μm frozen sections were stained with X-Gal (Gold Biotechnology, St. Louis, MO) (Hegedus et al., 2007).

Tamoxifen injection and recombination PCR

Tamoxifen was injected into lactating females (1 mg/50 μL i.p.) at PN1–3 or PN14–18 (Supplemental Experimental Procedures), and *Nf1* recombination determined by recombination PCR (Mayes et al., 2011).

Western blotting

Western blotting was performed as reported previously (Lee et al., 2010) (Supplemental Experimental Procedures).

Statistical analyses

Each experiment was performed with samples from at least three independent litters. Statistical significance ($p < 0.05$) was determined (Student's t -test) using GraphPad Prism 5.0 software (GraphPad, Inc.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance

Whereas some adult malignant cerebral hemispheric gliomas have been shown to arise from neural stem or progenitor cells residing in the subventricular zone of the lateral ventricle (lv-SVZ), the cellular origin of pediatric low-grade gliomas is unknown. Consistent with the propensity for childhood gliomas to develop in the optic nerve and chiasm, we demonstrate that third ventricle (TVZ) NSCs are molecularly and functionally distinct from their lv-SVZ counterparts and are the likely cell of origin for murine low-grade optic gliomas. These findings establish brain region NSC heterogeneity as a major determinant underlying the patterning of gliomagenesis in children and adults.

Highlights

- lv-SVZ and TVZ neural stem cells (NSCs) are molecularly-distinct populations
- TVZ and lv-SVZ NSCs differentially respond to glioma-associated mutations
- Third ventricle NSCs are the likely cell of origin for NF1 optic gliomas
- Innate brain region NSC heterogeneity partly dictates the pattern of gliomagenesis

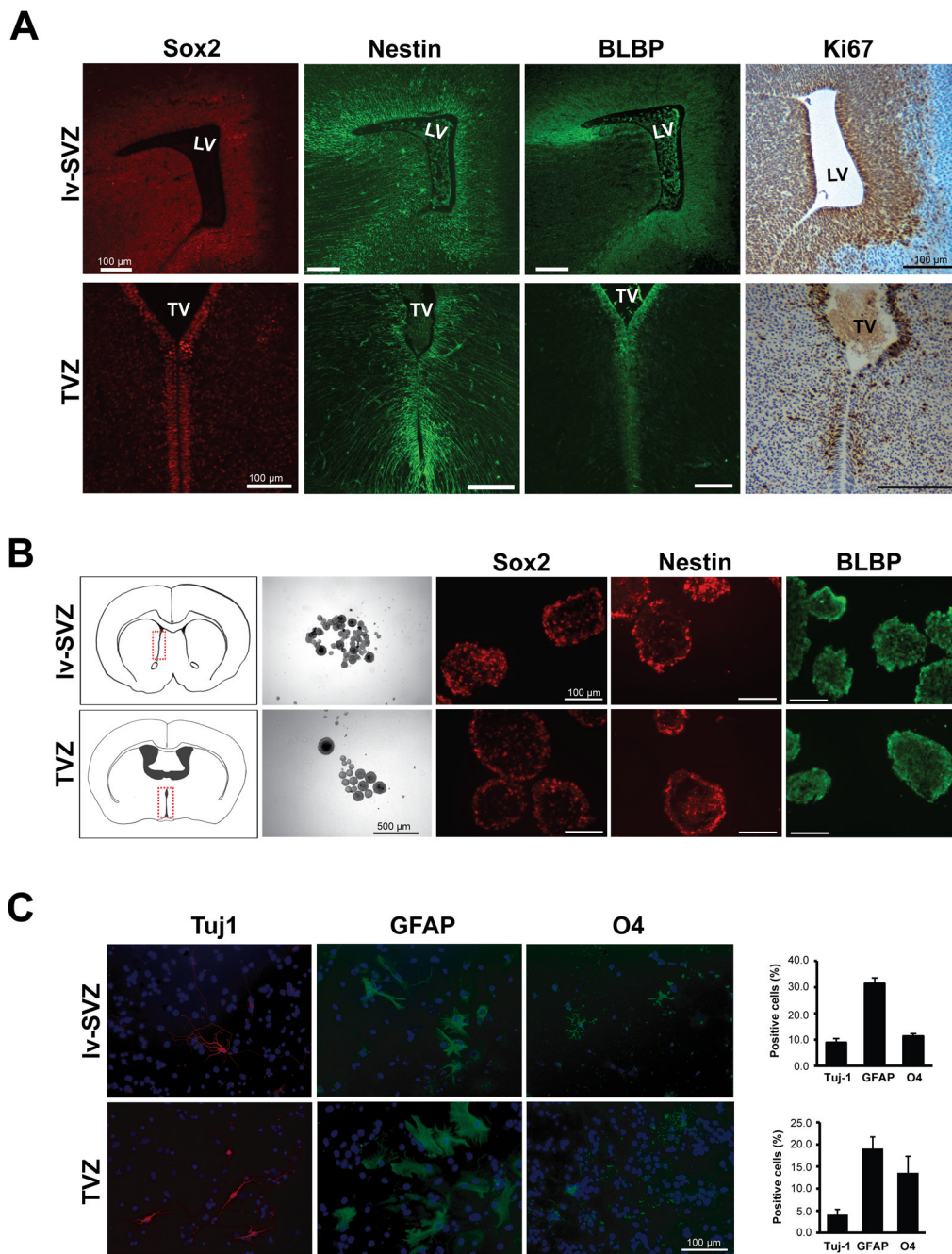
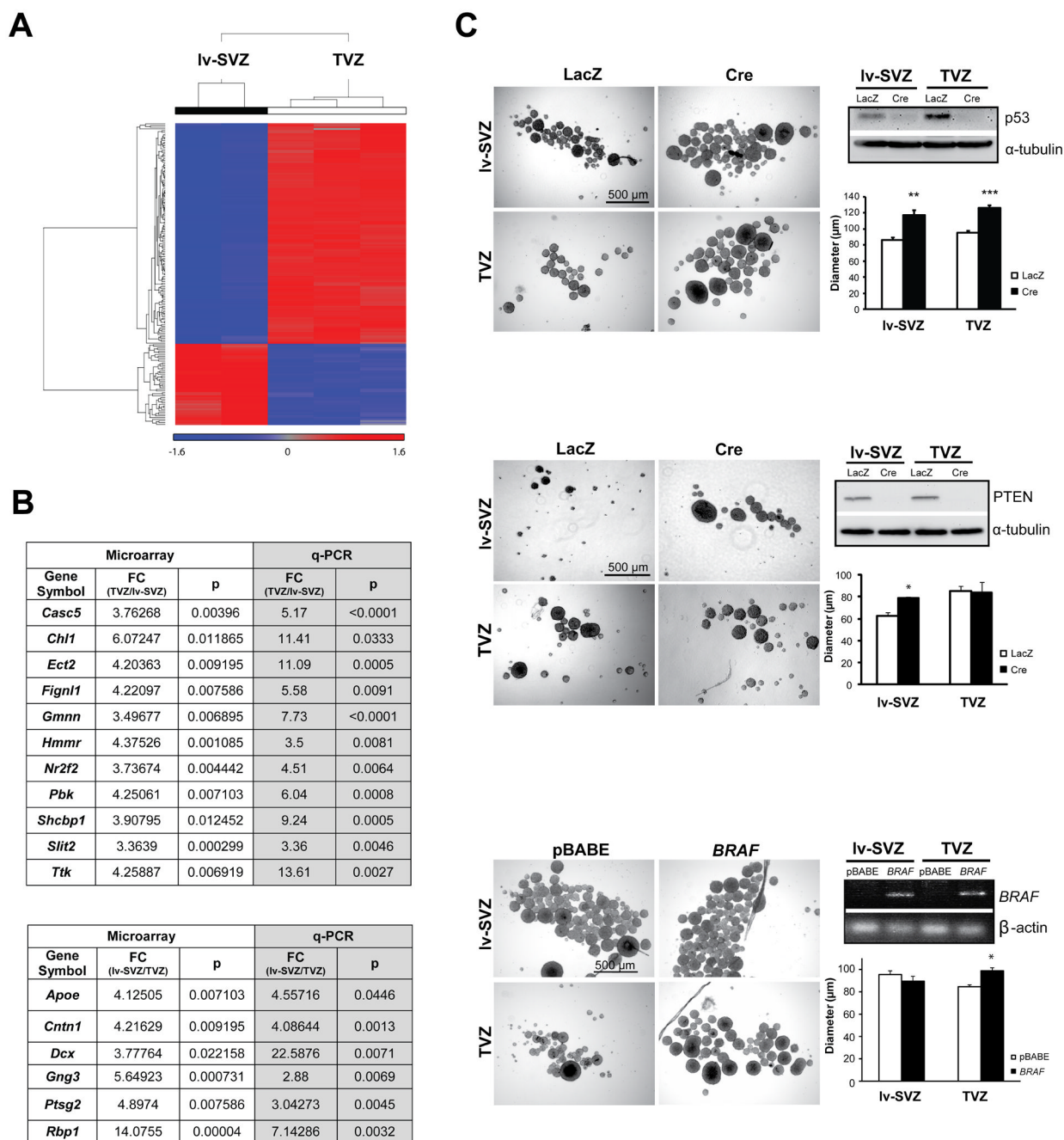


Figure 1. NSCs can be generated from the lv-SVZ and TVZ

(A) Immunostaining of the mouse E15.5 TVZ cells express the Sox2, nestin, and BLBP NSC markers. (B) Single neurospheres from the lv-SVZ and TVZ form secondary neurospheres *in vitro* and express Sox2, nestin and BLBP. The diagrams denote the regions used for NSC cultures. (C) TVZ NSCs can differentiate into neurons (Tuj-1), astrocytes (GFAP) and oligodendrocytes (O4). Values denote the mean \pm SEM.



KIAA1549:BRAF expression (RT-PCR). Values denote the mean \pm SEM. $p^* < 0.01$, $p^{**} < 0.001$, $p^{***} < 0.0001$. See also Figure S1.

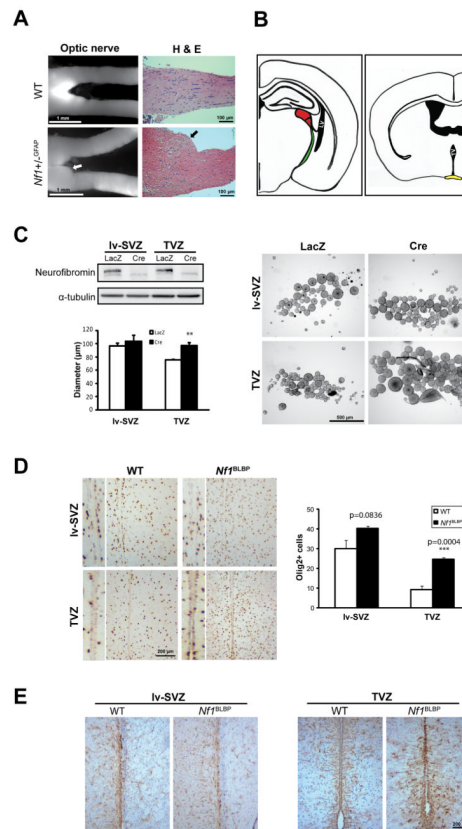


Figure 3. TVZ NSCs are preferentially sensitive to *Nf1* loss

(A) Increased optic nerve volume (white arrow) and abnormal cell clusters (black arrow) were observed in optic gliomas from 3-month-old *Nf1*^{+/-} GFAP mice. (B) The potential cellular origins (lv-SVZ and TVZ) of optic gliomas in *Nf1* mutant mice are illustrated. LV: lateral ventricle, red: lateral geniculate nucleus, green: optic tract, TV: third ventricle, yellow: optic chiasm. (C) Increased TVZ neurosphere proliferation was seen following *Nf1* loss, with little effect on lv-SVZ NSCs. (D) 3-fold more Olig2⁺ cells were found in the TVZ of *Nf1*^{BLBP} mice (p=0.0004), but not in the lv-SVZ (p=0.0836), compared to controls. (E) Increased numbers of GFAP⁺ cells were found in the TVZ, but not in the lv-SVZ, of *Nf1*^{BLBP} mouse compared to controls. Values denote the mean ± SEM. See also Figure S2.

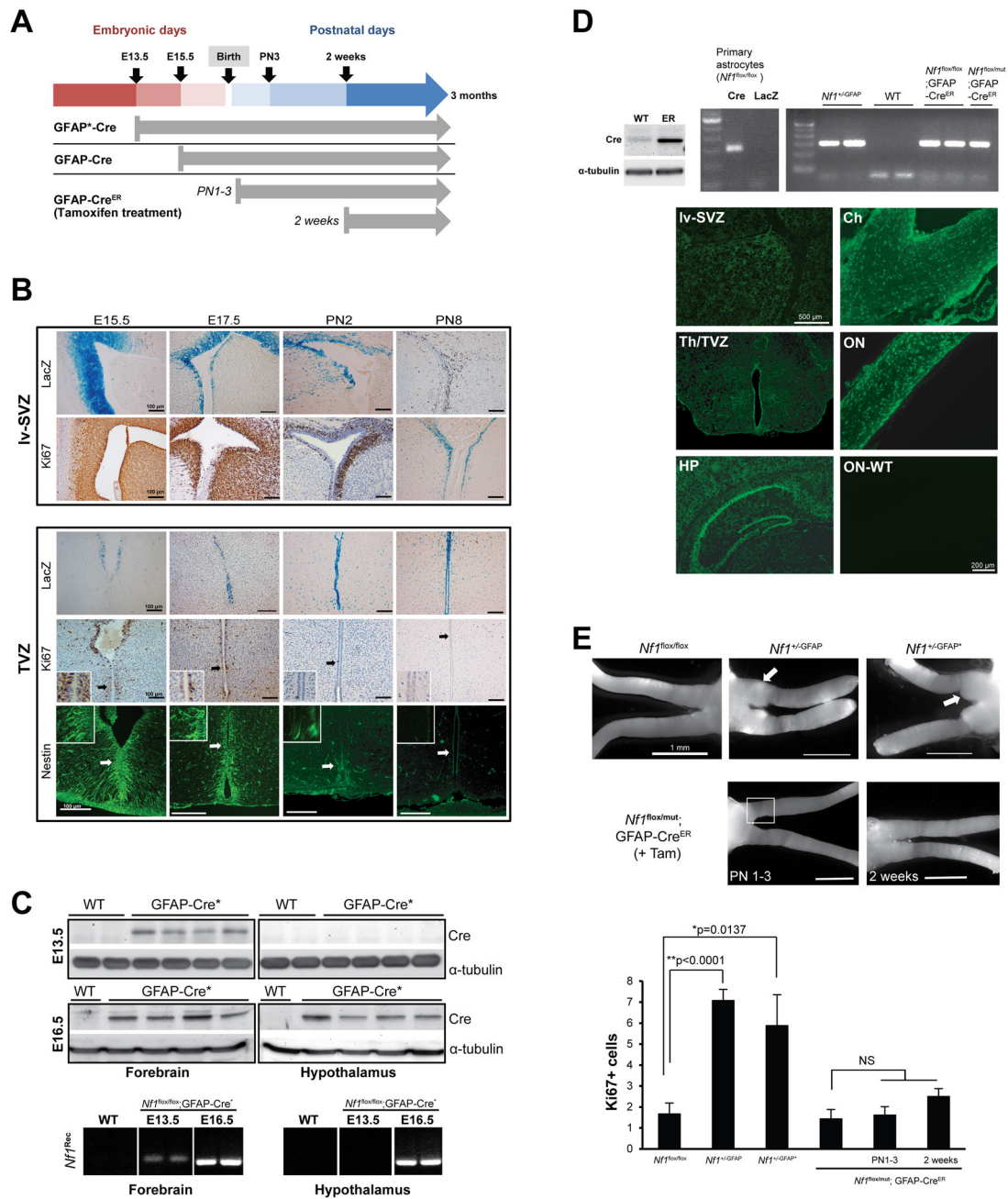


Figure 4. Embryonic *Nfl* inactivation is required for optic glioma formation

(A) The timing of *Nfl* inactivation by Cre-mediated excision is shown for each strain. (B) X-gal staining reveals GFAP-Cre transgene expression in the lv-SVZ and TVZ beginning at E15.5. Whereas lv-SVZ cells are Ki67⁺ from E15.5 through PN8, scant numbers of Ki67⁺ or nestin⁺ cells are detected in the TVZ by PN2. (C) Cre expression and *Nfl* gene recombination (*Nfl*^{Rec}) is detected by E13.5 in the anterior forebrain/lv-SVZ (“forebrain”) and by E16.5 in the hypothalamus/TVZ (“hypothalamus”) of GFAP-Cre* mice. (D) Cre-mediated *Nfl* gene recombination (PCR) in *Nfl*^{+/−}GFAP mice and in tamoxifen-treated *Nfl*^{lox/lox}; GFAP-Cre^{ER} and *Nfl*^{lox/mut}; GFAP-Cre^{ER} mice was seen. Wild-type (WT) C57BL/6 mouse brain was used as a negative control, whereas Ad5Cre-infected (Cre) and

Ad5LacZ-infected (LacZ) *Nfl*^{flox/flox} astrocytes served as positive and WT controls, respectively. Cre-ER fusion protein (~70 KDa) expression was detected in GFAP-Cre^{ER} (ER), but not in WT, mouse brains. EGFP was expressed in tamoxifen-treated ROSA-GREEN; GFAP-Cre^{ER} mouse brains and optic nerves at 1 month of age, but not in a WT mouse optic nerve (ON-WT). HP: hippocampus, Th: thalamus, Ch: chiasm, ON: optic nerve. (E) Whereas optic gliomas develop in *Nfl*^{+/-GFAP} and *Nfl*^{+/-GFAP*} mice, no gliomas formed in *Nfl*^{flox/flox} or *Nfl*^{flox/mut}; GFAP-Cre^{ER} treated with tamoxifen (+Tam) beginning at PN1 or PN14. Increased Ki67⁺ cells were found in the prechiasmatic and chiasm regions (square) of *Nfl*^{+/-GFAP} and *Nfl*^{+/-GFAP*} mice. In contrast, the number of Ki67⁺ cells in *Nfl*^{flox/mut}; GFAP-Cre^{ER} postnatally-treated with tamoxifen is indistinguishable from control *Nfl*^{flox/flox} mice. Values denote the mean ± SEM. See also Figure S3.