Neurofibromatosis-1 regulates neuroglial progenitor proliferation and glial differentiation in a brain region-specific manner

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Recent studies have shown that neuroglial progenitor/stem cells (NSCs) from different brain regions exhibit varying capacities for self-renewal and differentiation. In this study, we used neurofibromatosis-1 (NF1) as a model system to elucidate a novel molecular mechanism underlying brain region-specific NSC functional heterogeneity. We demonstrate that NF1 loss leads to increased NSC proliferation and gliogenesis in the brainstem, but not in the cortex. Using NF1 genetically engineered mice and derivative NSC neurosphere cultures, we show that this brain region-specific increase in NSC proliferation and gliogenesis results from selective Akt hyperactivation. The molecular basis for the increased brainstem-specific Akt activation in brainstem NSCs is the consequence of differential rictor expression, leading to region-specific mammalian target of rapamycin (mTOR)/rictor-mediated Akt phosphorylation and Akt-regulated p27 phosphorylation. Collectively, these findings establish mTOR/rictor-mediated Akt activation as a key driver of NSC proliferation and gliogenesis, and identify a unique mechanism for conferring brain region-specific responses to cancer-causing genetic changes.

[Keywords: Neurofibromin; neural stem cell; regional heterogeneity; gliogenesis; Akt; mTOR]

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The capacity of neural stem/progenitor cells (NSCs) to self-renew, proliferate, and give rise to differentiated cell types is essential for normal brain development and maturation. In addition, NSCs have critical roles in numerous pathological states, including the response to nervous system injury, neurodegenerative disease, and brain tumor formation (Corbin et al. 2008; Waldau and Shetty 2008; Alcantara Llaguno et al. 2009; Yadirgi and Marino 2009). Recent studies have shown that NSCs from different regions of the CNS exhibit varying abilities to divide and generate lineage-restricted progeny (Klein et al. 2005; Armando et al. 2007). In these studies, NSCs from the forebrain proliferated faster than those from the hindbrain (Horiguichi et al. 2004; Kim et al. 2006) or spinal cord (Fu et al. 2005) and exhibited differing abilities to produce radial glia (Kulbatski and Tator 2009) and glial fibrillary acidic protein (GFAP)-positive cells (astrocytes) in vitro (Hitoshi et al. 2002). However, it is not known whether these intrinsic region-restricted NSC properties have relevance to human disease pathogenesis.

While brain tumors can arise in any location in the CNS in adults, glial cell tumors (astrocytomas or gliomas) are most frequently observed in the cerebellum, brainstem (BS), and optic pathway/hypothalamus in children (Louis et al. 2007). This anatomic predisposition is best illustrated by the neurofibromatosis-1 (NF1) inherited cancer syndrome, in which low-grade astrocytomas are located predominantly in the optic pathway/hypothalamus and BS of young children, with rare tumors developing in the cerebral cortex (Listernick et al. 1994; Pollack et al. 1996; Guillamo et al. 2003). In light of studies implicating cells with stem cell-like properties in the genesis of astrocytoma (for review, see Stiles and Rowitch 2008), it is conceivable that this regional distribution of tumors partly reflects the intrinsic heterogeneity of stem cells from different regions of the brain to expand in response to cancer-associated genetic changes. Based on the unique spatial distribution of gliomas in children with NF1, we chose to employ NF1 genetically engineered mice and derivative NSCs to define the molecular basis for NSC heterogeneity in the brain.

NF1 is the most common cancer predisposition syndrome in which affected children develop gliomas. NF1-associated gliomas result from biallelic inactivation of
the NF1 tumor suppressor gene, leading to total loss of NF1 protein (neurofibromin) function. Neurofibromin is a large cytoplasmic protein that negatively regulates RAS pathway signaling by inactivating the RAS proto-oncogene such that loss of NF1 gene expression is associated with increased RAS and RAS effector (Akt and MAPK) activity [Basu et al. 1992; DeClue et al. 1992]. As predicted, examination of gliomas from individuals with NF1 confirmed loss of NF1 expression and increased RAS pathway activation [Gutmann et al. 2000; Lau et al. 2000]. Similarly, Nf1-deficient primary mouse forebrain astrocytes exhibit high levels of RAS activation, resulting in increased Akt and mammalian target of rapamycin (mTOR) signaling [Dasgupta et al. 2005b; Sandmark et al. 2007]. Inhibition of Akt/mTOR activity in Nf1−/− astrocytes restores their proliferation to wild-type levels in vitro and blocks optic glioma growth in vivo [Hegedus et al. 2008].

Previous studies have shown that neurofibromin is an important regulator of NSC function. In this regard, NF1 inactivation leads to an expansion in the number of proliferating spinal cord progenitors [Bennett et al. 2003], while Nf1−/− neural crest stem cells exhibit increased proliferation relative to their wild-type counterparts [Joseph et al. 2008]. Similarly, Nf1−/− embryonic telencephalic NSCs exhibit increased proliferation in vitro [Dasgupta and Gutmann 2005], while Nf1 inactivation in brain lipid-binding protein (BLBP)-expressing neuro-glial progenitors in vivo results in increased NSC proliferation and glial lineage differentiation [Hegedus et al. 2007]. Moreover, the effect of neurofibromin loss on NSC glial differentiation was dependent on RAS and Akt signaling, and could be recapitulated by the expression of either activated K-RAS or Akt in BLBP+ cells in vivo.

In this study, we describe regional differences in brain NSC proliferation and glial differentiation following NF1 gene inactivation in vitro and in vivo. Whereas Nf1−/− NSCs from the BS exhibit increased proliferation and glial cell differentiation in vitro and in vivo, we observed no effect of NF1 gene inactivation on neocortex (CTX) NSC proliferation or gliogenesis. We further show that these cell-autonomous differences reflect the ability of BS NSCs to activate Akt in an mTOR/riptor-dependent fashion and culminate in Akt-mediated p27 phosphorylation. Collectively, these results demonstrate that intrinsic differences in NSC signaling cause brain region-specific abnormalities in glial differentiation, which may partially account for the propensity of glial cell tumors to arise in the BS of children with NF1.

Results

NF1 inactivation results in increased BS NSC proliferation

Recently, we showed that NF1 inactivation results in the increased proliferation of astrocytes from some brain regions [BS, optic nerve [ON], and cerebellum], whereas CTX astrocyte proliferation is unaffected by NF1 loss [Yeh et al. 2009]. Since gliomas have been hypothesized to originate from cells with stem cell-like properties, we generated neurosphere cultures from several regions of the postnatal brain. The ON and BS were chosen to represent regions where gliomas arise in children with NF1, while the CTX was included as a region where glioma formation is far less common. We used these different neurosphere populations to determine whether NF1 loss had differential effects on NSC proliferation and gliogenesis as a function of brain region.

NSCs are defined as neurosphere cultures that express stem cell markers (e.g., BLBP and nestin) and retain the ability to undergo self-renewal and multi-lineage differentiation. For these experiments, we generated neurosphere cultures from postnatal day 1 [PN1] mouse CTX, BS, and ON. All of the resulting neurospheres from the CTX and BS were BLBP- and nestin-immunoreactive [Supplemental Fig. S1A]. However, ON neurosphere cultures had limited capacity for proliferation and self-renewal [Supplemental Fig. S1B,C], and failed to generate Tuj1+ or O4+ oligodendrocytes [Supplemental Fig. S1D]. In contrast, both CTX and BS neurospheres were capable of self-renewal, and generated neurons [Tuj1+], astrocytes [GFAP+], and oligodendrocytes [O4+] [Supplemental Fig. S1E]. Based on these results, ON neurospheres contained only glial progenitor cells and could not be classified as “true” NSCs. Therefore, we focused our analyses on NSCs from the BS and CTX as representative populations that derive from regions where gliomas commonly and rarely form, respectively, in children with NF1.

To determine whether NF1 inactivation has different effects on CTX versus BS NSC proliferation, we first measured neurofibromin levels to exclude brain region-specific differences in overall NF1 gene expression and found equivalent levels of neurofibromin expression [Supplemental Fig. S2A]. Second, using Nf1−/− and wild-type NSC cultures from PN1 Nf1+/− and wild-type neurosphere cultures from PN1 Nf1+/− and wild-type neurosphere cultures following Cre or LacZ gene adenovirus [Ad5] infection, respectively, Nf1−/− BS NSCs exhibited a 1.9-fold increased in proliferation compared with wild-type BS NSCs [Fig. 1A; Supplemental Fig. S2A]. In contrast, there was no increase in CTX NSC proliferation after NF1 inactivation. To demonstrate that these observations were not dependent on the developmental age or the method of NF1 inactivation, we used Nf1−/− NSCs generated from PN1 Nf1+/−; BLBP-Cre [Nf1BLBPCKO] mice as well as embryonic day 13.5 [E13.5] Nf1−/− NSCs following Ad5-Cre infection. Compared with their matched wild-type counterparts, these Nf1−/− NSCs from the BS, but not the CTX, also exhibited increased proliferation [Fig. 1A]. No change in self-renewal was observed in either BS or CTX NSCs following NF1 inactivation [Supplemental Fig. S2B].

Next, to assess the impact of NF1 inactivation on NSCs in the subventricular zone [SVZ], an important germinal zone for neurogenesis in both adults and children, we isolated NSCs from the SVZ of PN1 mice. Whereas neurofibromin loss in BS NSCs resulted in a 1.8-fold increase in NSC proliferation, NSC proliferation was increased by
1.4-fold in SVZ NSCs in vitro (Supplemental Fig. S2C). Similarly, the number of proliferating (Ki67 +) cells was increased by 2.8-fold in the BS of Nf1BLBPCKO mice compared with wild-type controls in vivo, while the number of Ki67 + cells in the SVZ was increased by only 1.3-fold (Supplemental Fig. S2D).

Finally, to demonstrate that this effect on NSC proliferation was also seen in vivo, the impact of Nf1 inactivation on the numbers of BLBP + cells in PN1 and PN8 Nf1BLBPCKO mice was examined. Similar to the in vitro results, increased numbers of BLBP + cells were observed in the BS of Nf1BLBP CKO mice at PN1 (1.5-fold) (Fig. 1B) and PN8 (1.3-fold) (Fig. 1C), whereas no significant change in BLBP + cell number was observed in the C TX of Nf1BLBP CKO mice relative to wild-type mice. Cre-mediated Nf1 gene inactivation was confirmed by Cre immunohistochemistry and neurofibromin Western blotting (Supplemental Fig. S2E). Collectively, these data demonstrate that Nf1 inactivation increases NSC proliferation in a brain region-specific fashion.

Nf1 inactivation increases gliogenesis in the BS

To determine whether Nf1 loss in NSCs results in increased glial lineage differentiation in a brain region-specific manner, we first quantified the number of Olig2 + glial progenitor cells in PN1 Nf1−/− and wild-type NSCs in vitro. The number of Olig2 + cells was increased by 2.4-fold to fourfold in dissociated Nf1−/− BS NSCs compared with wild-type BS NSCs (Supplemental Fig. S3A). Similar results were also observed using NSCs isolated from the BS and C TX of E16 pups (Supplemental Fig. S3B). We also noted a 1.2-fold increase in the number of Olig2 + cells following Nf1 loss in SVZ NSCs relative to wild-type SVZ NSCs (Supplemental Fig. S3A). In contrast, there was no significant change in Olig2 + cell number in Nf1−/− C TX NSCs relative to wild-type C TX NSCs. Next, we examined the number of Olig2 + cells in Nf1BLBP CKO mice and control littermates in vivo. The brain regions used for direct cell counting are illustrated in Figure 2B. As observed in vitro, the number of Olig2 + cells was increased in the BS of PN8 (1.9-fold) and PN18 (1.5-fold) Nf1BLBP CKO mice compared with wild-type littermates in vivo (Fig. 2C,D).

To determine whether these Olig2 + cells represented glial progenitor cells or committed oligodendrocyte lineage cells, we performed double-immunofluorescence staining with Olig2 and established oligodendrocyte cell lineage markers, including CC1 (APC), PDGFR-α, and O4. First, we found that the number of APC (CC1)/Olig2

Figure 1. Nf1 inactivation increases BS NSC proliferation. (A) Nf1 loss results in increased BS NSC neurosphere size, with no effect on C TX NSCs. Identical results were obtained with PN1 NSCs following acute Nf1 inactivation with Ad5-Cre (Cre), PN1 NSCs from Nf1BLBP CKO mice, or E13.5 NSCs after Nf1 inactivation. For controls, Nf1flx/flx NSCs were infected with Ad5-LacZ [LacZ]. Phase contrast images show representative neurospheres, which are graphically illustrated below. Similarly, increased numbers of BLBP + cells by immunofluorescence were observed in the BS of PN1 [B] and PN8 [C] mice, with no increase seen in the C TX. Sections were counterstained with DAPI. Graphs show the number of BLBP + cells per surface area (0.1 mm²). Values denote the mean ± SEM. (*) P < 0.05. Bars: A, 300 μm; B, C, 100 μm.
double-positive cells was not increased by Nf1 inactivation [Supplemental Fig. S3C]. Second, the majority of the Olig2+ cells increased in the BS of Nf1BLBPCKO mice were not PDFR-α-immunoreactive (Supplemental Fig. S3D). Third, following Nf1-deficient NSC differentiation in vitro, the Olig2+ cells were not O4+ [Supplemental Fig. S3E]. Fourth, while we were unable to perform NG2/Olig2 double-labeling experiments to determine whether the Olig2+ cells were also NG2+, we found that the total number of NG2+ cells was increased in both CTX and BS of Nf1BLBPCKO mice compared with wild-type controls [Supplemental Fig. S3F]. Together, these data demonstrate that the Olig2+ cells increased following BS NSC Nf1 inactivation are glial progenitor cells, rather than committed oligodendrocyte progenitors.

To determine whether the increase in Olig2+ cells reflected an overall increase in gliogenesis, we quantified the number of GFAP+ cells (astrocytes) and found increased numbers of GFAP+ cells in the BS of Nf1BLBPCKO mice at both PN8 and PN18 compared with wild-type mice. There was no increase in the number of GFAP+ cells in the CTX of Nf1BLBPCKO mice. Graphs show the number of GFAP+ cells per surface area [0.1 mm²] and fold change (Nf1BLBPCKO/WT) of GFAP+ cells are graphically represented. Values denote the mean ± SEM. (*) P < 0.005; (**) P < 0.001; (***) P < 0.0001. Bars, 100 μm.

Figure 2. Nf1 inactivation increases the number of BS Olig2+ glial progenitors and GFAP+ astrocytes. (A) The number of Olig2+ cells [red] was increased in Nf1/−/− BS NSCs (Cre) compared with wild-type NSCs [LacZ]. No increases were observed in CTX NSCs. Cells were counterstained with DAPI [blue]. (B) Schematic shows the brain regions used for direct cell counting [blue]. (CC) Corpus callosum; [LV] lateral ventricle; [AC] anterior commissure; [VN] ventricle; [PnC] pontine nucleus, caudal. (C) The number of Olig2+ glial progenitor cells was increased in the BS of Nf1BLBPCKO mice at both PN8 and PN18 compared with wild-type [WT] mice. There was no increase in the number of Olig2+ cells in the CTX of Nf1BLBPCKO mice. Graphs show the number of Olig2+ cells per surface area [0.1 mm²] and fold change (Nf1BLBPCKO/WT) of Olig2+ cells are graphically represented. Values denote the mean ± SEM. (*) P < 0.005; (**) P < 0.001; (***) P < 0.0001. Bars, 100 μm.
Ras activation increases NSC proliferation and gliogenesis in the BS

Because Ras activity is increased in Nf1−/− embryonic NSCs (Dasgupta and Gutmann 2005), we examined the activation state of the three different Ras isoforms (K-Ras, H-Ras, and N-Ras) expressed in E11.5 NSCs following Nf1 loss, and found increased activation of all three Ras isoforms in Nf1−/− NSCs relative to wild-type NSCs (Supplemental Fig. S5A). To recapitulate Ras activation by Nf1 loss in vitro, we infected PN1 NSCs from the CTX and BS with either constitutively active K-Ras (KRas*) or H-Ras (HRas*). The expression and activation of these transduced Ras molecules were confirmed by Western blotting and Ras activity assays (Supplemental Fig. S5B-D). Similar to Nf1−/− NSCs, only BS NSCs exhibited increased proliferation following either activated K-Ras (1.9-fold) (Fig. 3A) or H-Ras (1.5-fold) (Fig. 3B) transduction. No increased proliferation was observed in CTX NSCs following either K-Ras or H-Ras expression. To provide an in vivo correlate for these in vitro observations, we also examined the effect of K-Ras activation on gliogenesis in PN18 Lox-stop-lox (LSL)-K-RasG12D; BLBP-Cre (KRas*) mice. Similar to Nf1−/− mice, KRas* mice exhibited increased numbers of Olig2+ glial progenitor cells (1.9-fold) (Fig. 3C) and GFAP+ astrocytes (1.8-fold) (Fig. 3D) in the BS, but not the CTX, compared with wild-type mice. These data show that Ras activation in NSCs recapitulates the brain region-specific effects of Nf1 loss on NSC proliferation and gliogenesis.

The brain region-specific effects of Nf1 loss on NSC proliferation and gliogenesis are Akt-dependent

Because MAPK and Akt are the two major Ras downstream targets relevant to neurofibromin growth regulation, we measured their activation status in PN1 Nf1−/− and wild-type NSCs using phospho-specific antibodies. Whereas MAPK activation was increased in both CTX and BS Nf1-deficient NSCs relative to wild-type NSCs (Fig. 4A,B), Akt activation was increased only in Nf1−/− NSCs from the BS. In contrast, S6 activation [phospho-S6 (p-S6)] was not increased in either BS or CTX NSCs following Nf1 inactivation (Fig. 4A,B). Similar to Nf1−/− loss, increased Akt activation was observed in BS, but not CTX, NSCs expressing either constitutively active K-Ras (KRas*) or H-Ras (HRas*) compared with wild-type NSCs (Fig. 4C).

To determine whether induced expression of an activated Akt in brain NSCs would result in increased gliogenesis in both the BS and the CTX, we generated mice with activated Akt (myr-Akt) expression in BLBP+ cells (BLBP-Cre × LSL-myr-Akt mice [Akt+ mice]) (Hegedus et al. 2007). In contrast to Nf1−/−BLBPCKO and KRas* mice, Akt+ mice exhibited increased numbers of Olig2+ cells in both the CTX (1.7-fold) and the BS (1.4-fold) compared with wild-type mice (Fig. 4D,G). Similarly, the number of GFAP+ cells was also increased in both the CTX (threefold) and the BS (twofold) (Fig. 4E,G). Transgene expression was confirmed by GFP immunohistochemistry (Fig. 4F). Together, these data demonstrate that Akt activation is the major determinant underlying the region-specific effects of Nf1 loss on NSC proliferation and gliogenesis.
effects of \textit{Nf1} inactivation on brain NSC proliferation and gliogenesis.

\textbf{Differential regulation of Akt by mTOR underlies the brain region-specific effects of \textit{Nf1} loss on NSC proliferation and gliogenesis}

To determine the mechanism of differential Akt regulation in BS versus CTX NSCs, we first measured the levels of numerous upstream regulators of Ras and Akt by Western blotting (Fig. 5A) and quantitative RT–PCR (qRT–PCR) (Supplemental Fig. S6A). We found no significant differences in p120 Ras-GAP, PTEN, or phospho-PDK1 expression in NSCs from the CTX versus the BS (Fig. 5A), as well as no differences in \textit{Ppp2r2b} (protein phosphatase 2), \textit{Prkce} (PKCe), \textit{Prkcq} (PKCo), \textit{Tenc1} (C1-TEN), \textit{Rasal2} (Ras protein activator-like 2), or \textit{Rasgrp1} (Ras-GRP) mRNA expression in NSCs from the CTX versus the BS (Supplemental Fig. S6A).

Next, we explored the possibility that mTOR complexes might differentially regulate Akt activity through raptor or rictor function (Guertin and Sabatini 2007). Using rapamycin to inhibit mTOR function in vitro, \textit{Nf1}\textsuperscript{flox/flox}/\textit{C0}/\textit{C0} BS NSC proliferation was reduced by twofold in response to rapamycin treatment (0.5–1 nM), whereas CTX NSC proliferation was not affected (Fig. 5B). In these experiments, rapamycin treatment (1 nM) reduced Akt hyperphosphorylation exclusively in BS NSCs, whereas S6 activation was inhibited in \textit{Nf1}\textsuperscript{flox/flox}/\textit{C0}/\textit{C0} NSCs from both the BS and CTX (Fig. 5B). To provide an in vivo correlate for these in vitro findings, pregnant females and their resulting pups were treated with rapamycin, and the numbers of Olig2\textsuperscript{+} and GFAP\textsuperscript{+} cells were quantified at PN8. Consistent with the hypothesis that mTOR regulates Akt-mediated gliogenesis, we found that rapamycin treatment reduced the number of Olig2\textsuperscript{+} (1.4-fold) and GFAP\textsuperscript{+} cells (2.1-fold) in the BS of \textit{Nf1}\textsuperscript{flox/flox}/\textit{C0}/\textit{C0} mice (Fig. 5C). Similar to \textit{Nf1}\textsuperscript{flox/flox}/\textit{C0}/\textit{C0} NSCs, we found that Akt hyperactivation in the BS of \textit{Nf1}\textsuperscript{flox/flox}/\textit{C0}/\textit{C0} mice was reduced following rapamycin treatment in vivo, while S6 activation was inhibited in both the BS and CTX in vivo (Fig. 5D).

Based on reports demonstrating that rictor-containing mTOR complexes positively regulate Akt through Ser 473 phosphorylation (Hresko and Mueckler 2005), we measured raptor and rictor expression in vitro and in vivo. Surprisingly, rictor expression was higher in BS compared with CTX in both NSC cultures (threefold) and brain tissue (2.6-fold), whereas no differences in raptor expression were observed (Fig. 6A). To determine whether increased rictor expression in BS NSCs reflected differential rictor/raptor transcription, we examined the mRNA expression levels of rictor and raptor in CTX and BS NSCs using qRT–PCR. No differences were observed in the mRNA expression of rictor (\(P = 0.3962\) or raptor (\(P = 0.7442\)) between CTX and BS NSCs.
To determine whether mTOR/riCTOR function mediates the increased proliferation and gliogenesis observed in Nf1<sup>-/-</sup> NSCs, we performed shRNAi knockdown (KD) experiments. Rictor KD decreased the proliferation (as assessed by direct counting and neurosphere diameter measurements) and Akt activation of Nf1<sup>+/+</sup> BS NSCs to wild-type levels, with no effect on Nf1<sup>+/+</sup> CTX NSCs (Fig. 6B,C). Consistent with its role in TORC2 signaling, rictor KD had no effect on S6 phosphorylation (Fig. 6C). In contrast, raptor KD had no effect on Nf1<sup>+/+</sup> BS NSC proliferation (neurosphere diameter and cell number) or gliogenesis (Olig2<sup>+</sup> cell number) and did not change Akt activation (data not shown). Together, these results demonstrate that the ability of BS NSCs to proliferate following Nf1 loss results from mTOR/riCTOR-mediated Akt activation.

Since we observed no differential changes in the activation status of mTOR downstream effectors [PKCa, S6 kinase, S6, and 4EBP1] after Nf1 inactivation in the BS versus the CTX [Supplemental Fig. S6B], we reasoned that downstream targets of Akt, other than mTOR, must be responsible for the increased proliferation of BS NSCs following Nf1 loss. We first measured the activation status of STAT3, FOXO1, and GSK3β in PN1 Nf1<sup>BLBPCKO</sup> and wild-type NSCs from CTX and BS using phospho-specific antibodies, but found no differential activation of these signaling molecules. However, p27 expression was increased in Nf1<sup>BLBPCKO</sup> CTX NSCs relative to wild-type CTX NSCs, whereas no such increase was observed in Nf1<sup>BLBPCKO</sup> BS NSCs (Fig. 7A). Previous studies have shown that p27 expression can be regulated at both the transcriptional and post-translational levels [Lin et al. 2009; Prasad et al. 2009]. Whereas there were no differences in p27 mRNA expression between Nf1-deficient BS and CTX NSCs by qRT–PCR in vitro or in vivo (P = 0.46), we found that p27 phosphorylation on both Ser 10 and Thr 198 residues was increased following Nf1 loss exclusively in BS NSCs (Fig. 7B). Since increased p27 phosphorylation on Thr 198 and Ser 10 leads to its nuclear export and subsequent degradation [Fujita et al. 2002; Ishida et al. 2002], we ectopically expressed p27 in Nf1<sup>BLBPCKO</sup> BS NSCs by viral transduction to mimic the increased p27 levels seen in Nf1<sup>BLBPCKO</sup> CTX NSCs, and found that p27 expression reduced Nf1<sup>BLBPCKO</sup> BS NSC proliferation to wild-type levels (Fig. 7C).
To determine whether p27 phosphorylation was regulated by mTOR and Akt in Nf1−/− BS NSCs, we examined Thr 198 p27 phosphorylation following mTOR (rapamycin) and PI3-kinase (LY294002) inhibition. We found that p27 phosphorylation was inhibited by rapamycin and LY294002 in Nf1−/− BS NSCs (Fig. 7D). Similarly, p27 phosphorylation at Ser 10 was also inhibited by rapamycin and LY294002 treatment in Nf1−/− BS NSCs (data not shown). Finally, using the identical lysates from the experiment shown in Figure 6, B and C, we found that, following rictor KD (but not raptor KD) (data not shown), p27 expression was increased and p27 phosphorylation (Thr 198) was decreased in Nf1−/− BS NSCs (Fig. 7E).

These results demonstrate that mTOR/rictor/Akt regulates p27 function through phosphorylation in Nf1−/− NSCs (Fig. 7F), and collectively establish mTOR/rictor-mediated positive regulation of Akt activity as the major determinant responsible for mediating the brain region-specific effects of neurofibromin loss on NSC proliferation and gliogenesis.

Figure 6. Differences in mTOR regulation of Akt underlie the brain region-specific differences in Nf1−/− NSC proliferation. [A] Rictor expression was higher in the BS compared with the CTX in vitro (NSCs, three-fold) and in vivo (brain, 2.6-fold). There was no difference in rapamycin expression. Results were quantitated by scanning densitometry using α-tubulin as an internal loading control. [B] Rictor silencing by lentiviral rictor siRNA (siRNA) decreased neurosphere size in Nf1−/− BS NSC cultures. No change was observed in Nf1−/− CTX NSCs following rictor silencing. Lentivirus-GFP (GFP) served as a control for viral infection. [C] The fold change in p-Akt and p-S6 (after normalization to total Akt and S6, respectively) relative to wild-type (WT) controls is indicated below the blots. Error bars denote the mean ± SEM. (**) \( P < 0.01 \). Bar, 500 μm.

Figure 7. Differential phosphorylation of p27 results from mTOR/rictor/Akt-mediated activation in Nf1−/− BS NSCs. [A] p27 expression was increased exclusively in Nf1−/− BS NSCs compared with wild type, whereas no regional difference in the level of phospho-STAT3, FoxO1, or GSK was observed following Nf1 loss. Ad5-LacZ (LacZ) served as a control for viral infection. [B] Nf1 loss resulted in increased p27 phosphorylation (p-p27; Ser 10 and Thr 198) in Nf1−/− BS NSCs. [C] Ectopic p27 expression in Nf1−/− BS NSCs restored proliferation to wild-type levels. (D) p27 phosphorylation (p-p27; Thr 198) in Nf1−/− BS NSCs was reduced by rapamycin [1 nM] and LY294002 [20 μM] treatment. [E] p27 expression in Nf1−/− BS NSCs was increased following rictor siRNA silencing. In contrast, p27 phosphorylation (Thr 198) in Nf1−/− BS NSCs was reduced following rictor siRNA treatment. No change in p27 expression or phosphorylation was observed following rictor KD in Nf1−/− CTX NSCs. α-Tubulin was included as an internal loading control for Western blotting. [F] Rictor-mediated Akt activation regulates p27 phosphorylation and function in Nf1−/− BS NSCs. Error bars denote the mean ± SEM. (**) \( P < 0.01 \).
Discussion

Neural stem cell functional diversity in the CNS is dictated by the developmental age of the animal as well as by spatially restricted signals present in specific brain regions. In addition, NSCs from different regions of the brain may be intrinsically distinct by virtue of cell-autonomous properties innate to these unique NSC populations. These brain region-specific cell-autonomous differences are well illustrated by the NF1 tumor predisposition syndrome, in which gliomas most commonly arise in the ON and BS, but rarely occur in the cortex. In this study, we demonstrate that NSCs from the BS proliferate faster and differentiate into glial lineage cells following Nf1 gene inactivation, whereas Nf1 loss in CTX NSCs does not result in increased proliferation or gliogenesis. These differences do not reflect the mouse developmental age, as similar region-specific effects of Nf1 loss were observed in both embryonic and early postnatal NSCs. Moreover, these differences were also found in young mice in vivo following Nf1 inactivation in BLBP+ NSCs at E9.5. It is also unlikely that these differences result from unique microenvironmental influences present in the BS or CTX, since these brain region-specific differences were observed in isolated NSC populations in vitro.

Recently, we showed that astrocytes from the BS exhibit increased proliferation following Nf1 inactivation, whereas no such increase in proliferation was observed in Nf1-deficient neocortical astrocytes [Yeh et al. 2009]. To account for these brain region-specific responses to neurofibromin loss, we found that astrocytes from the CTX expressed significantly less neurofibromin relative to astrocytes from the BS and ON. Furthermore, Nf1 loss in GFAP+ cells had no effect on neocortical astrocyte proliferation in vitro or astrocyte numbers in vivo. In contrast to astrocytes, we found no differences in Nf1 gene or neurofibromin protein expression between NSCs from the CTX and the BS. This finding implicates other mechanisms in dictating the response of NSCs from different brain regions to Nf1 inactivation.

To identify the mechanism underlying the brain region-specific effect of Nf1 loss on NSC function, we sought to determine whether constitutive RAS activation in NSCs in vitro and in vivo could mimic the effects of neurofibromin loss. The finding that RAS activation in NSCs similarly increases NSC proliferation and gliogenesis in an identical brain region-specific manner supports the notion that RAS hyperactivation is the primary driver of NSC proliferation and glial differentiation following Nf1 gene inactivation. In contrast to Nf1−/− astrocytes in which only K-RAS hyperactivation was observed [Dasgupta et al. 2005a], neurofibromin loss in NSCs results in increased activity of all three RAS isoforms (H-RAS, K-RAS, and N-RAS). While we found that both activated K-RAS and H-RAS expression in NSCs led to increased BS (but not CTX) NSC proliferation, it is not clear whether the three RAS isoforms perform overlapping redundant functions with respect to NSC function. Current studies are focused on exploring the consequences of N-RAS activation on NSC function in the BS and CTX in vitro and in vivo.

Next, we determined whether differential Akt activation was responsible for the observed differences in NSC behavior. Akt is a potent regulator of NSC proliferation in many brain regions [Otaegi et al. 2006; Peltier et al. 2007], such that hyperactivation of PI3-kinase and Akt increases the proliferation of Pten-deficient NSCs [Groszer et al. 2001]. We showed previously that Akt activation was increased in Nf1-deficient embryonic NSCs [Dasgupta and Gutmann 2005], and that activated Akt expression in BLBP+ cells resulted in increased gliogenesis in vivo [Hegedus et al. 2007]. In the present study, we found differential Akt activation in BS NSCs relative to NSCs from the CTX. This brain region-specific Akt activation was also observed following K-RASG12D and H-RASG12V expression in NSCs in vitro or K-RASG12D expression in BLBP+ cells in vivo (data not shown), suggesting that deregulated neurofibromin/RAS signaling in NSCs is not responsible for the differences in brain region-specific NSC Akt activation. In contrast, expression of activated Akt in BLBP+ cells in vivo was sufficient to increase BLBP+ NSC numbers and gliogenesis, arguing that Akt activation alone accounts for the brain region-restricted differences in Nf1−/− NSC behavior. The observation that Akt is a central driver of gliogenesis is consistent with prior reports demonstrating that inhibition of PI3-kinase/Akt activation with LY294002 decreased the number of GFAP+ cells in the olfactory bulb [Otaegi et al. 2006] as well as changed the subcellular localization of Olig2, which is critical for the induction of astrocyte differentiation [Setoguchi and Kondo 2004].

In this study, we determined that BS-specific NSC Akt activation results from differences in mTOR regulation. mTOR forms complexes with raptor and rictor to negatively and positively regulate Akt activity, respectively [Guertin and Sabatini 2007]. The mTOR complex containing rictor [TORC2] positively regulates Akt activity through phosphorylation on Ser 473 [Hresko and Mueckler 2004]. Next, we showed that BS-specific NSC Akt activation results from differences in mTOR regulation. mTOR forms complexes with raptor and rictor to negatively and positively regulate Akt activity, respectively [Guertin and Sabatini 2007]. The mTOR complex containing rictor [TORC2] positively regulates Akt activity through phosphorylation on Ser 473 [Hresko and Mueckler 2004].
tumors. In this regard, rictor is overexpressed in gliomas, resulting in Akt hyperactivation [Masri et al. 2007], and PI3-kinase/Akt, but not mTOR, signaling regulates stem cell-like phenotypes in gliomas [Bleau et al. 2009].

Based on the observation that mTOR acts solely as a regulator of Akt function in NSCs, we examined Akt downstream targets that are regulated by Akt. No changes in GSK3β, FOXO1, S6, S6K, 4EBP1, PKCα, and STAT3 phosphorylation were found in Nf1+/− BS NSCs relative to those from the CTX. In contrast, we found increased expression of the p27 cyclin-dependent kinase inhibitor following Nf1 inactivation in CTX NSCs, but not in BS NSCs. p27 function is regulated by Akt-mediated phosphorylation at residues Thr 198 and Ser 10, resulting in cytoplasmic localization and inhibition of its activity [Fujita et al. 2002; Motti et al. 2004; Crean et al. 2006]. Akt also regulates Skp2 [S-phase kinase-associated protein-2], a SCF F-box protein, which binds to p27 and targets it for ubiquitination and degradation [Lin et al. 2009]. Consistent with a model in which mTOR/riCTOR-mediated Akt activation in Nf1+/− BS NSCs results in down-regulation of p27 expression, we identified an Akt-dependent increase in p27 phosphorylation [Ser 10 and Thr 198] and a reduction in nuclear (active) p27 in Nf1+/− BS, but not CTX, NSCs [DY Lee and DH Gutmann, unpubl.]. Second, p27 re-expression in Nf1+/− BS NSCs restored proliferation to levels observed in wild-type BS NSCs. Third, p27 induction [as seen in Nf1+/− CTX NSCs] was observed only in Nf1+/− BS NSCs following rictor shRNAi silencing. These findings establish mTOR/riCTOR/Akt regulation of p27 function as a key determinant of Nf1-deficient brain region-specific NSC proliferation and differentiation.

The relevance of p27 to gliomagenesis is further underscored by several observations: First, p27 loss in O2A glial progenitors results in a 2.6-fold increase in the number of GFAP+ astrocytes generated following in vitro differentiation and a 2.8-fold increase in p27-null mice in vivo [Casaccia-Bonnefil et al. 1997]. Second, p27 loss in mice increases the number and proliferation of transient amplifying [type C] neural progenitors in the SVZ in vivo [Doetsch et al. 2002]. Third, mutant epidermal growth factor receptor [EGFR] signaling, as observed in some high-grade gliomas, has been shown to regulate glioma cell growth by suppressing p27 expression through activation of the PI3-kinase/Akt pathway [Narita et al. 2002]. While these findings provide compelling support for p27 as a critical downstream Akt effector that controls NSC proliferation and perhaps gliomagenesis, it is also possible that other molecules are involved in specifying glial differentiation. In this regard, Akt is known to regulate gliogenesis by modulating the phosphorylation/expression of specific transcription factors important for glial differentiation, including N-CoR and Olig2 [Hermanson et al. 2002; Setoguchi and Kondo 2004]. Future studies will be required to address the role of these Akt-regulated transcription factors in gliogenesis and glioma formation.

Our finding that Nf1 loss results in increased numbers of Olig2+ cells is important in light of several recent reports implicating Olig2 in gliomagenesis. First, Olig2 is expressed universally in diffuse gliomas [Ligon et al. 2004], including low-grade pilocytic astrocytomas, such as those arising in children with NF1 [Takei et al. 2008]. Second, the vast majority of the proliferating cells in diffuse gliomas are Olig2+ cells [Rhee et al. 2009]. Third, Olig2 function is required for neural progenitor proliferation as well as astrocytoma formation in genetically engineered mouse glioma models in vivo [Ligon et al. 2007]. Collectively, these observations, coupled with our findings, suggest a critical role for this population of neural progenitors in glioma development.

Over the last several years, it has become widely recognized that neural stem cells residing in the SVZ of adults may be the cell of origin for gliomas [Jackson et al. 2006; Alcantara Llaguno et al. 2009]. In these studies, targeting NSCs in the SVZ niche with glioma-associated oncogenic mutation or tumor suppressor gene inactivation is sufficient for gliomagenesis. It was therefore anticipated that Nf1 loss in SVZ NSCs in vitro and in vivo resulted in more modest increases in proliferation and Olig2+ progenitor cell generation. While further experimentation will be required, it is possible that different stem cell niches are uniquely affected by both the specific cancer-associated genetic change (Nf1 loss vs. combined Nf1, p53, and Pten inactivation) and the developmental age of the animal [young vs. adult mice]. These determinants may be particularly relevant to the distinct spatial and temporal pattern of gliomagenesis in patients with NF1 [optic pathway and BS gliomas arising in children <10 yr of age] [Listernick et al. 2007].

In summary, we provide a mechanistic explanation for the differential response of spatially distinct populations of brain NSCs to neurofibromin loss. These findings are particularly germane to glioma formation in children with NF1, whose brain tumors usually develop in the optic pathway and BS, but rarely in the cortex. We showed recently that stromal signals expressed by microglia [meingioma-expressed antigen-5 [MGEA5]] or multiple other cellular elements [CXCL12] in a spatially restricted fashion along the optic pathway uniquely promote the proliferation and survival of Nf1-deficient, but not wild-type, astrocytes [Daginakatte and Gutmann 2007; Warrington et al. 2007]. In this regard, we propose that tumor formation requires a permissive microenvironment composed of cell types and molecular signals that facilitate cell growth in receptive preneoplastic cell populations. Our demonstration of a receptive preneoplastic cell type defined by differential mTOR-mediated Akt activation broadens our understanding of the mechanisms underlying NSC heterogeneity, and offers new insights relevant to the design of future therapeutics that target specific populations of cancer cells.

Materials and methods

Mice

BLBP-Cre transgenic mice were intercrossed with Nf1<sup>lox/lox</sup> mice to generate BLBP-Cre; Nf1<sup>lox/lox</sup> [N1]<sup>BLBP<sub>CKO</sub></sup> mice. To generate transgenic mice that express Kras<sup>+</sup> or Akt<sup>+</sup>, BLBP-Cre transgenic mice were intercrossed with LSL-K-Ras<sup>G12D</sup> mice and LSL-myr-Akt mice, respectively. All strains were maintained on
a C57BL/6 background and used in accordance with approved animal studies protocols at Washington University.

Culture of NSCs and generation of Nf1<sup>−/−</sup> neurospheres

CTX and BS regions were dissected from PN1 mouse pups as well as E13.5 mouse embryos of each genotype, and NSCs were generated as reported previously [Dasgupta and Gutmann 2005]. Briefly, CTX tissues were derived from the somatosensory cortex, excluding the white matter tracts and the corpus callosum, while BS tissues included the midbrain, pons, and medulla after careful removal of the cerebellar peduncles [Fig. 2B]. NSCs from Nf1<sup>Flox/Flox</sup> mouse brains were treated with Ad5-Cre or Ad5-LacZ diluted in NSC medium. After loss of neurofibromin, expression was verified by Western blotting.

Measurement of NSC proliferation

At least 10 neurospheres from each genotype were trypsinized and plated into individual wells of ultralow-binding 24-well plates with complete NSC medium containing EGF and FGF. After 10–12 d, the size (diameter) of the resulting secondary neurospheres was determined using Metamorph 7.1 [Molecular Devices].

Generation of NSCs expressing constitutively active K-Ras and H-Ras

K-Ras-expressing NSCs were generated by infecting PN1 LSL-K-Ras<sup>G12D</sup> neurospheres with Ad5-Cre. As a control, NSCs isolated from wild-type mouse brains were infected with Ad5-Cre. H-Ras-expressing NSCs were generated following infection with MSCV containing H-Ras<sup>G12V</sup> [MSCV-H-Ras<sup>G12V-GFP</sup>]. MSCV-GFP was used as a control for these experiments.

Immunohistochemistry and immunocytochemistry

Brain tissues were obtained following intracardiac perfusion with PBS and 4% PFA solution, and paraffin sections prepared as described previously [Hegedus et al. 2007]. Sections were treated with antigen retrieval solution and stained with appropriate antibodies [Supplemental Table S1]. Immunocytochemistry was performed on trypsinized NSCs plated onto 50 μg/mL poly-D-lysine-coated and 10 μg/mL fibronectin-coated 24-well plates in defined NSC medium containing N2 and B27 without growth factors. After 5 d, cells were fixed and stained with appropriate primary antibodies [Supplemental Table S1] overnight at 4°C. For fluorescence detection, suitable Alexa Fluor-tagged secondary antibodies [Molecular Probes] were used, and cells were counterstained with DAPI.

Western blotting

Western blots were performed as described previously [Dasgupta and Gutmann 2005; Hegedus et al. 2007] using the primary antibodies listed in Supplemental Table S2. Appropriate HRP-conjugated secondary antibodies [Cell Signaling] were used for detection by enhanced chemiluminescence [New England Biolabs]. Each experiment was performed with samples from at least three independent groups.

Pharmacologic inhibition studies

Neurospheres were dissociated into single cells by trypsinization and plated onto ultralow-binding 24-well plates at a density of 1 × 10<sup>3</sup> cells per well. Cells were treated with rapamycin [0.5–1 nM] and LY294002 [20 μM] for 5–6 d in vitro.

For the in vivo experiments, rapamycin [1 mg/kg per day] was injected intraperitoneally into pregnant females from E16 to E18. Postnatally, 0.5 mg/kg rapamycin was injected subcutaneously at PN1 and PN3, and the pups were perfused at PN8.

Retrovirus infection

Rictor siRNA [NM0310168.2, TRCN0000123397] or GFP-containing plKO.1 plasmid was cotransfected with VSV-G and Δ8.9 helper DNA into HEK293T cells using the FuGENEHDI transfection reagent [Roche]. p27-MSCV was cotransfected with T-helper DNA into HEK293T cells. Nf1<sup>−/−</sup> neurospheres [Ad5-Cre-infected Nf1<sup>Flox/Flox</sup> cells] were dissociated into single cells by trypsin and transduced with viral supernatants from HEK293T cells. Transfection efficiency was evaluated by Western blotting. Each experiment was performed at least two times with identical results.

Statistical analysis

Each experiment was performed with samples from at least three independent groups. Statistical significance (P < 0.05) was determined using the Student’s t-test and GraphPad Prism 4.0 software [GraphPad, Inc.].

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