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ORIGINAL ARTICLE

NG2-cells are not the cell of origin for murine neurofibromatosis-1 (*Nf1*) optic glioma

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Low-grade glial neoplasms (astrocytomas) represent one of the most common brain tumors in the pediatric population. These tumors frequently form in the optic pathway (optic pathway gliomas, OPGs), especially in children with the neurofibromatosis type 1 (NF1)-inherited tumor predisposition syndrome. To model these tumors in mice, we have previously developed several *Nf1* genetically-engineered mouse strains that form optic gliomas. However, there are three distinct macroglial cell populations in the optic nerve (astrocytes, NG2+ (nerve/glial antigen 2) cells and oligodendrocytes). The presence of NG2+ cells in the optic nerve raises the intriguing possibility that these cells could be the tumor-initiating cells, as has been suggested for adult glioma. In this report, we used a combination of complementary *in vitro* and novel genetically-engineered mouse strains *in vivo* to determine whether NG2+ cells could give rise to *Nf1* optic glioma. First, we show that *Nf1* inactivation results in a cell-autonomous increase in glial fibrillary acidic protein+ (GFAP+), but not in NG2+, cell proliferation *in vitro*. Second, similar to the GFAP-Cre transgenic strain that drives *Nf1* optic gliomagenesis, NG2-expressing cells also give rise to all three macroglial lineages *in vivo*. Third, in contrast to the GFAP-Cre strain, *Nf1* gene inactivation in NG2+ cells is not sufficient for optic gliomagenesis *in vivo*. Collectively, these data demonstrate that NG2+ cells are not the cell of origin for mouse optic glioma, and support a model in which gliomagenesis requires *Nf1* loss in specific neuroglial progenitors during embryogenesis.

INTRODUCTION

Brain tumors are the most common solid tumor in the pediatric population, with low-grade glial neoplasms (pilocytic astrocytoma) representing the major histologic subtype in children between the ages of five and fifteen (Central Brain Tumor Registry of the United States (CBTRUS) 2012; www.cbtrus.org). In contrast to adults, low-grade gliomas tend to arise in distinct brain regions, including the cerebellum, brainstem and optic pathway. This unique geographic predilection is nicely illustrated by the pattern of glioma formation in individuals with the neurofibromatosis type 1 (NF1)-inherited tumor syndrome. Fifteen to twenty percent of children with NF1 will develop a low-grade glioma, the majority of which arise in the optic pathway (optic pathway glioma, OPG).[1,2] These OPGs can arise anywhere along the optic pathway, from the retro-orbital optic nerve to the post-chiasmatic optic tract.[3,4] Most OPGs are slow-growing tumors composed of glial fibrillary acidic protein (GFAP)-immunoreactive cells with low proliferative indices (<1%). Whereas death from these tumors is rare, there is significant associated morbidity, including hypothalamic dysfunction and visual loss. In this regard, nearly half of children with NF1-associated OPGs will have visual impairment at initial presentation.[5]

To gain insights into the molecular and cellular pathogenesis of NF1-associated optic glioma, we and others have developed *Nf1* genetically-engineered mouse strains. Based on their glial histology, *Nf1* loss in GFAP-immunoreactive cells has been modeled using GFAP-Cre mouse lines. In these experiments, *Nf1*+/− mice with GFAP-Cre-mediated *Nf1* inactivation develop optic glioma.[6,7] Careful analysis of the GFAP-Cre strains used in these studies has revealed that Cre expression first occurs in GFAP+ neuroglial progenitor cells either at E11.5[7] or E14.5,[8] rather than in differentiated astrocytes. These findings support a model in which *Nf1* loss must occur in specific neuroglial progenitors during embryonic development in order for gliomagenesis to ensue.

In the optic nerve and relevant ventricular (germinal) zones, there are two types of potential neuroglial progenitors, GFAP+[9,10] and nerve/glial antigen 2+ (NG2+) cells.[11] This latter population has been shown to represent a potential cell of origin for rat malignant gliomas,[12,13] suggesting that NG2+ progenitors may represent the initiating cell for *Nf1* optic glioma. To determine whether NG2+ neuroglial progenitors could serve as the cell of origin for *Nf1* genetically-engineered mouse optic glioma, we employed a combination of *in vitro* and *in vivo* strategies. In this report, we demonstrate that *Nf1* loss in NG2+ cells *in vitro* does not increase glial cell proliferation and that *Nf1* loss in NG2+ progenitor cells *in vivo* is insufficient for optic gliomagenesis. Together, these data exclude NG2+ cells as the likely cell of origin for NF1-associated optic glioma and establish a model of gliomagenesis in which *Nf1* loss occurs in specific progenitors during embryonic development.

RESULTS

The mouse optic nerve is composed of three distinct types of macroglial cells

In order to better characterize the macroglial compartment that contributes to optic gliomagenesis, we performed immunostaining

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with antibodies that recognize GFAP (astrocytes), nerve/glial antigen 2 (NG2 cells) and adenomatous polyposis coli (APC; oligodendrocytes). We found that the majority of macroglia in both wild type (WT) and optic glioma-bearing (*Nf1*^{flox/mut}; GFAP-Cre; OPG mice) mouse optic nerves are APC+ oligodendrocytes at both 3 weeks and 3 months of age. In contrast, GFAP+ and NG2+ cells compromise a smaller percentage of optic nerve macroglial cells (Figure 1a and Supplementary Figure 1). Importantly, upon *Nf1* loss, we observed a two-fold increase in the number of GFAP+ astrocytes in the optic nerves of OPG mice relative to their WT counterparts. The number of NG2+ cells and oligodendrocytes did not change after *Nf1* inactivation (Figure 1a).
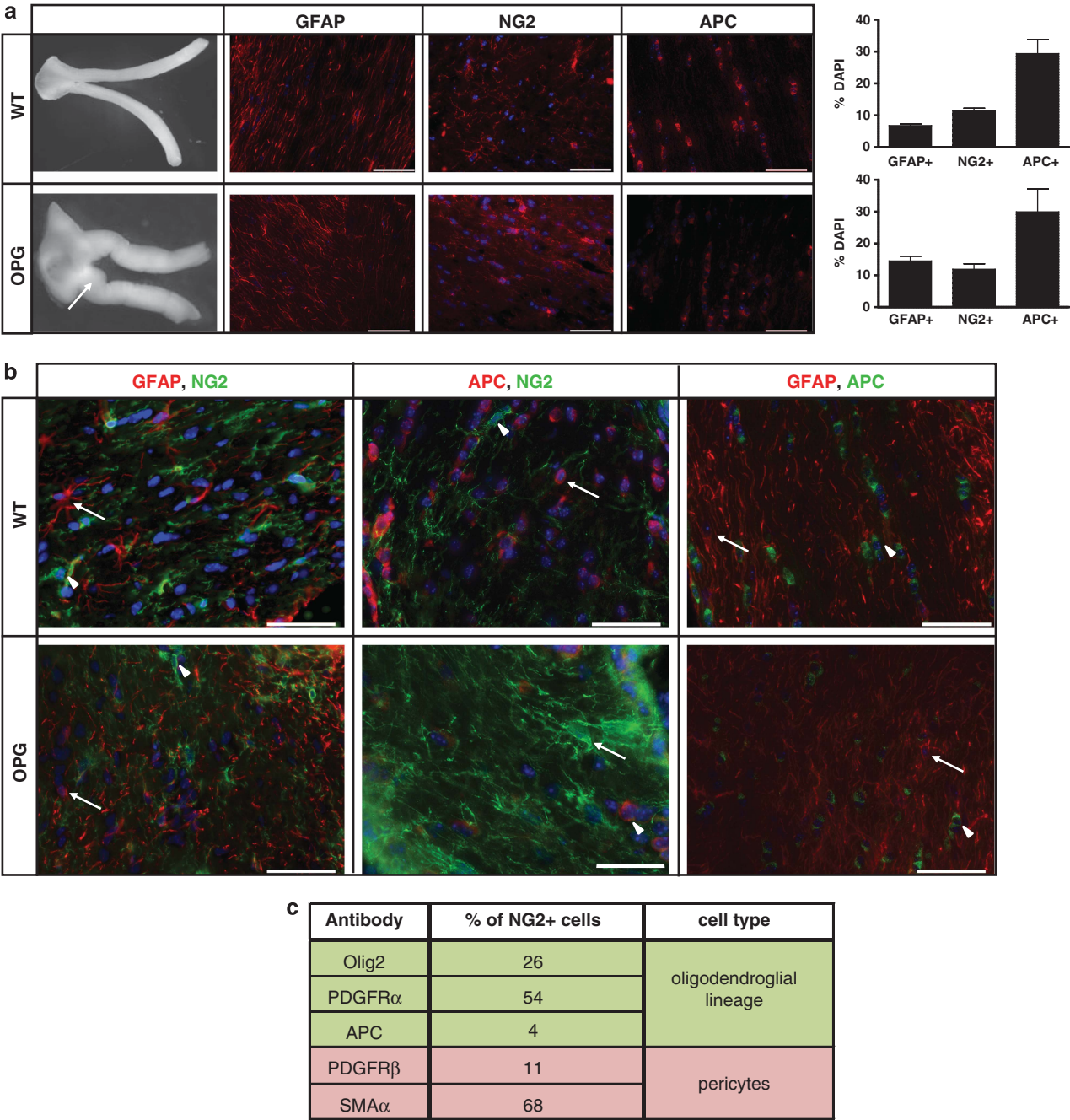

Figure 1. Optic nerve astroglial cell populations in WT and *Nf1* OPG mice. (**a**) *Nf1*^{flox/mut}; GFAP-Cre (OPG) mice develop optic nerve gliomas. Representative images of the optic nerves from 3-month old WT and OPG mice are shown. The arrow denotes an enlarged optic nerve and chiasm in one representative OPG mouse, not seen in WT mice. Histological comparison of cell type-specific markers demonstrates that ~30% of the cells are APC+, 11% of the cells are NG2+ and 7% of the cells are GFAP+. Increased numbers of GFAP+ astroglial cells are found in the optic nerves of OPG mice compared to WT mice. Each error bar represents mean ± s.e.m. (**b**) Double-labeling of 3-month-old WT and OPG nerves shows that these three glial cell populations are distinct. APC/NG2 double-positive cells account for fewer than 5% of the cells in the optic nerve. Representative images are shown. Scale bar 50 μm. DAPI (blue) was used as a counterstain to identify all cells in the sections. (**c**) NG2 double-labeling experiments revealed that 68 and 11% of the NG2 cells are SMAα+ or PDGFRβ+, respectively (pericyte markers), whereas 26 and 54% of the NG2+ cells are Olig2+ and PDGFRα+, respectively (oligodendroglial lineage markers).

To establish that these macroglia represent distinct cell types, we performed double-labeling immunohistochemistry. In these experiments, there were no GFAP+/APC+ or GFAP+/NG2+ cells, and fewer than 5% of the APC+ cells were NG2-immunopositive (Figures 1b and c). Next, we demonstrated that nearly 100% of GFAP+ cells also co-expressed aldehyde dehydrogenase 1 family, member L1 (ALDH1L1), previously reported as a marker of adult rat astrocytes.[14] Similar to the GFAP immunostaining, we did not detect ALDH1L1+/NG2+ double-positive cells (Supplementary Figure 2).

To better characterize the NG2+ cell population in the optic nerve, we performed additional experiments based on previous studies on NG2+ cells from other brain regions, suggesting that NG2+ cells can be either pericytes[15] or oligodendrocyte precursors (OPCs).[16] First, we demonstrated that the majority of NG2+ cells (68%) in the normal optic nerve co-label with smooth muscle actin α (SMAα; pericyte marker), whereas only 11% co-label with platelet-derived growth factor receptor β (PDGFRβ; pericyte progenitor marker). However, 26% of the NG2+ cells in the normal optic nerve co-labeled with Olig2, while 54% of the NG2+ cells were also PDGFRα+. Few of the NG2+ cells were APC immunopositive (Figure 1c and Supplementary Figure 3A). These findings highlight the issues related to confidently assigning a cellular identity to NG2+ cells using available antibodies.

Second, to determine the identity of the NG2+ cells in $Nf1^{flox/mut}$; GFAP-Cre mouse optic gliomas, we performed NG2 and SMAα double labeling (Supplementary Figure 3B). Nearly 100% of the NG2+ cells were SMAα+. A similar SMAα-staining pattern was also observed in a representative human NF1-associated OPG (Supplementary Figure 3C); however, NG2/SMAα double labeling could not be performed in human tissue owing to technical limitations of available NG2 antibodies for human tissue.

Third, as pericytes are often clustered around blood vessels, we performed NG2/endoglin double labeling to determine whether there was preferential clustering of NG2+ cells near endothelial cells in $Nf1^{flox/mut}$; GFAP-Cre mouse optic gliomas (Supplementary Figure 3D). In these experiments, we observed a uniform distribution of NG2+ cells in the optic nerve with no preferential association near endoglin+ blood vessels.

To define the populations of macroglial cells that increase their proliferation following Nf1 gene inactivation in OPG mice, we performed Ki67 double-labeling experiments. Following GFAP-Cre-mediated Nf1 loss, we observed a three-fold increase in cell proliferation (Ki67-labeling index) relative to WT mice (Figure 2a). To determine which macroglial cells undergo GFAP-Cre-mediated Nf1 inactivation, we performed lineage tracing experiments using the Rosa-GREEN reporter strain. We found that all three macroglial cells (APC+ oligodendrocytes, NG2+ glia, and GFAP+ astrocytes) exhibited Cre-mediated enhanced green fluorescent protein (EGFP) expression (Supplementary Figure 4), and therefore, could represent the Nf1-deficient preneoplastic/neoplastic cell population relevant to optic gliomagenesis in mice.

Based on these findings, to determine which macroglial cell in the murine Nf1 optic gliomas had increased proliferation following Nf1 gene inactivation, double immunofluorescence studies were performed. Consistent with the designation of these tumors as astrocytomas, there were no proliferating APC+ cells (oligodendrocytes). However, both NG2+ and GFAP+ cells exhibited increased proliferation in the optic nerves of OPG mice relative to littermate controls at 3 months of age (Figure 2b). Similar results were obtained using ALDH1L1 as an additional astrocyte marker (Supplementary Figure 5). The small number of proliferating cells in prechiasmatic optic nerve and chiasm of WT mice (<2 Ki67+ cells per specimen) precluded a meaningful comparison. Together, these findings demonstrate that only NG2+ and GFAP+, but not APC+, macroglial cells hyperproliferate following Nf1 loss in OPG mice in vivo.

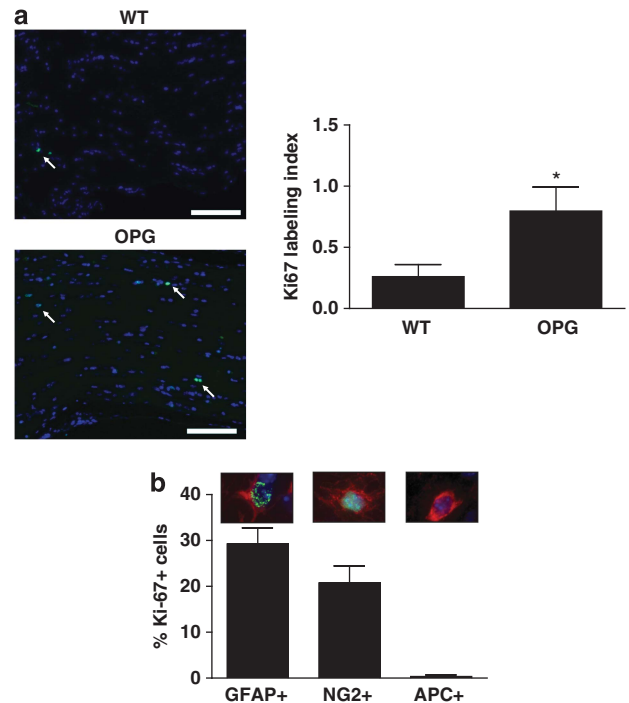
Figure 2. Neurofibromin loss results in increased NG2 and GFAP cell proliferation in vivo. (a) Proliferation was measured by Ki-67 labeling. Nf1 inactivation results in a three-fold increase in the percent of Ki-67 positive cells (labeling index) in the optic nerves of OPG mice compared to WT mice at 3 months of age. Representative images are shown. Arrows denote representative Ki-67-immunopositive cells. Scale bar 100 μm. (b) The percentages of GFAP/Ki-67, NG2/Ki-67 and APC/Ki-67 cells in the optic nerves of OPG mice at 3 months of age are shown. The images above each bar in the graph depict representative GFAP/Ki-67 double-positive, NG2/Ki-67 double-positive, or APC-positive/Ki-67-negative cells, respectively. Each error bar represents mean ± s.e.m. Asterisks denote statistically significant differences (*) $P = 0.0482$.

Only GFAP+ optic nerve astrocytes exhibit increased proliferation in response to Nf1 inactivation in vitro

Previous studies from our laboratory revealed that astrocyte cultures from the optic nerve are cellularly- and molecularly-distinct macroglial cell populations.[11] Whereas primary postnatal (PN) day 1 astrocyte cultures from the brainstem, neocortex, and cerebellum are composed of >98% GFAP+ cells with fewer than 5% NG2+ cells, optic nerve astrocyte cultures under the identical in vitro conditions are composed of ~30% GFAP+ and ~70% NG2+ cells (Figure 3a). Similar to macroglial cells in the intact mouse optic nerve, there were no glial cells expressing both GFAP and NG2. Under these culture conditions, no O4+ oligodendrocytes were generated.

To determine which glial cell population had the capacity to hyperproliferate in response to Nf1 gene inactivation, PN1 $Nf1^{flox/flox}$ optic nerve astroglial cultures were generated (passage 0) and infected with either Ad5-Cre or Ad5-LacZ virus to generate Nf1-deficient (Nf1−/−) and WT astroglial cells (passage 2), respectively. Following Cre-mediated Nf1 inactivation, neurofibromin was undetectable in these cultures (Figure 3b) concomitant with an overall 1.5-fold increase in proliferation (Figure 3c). Whereas Nf1 loss resulted in a ~1.5-fold increase in proliferation of GFAP+ cells, no increased proliferation was observed in the NG2+ cell population relative to Ad5–LacZ–infected cultures (Figure 3d). It is possible that differences in Nf1 gene expression underlie the failure of NG2+ cells to increase their proliferation
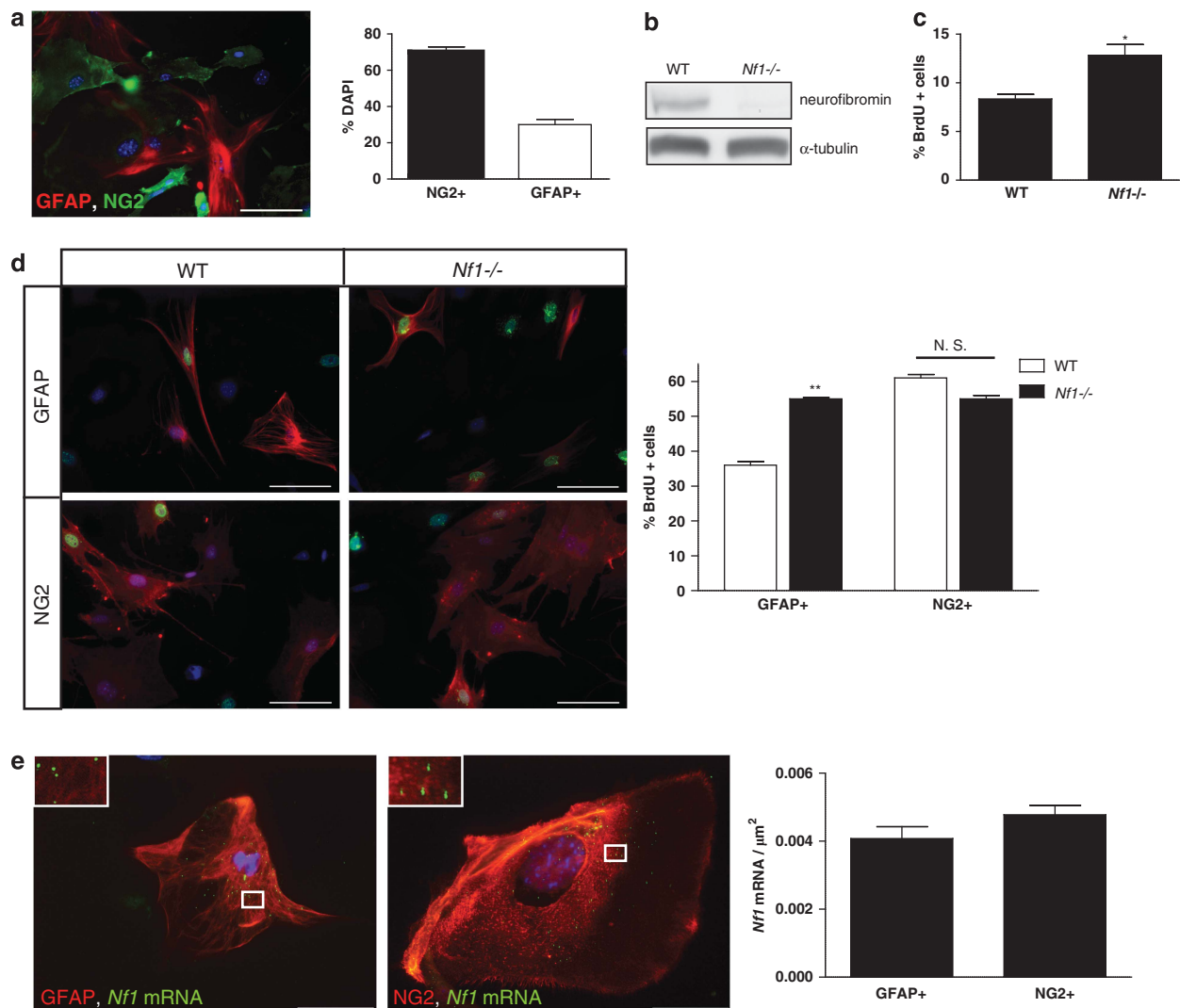
Figure 3. Neurofibromin loss results in increased optic nerve GFAP + cell proliferation *in vitro*. (**a**) Double-labeling immunocytochemistry reveals that 30% of the cells in optic nerve glial cell cultures are GFAP +, whereas 70% of the cells are NG2 + . There are no GFAP/NG2 double-positive cells. Following *Nf1* inactivation by Ad5-Cre expression, loss of neurofibromin expression was observed (**b**) coincident with an increase in proliferating (BrdU +) cells (**c**). α-tubulin is used as an internal control for protein loading. (**d**) Following *Nf1* inactivation by Ad5-Cre expression, only the GFAP + population of cells exhibited increased proliferation. Representative BrdU + proliferating cells (green) and DAPI + nuclei (blue) are shown. The percentage of BrdU + /NG2 + and BrdU + /GFAP + cells are represented as the mean ± s.e.m. Scale bar, 100 μm. N. S. = not significant, (**) $P = 0.0055$, (*) $P = 0.0129$. (**e**) FISH revealed comparable levels of *Nf1* mRNA expression (green particles; inset) in GFAP + and NG2 + cells. The number of signals is the average of 10 counted cells per area and is displayed in the graph. Nuclei were counterstained with DAPI (blue). Representative images are shown. Scale bar, 50 μm.

following *Nf1* loss. To determine whether NG2 + and GFAP + cells have different relative levels of *Nf1* mRNA expression, we employed *Nf1* RNA fluorescence *in situ* hybridization (FISH). In these experiments, GFAP + cells and NG2 + cells had comparable levels of *Nf1* mRNA expression (Figure 3e). Collectively, these results demonstrate that GFAP + astrocytes are the macroglial population with the greatest ability to hyperproliferate following neurofibromin loss *in vitro*.

NG2 + cells give rise to oligodendrocytes and astrocytes *in vivo*

To further analyze the NG2 + cell population in mouse optic nerve glial cell cultures, we performed several additional experiments. First, we used double-labeling immunofluorescence to demonstrate that nearly all NG2 + cells co-label with PDGFRβ, while 64% of the NG2 + cells are SMAα + (pericyte markers). Moreover, 40 and 67% of the NG2 + cells are double-positive for A2B5 and

PDGFRα, respectively (OPC markers) (Figure 4a). Second, to determine the capacity of these NG2 + cells to differentiate into distinct glial cell populations, we employed immunopanning to obtain highly purified NG2 + cells from the optic nerve. This purified NG2 + cell population remained 100% NG2 + after one day in culture, without any contaminating GFAP + astrocytes or O4 + oligodendrocytes, but did not generate either O4 + or GFAP + cells under either serum or serum-free conditions after 3 and 7 days in culture (Figure 4b). These findings again underscore the problems associated with confidently distinguishing NG2 + cell populations using cell type-specific antibodies, and demonstrate that NG2 + optic nerve cells are unlikely to be glial progenitors *in vitro*.

Third, to determine whether NG2 + cells could give rise to the three major macroglial populations in the optic nerve as has been reported for the forebrain,[17] we leveraged a mouse strain in which Cre recombinase is expressed from the endogenous NG2
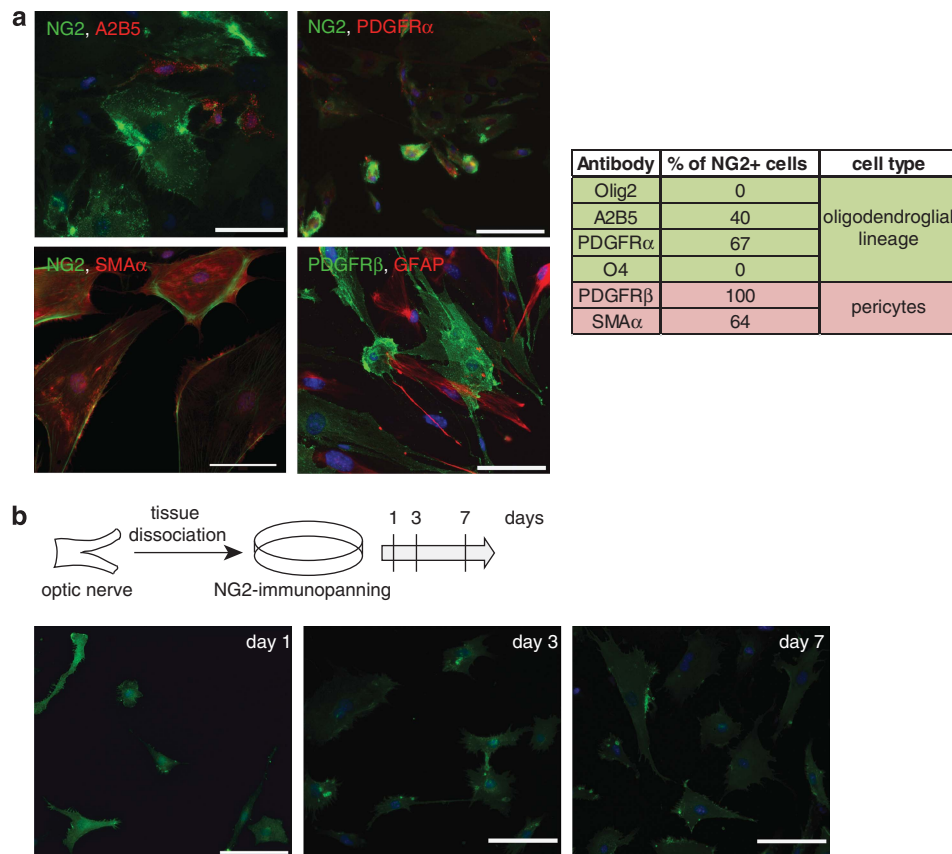
Figure 4. Characterization of NG2+ optic nerve cells *in vitro*. (**a**) 64% of the NG2 cells are double-positive for SMAα and almost all are PDGFRβ+ (pericyte markers). None of the GFAP+ cells are SMAα+ or PDGFRβ+. In contrast, 40 and 67% of the NG2+ cells are A2B5+ or PDGFRα+, respectively (oligodendroglial lineage markers). (**b**) Following NG2 antibody immunopanning and replating, PN1 optic nerve NG2+ cells grown for 1, 3 and 7 days do not differentiate into either GFAP+ or O4+ cells *in vitro*. Nuclei were counterstained with DAPI (blue). Representative images are shown. Scale bar, 100 μm.

promoter.[17] NG2-Cre mice were crossed with Rosa-GREEN reporter mice, and at 3 weeks of age, we detected EGFP expression in NG2+, APC+ and ALDH1L1+ cells (Figure 5), similar to that observed with the GFAP-Cre strain used to generate *Nf1* optic gliomas (Supplementary Figure 4). Similar to the *in vitro* findings, we also found SMAα+ cells with NG2-mediated EGFP expression in the optic nerve (Supplementary Figure 6A). Since A2B5 cannot be employed for immunohistochemistry, we derived optic nerve cultures from Rosa-GREEN × NG2-Cre mice to demonstrate that NG2+ cells can give rise to A2B5+ cells in the optic nerve (Supplementary Figure 6B). Collectively, these results demonstrate that NG2+ cells give rise to all macroglial cell lineages *in vivo*.

Nf1^flox/mut^; NG2-Cre mice do not develop optic glioma
To determine whether *Nf1* loss in NG2+ progenitor cells is sufficient for optic gliomagenesis in mice, we generated *Nf1*+/− mice with NG2-specific *Nf1* inactivation. This genetic configuration is similar to the *Nf1* optic glioma mice in which *Nf1*+/- mice harbor neurofibromin loss in GFAP+ cells.[6] Cohorts of ten *Nf1*^flox/mut^; NG2-Cre mice and ten control littermates (NG2-Cre and *Nf1*^flox/WT^; NG2-Cre mice; n = 10 each) were collected. These mice were healthy and bred successfully.

Analysis of the optic nerves from *Nf1*^flox/mut^; NG2-Cre mice revealed no evidence of optic glioma formation at 3 months of age. Specifically, we observed no gross morphological or histological differences and only a slight increase in optic nerve volume.

There was no change in mitotic indices, microglia numbers, GFAP+ astrocyte numbers or NG2+ glial cell numbers (Figure 6). Moreover, no optic gliomas were observed using the above criteria in a cohort of *Nf1*^flox/mut^; NG2-Cre mice at 6 months of age (data not shown; n = 7).

The failure to generate optic gliomas in *Nf1*^flox/mut^; NG2-Cre mice could reflect a number of possibilities, including the efficiency of Cre-mediated recombination as well as the timing and specific cell type in which *Nf1* loss occurs. To demonstrate *Nf1* inactivation in the optic nerve, we first employed recombination PCR on whole tissue (Figure 7a). Both *Nf1*^flox/mut^; NG2-Cre and *Nf1*^flox/mut^; GFAP-Cre mouse optic nerves exhibited *Nf1* gene inactivation. Second, we employed the Rosa-GREEN reporter mice to show that both NG2-Cre and GFAP-Cre mouse optic nerves had equivalent numbers of EGFP+ cells, indicative of Cre-mediated excision (Figure 7b). Third, we used RNA *in situ* hybridization to measure *Nf1* mRNA expression *Nf1*^flox/mut^; NG2-Cre and *Nf1*^flox/mut^; GFAP-Cre mouse optic nerves. There was a similar reduction in total *Nf1* mRNA expression in these mice relative to their WT counterparts (42% decrease in *Nf1*^flox/mut^; NG2-Cre and 67% decrease in *Nf1*^flox/mut^; GFAP-Cre mouse optic nerves) (Figure 7c). As we could not confidently localize the *Nf1* mRNA FISH signal to a specific cell type by double-labeling immunofluorescence, we performed GFAP/neurofibromin and NG2/neurofibromin double labeling. In optic nerve specimens from both *Nf1*^flox/mut^; NG2-Cre and *Nf1*^flox/mut^; GFAP-Cre mice, there was a 30–37% reduction in the percent of GFAP/neurofibromin double-positive cells relative to WT mice, with no change in the percent of NG2/neurofibromin
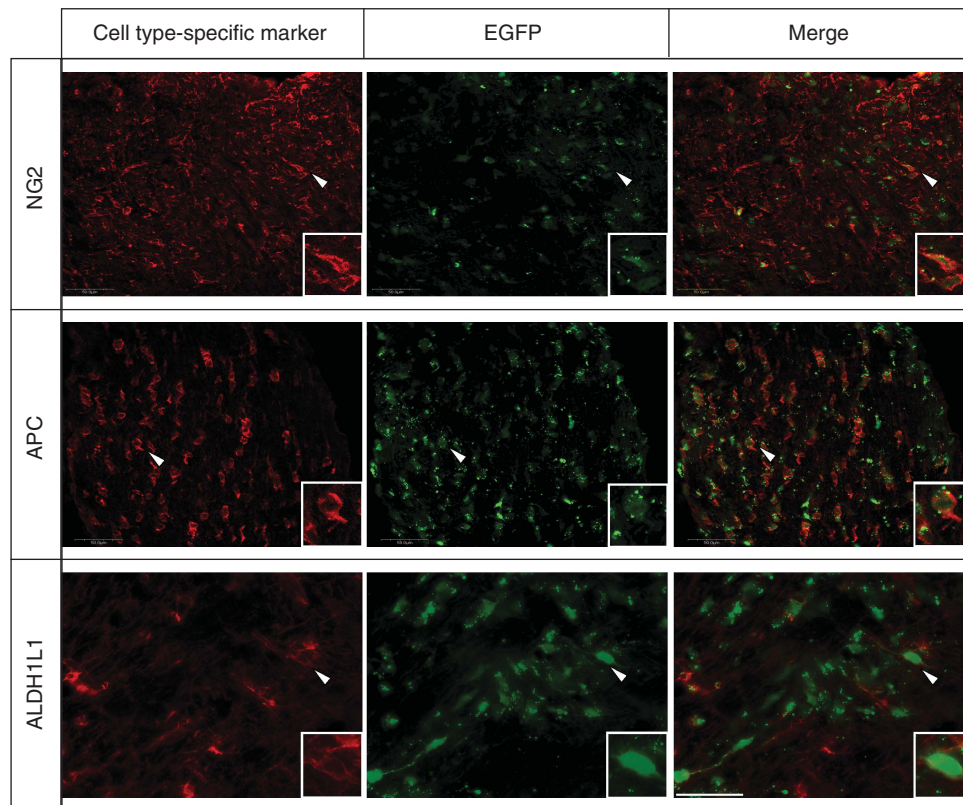
Cell type-specific marker	EGFP	Merge

Figure 5. NG2-Cre cells give rise to NG2+, ALDH1L1+ and APC+ glial cell populations *in vivo*. Optic nerve sections from Rosa-GREEN × NG2-Cre mice reveal EGFP+/NG2+ cells, EGFP+/APC+ (oligodendrocytes) and EGFP+/ALDH1L1+ (astrocytes). Scale bar, 50 μm. Representative images are shown with insets of immunopositive cells.

double-positive cells (Figure 7d and Supplementary Figure 7). These data exclude differences in *Nf1* gene expression or inactivation as the likely reason for the failure of *Nf1*^{*flox/mut*}; NG2-Cre mice to develop optic glioma.

Next, to determine when and where the NG2-Cre transgene is first expressed relative to the GFAP-Cre transgene used to generate *Nf1* optic glioma mice, NG2-Cre and GFAP-Cre mice were crossed to Rosa-GREEN reporter mice and their brains examined at various developmental times. In these experiments, Cre activity was first detected in the third (TVZ) and lateral ventricular zones (lv-SVZ) at embryonic day 14.5 (Figure 8a), similar to the GFAP-Cre mouse strain (Figure 8b), excluding differences in the timing of *Nf1* loss.

Finally, we sought to determine where the NG2-Cre transgene was expressed in the lateral and third ventricle germinal zones *in vivo*. Whereas both NG2-Cre and GFAP-Cre mice generate EGFP+/nestin+ cells, the EGFP+/nestin+ cells in the NG2-Cre strain were not preferentially localized to the periventricular areas around the lv-SVZ and TVZ (Figure 8a). Moreover, there were EGFP+/PDGFRα+ and EGFP+/PDGFRβ+ cells similarly distributed throughout the TVZ (Supplementary Figure 6C). In contrast, EGFP+/nestin+ cells in the GFAP-Cre mouse were preferentially localized to the periventricular zones along the wall of the third ventricle (Figure 8b), consistent with their designation as potential neural stem cells relevant to optic glioma.[18] Collectively, these findings argue that NG2+ progenitor cells are not identical to GFAP-Cre optic glioma progenitors with respect to their ventricular location, and support a model in which *Nf1* inactivation likely occurs in specific progenitors (GFAP-Cre-expressing stem cells) to result in optic gliomagenesis.

DISCUSSION

Numerous studies have shown that oligodendrocytes, astrocytes and NG2+ cells comprise the three types of macroglial cells in the mammalian central nervous system. In most brain regions, astrocytes are the major macroglial cell population, whereas NG2-glia comprise 8–9% of the total cell population in white matter regions, and 2–3% of the total cells in gray matter regions.[19] Relevant to low-grade gliomas in children, similar macroglial cell populations exist in the optic nerve. The optic nerve is a heavily myelinated structure composed of axons carrying visual information from the retina to the lateral geniculate bodies. For this reason, oligodendrocytes constitute the majority of the macroglial cells in the optic nerve.

However, optic gliomas are not oligodendroglial neoplasms (oligodendrogliomas), and are instead composed of GFAP-immunoreactive cells. Consistent with observations in human tumors, we found that the majority of the proliferating cells in the *Nf1* mouse optic glioma were GFAP+ or NG2+ cells, rather than APC+ cells (oligodendrocytes). In addition, we found that glial cell cultures from the normal optic nerve were composed of two distinct non-oligodendrocyte cell types that express either NG2 or GFAP, but not both markers,[11] analogous to studies performed on cells from other regions of the central nervous system. For this reason, we focused on the relative contributions of GFAP+ and NG2+ cells to *Nf1* murine optic gliomagenesis.

Early landmark studies examining rat optic nerve glia revealed two types of astrocytes (type-1 and type-2).[20] In contrast to type-1 astrocytes, type-2 astrocytes are defined by A2B5 expression[20,21] and are thought to arise from A2B5+, but GFAP- and galactocerebrosidase-negative, oligodendrocyte-type-2 astrocyte precursor cells.[20–22] Unfortunately, A2B5+ cells cannot be reliably
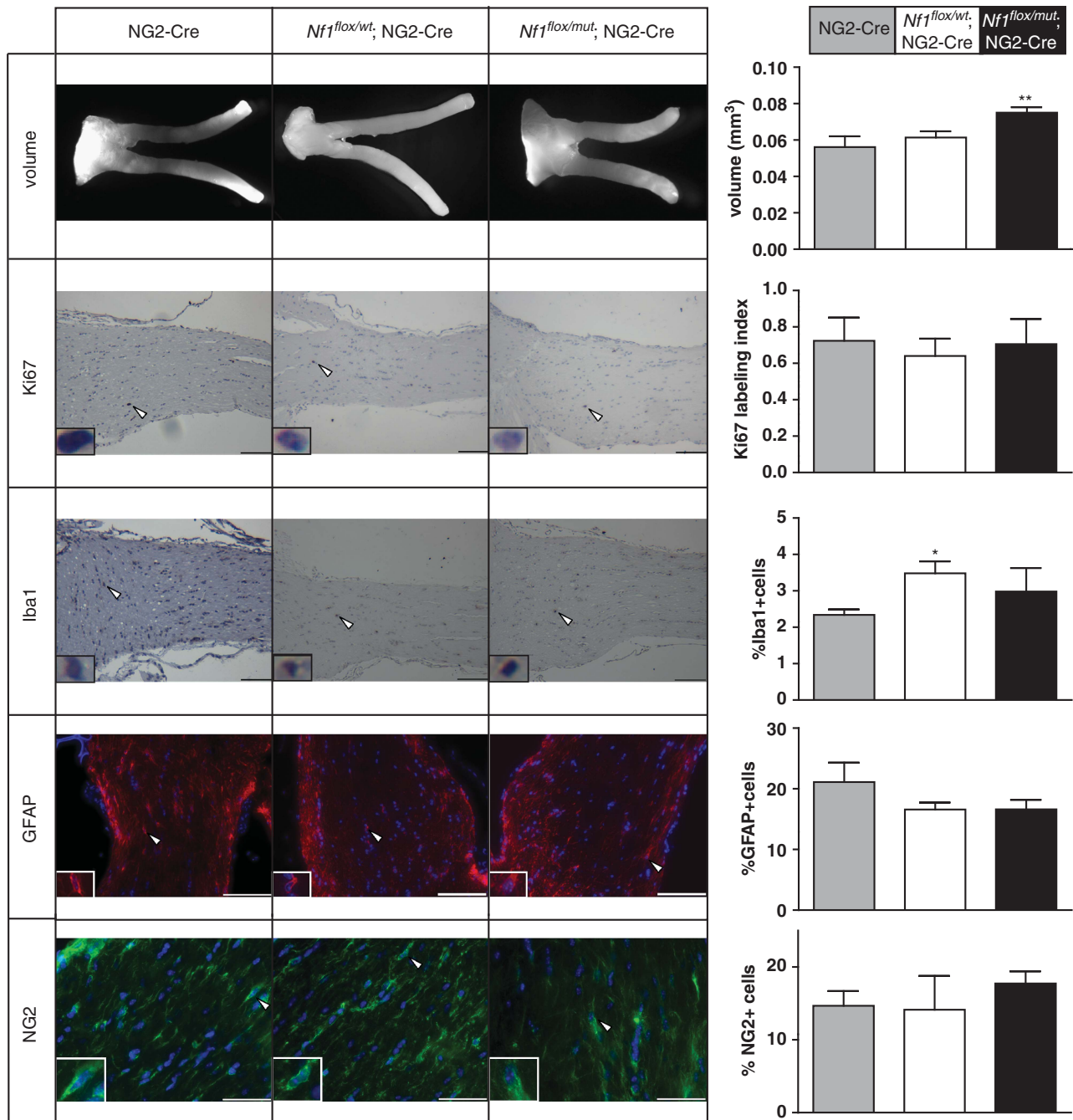
Figure 6. $Nf1^{flox/mut}$; NG2-Cre mice do not develop optic glioma. Representative images of the optic nerves from all three genotypes are shown. $Nf1^{flox/mut}$; NG2-Cre mice optic nerve volumes are slightly increased relative to control (NG2-Cre and $Nf1^{flox/WT}$; NG2-Cre) mice. However, no increases in the Ki67-labeling index, percent of Iba1+ microglia, or percent of GFAP+ or NG2+ cells were found in $Nf1^{flox/mut}$; NG2-Cre mice relative to control mice. Scale bar, 100 μm, 50 μm NG2 panel. Error bars represent mean ± s.e.m. Asterisks denote statistically significant differences (*) $P = 0.0119$, (**) $P < 0.0052$.

detected *in vivo* owing to antibody technical limitations. Based on the fact that some rat optic nerve NG2+ cells co-express A2B5 and are capable of generating GFAP+ astrocytes[23] and A2B5+ cells[24] *in vitro*, it has been postulated that a population of NG2+ cells may serve as O2A progenitors. The progenitor properties of NG2+ cells is reinforced by studies in the spinal cord in which some NG2+ cells express nestin or A2B5, and can give rise to oligodendrocytes and astrocytes.[25,26] Additionally, embryonic-mouse forebrain neurospheres contain a small population of NG2/A2B5 double-positive cells.[27] However, using

NG2-Cre mice, none of the GFAP+ cells originated from NG2+ cells in white matter regions *in vivo*.[17]

In support of the role for NG2+ cells as glial progenitors in the optic nerve, we found that 40% of the NG2+ cells in the mouse optic nerve express A2B5 *in vitro*, and that NG2+ cells give rise to astrocytes *in vivo*. Moreover, we found that 26 and 54% of the NG2+ cells in the optic nerve co-express either Olig2 or PDGFRα, respectively, markers of glial progenitor cells. The ability of NG2+ cells to function as progenitors for astrocytes in some regions of the central nervous system (optic nerve, spinal cord), but not in
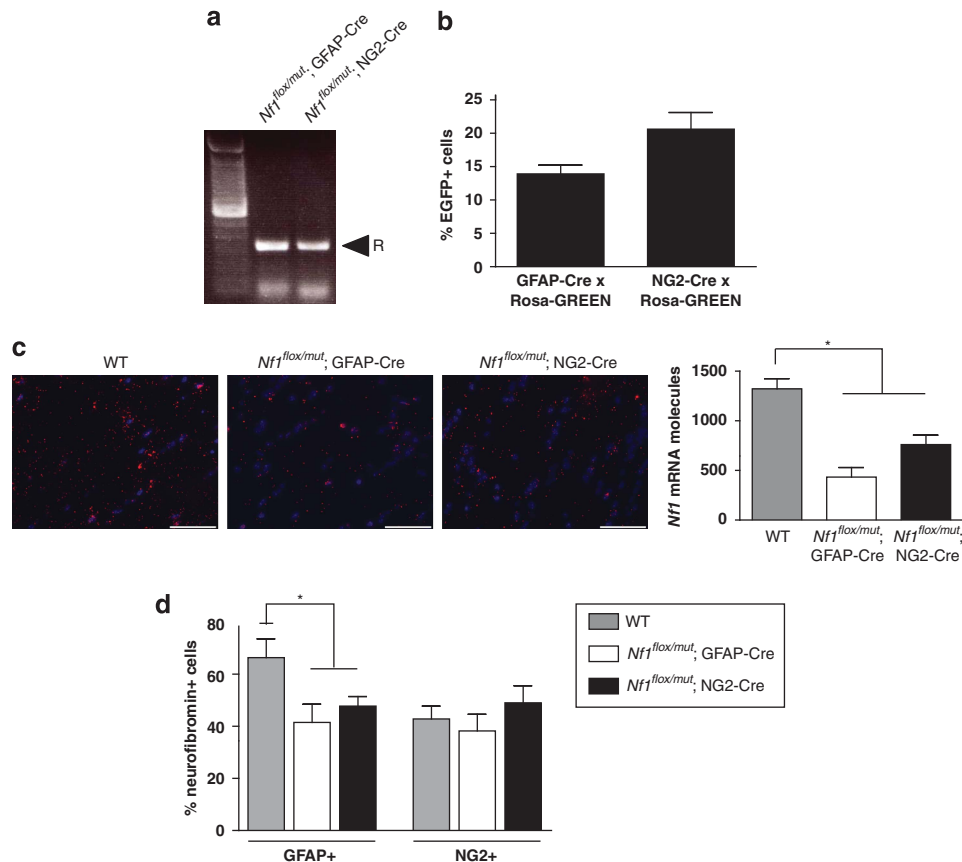
Figure 7. *Nf1*$^{flox/mut}$; NG2-Cre mice. (**a**) Recombination PCR demonstrates Cre-mediated *Nf1* gene recombination (R) in both *Nf1*$^{flox/mut}$; GFAP-Cre and *Nf1*$^{flox/mut}$; NG2-Cre mice. (**b**) The efficiency of Cre-mediated recombination was determined following intercrossing of GFAP-Cre and NG2-Cre with Rosa-GREEN reporter mice. The percent of EGFP + cells relative to the total number of cells in Rosa-GREEN × GFAP-Cre mice (13%) is similar to that observed in Rosa-GREEN × NG2-Cre mice (20%). (**c**) FISH analysis reveals similar reductions in *Nf1* mRNA (red) expression in the optic nerves of *Nf1*$^{flox/mut}$; GFAP-Cre mice (67%) and *Nf1*$^{flox/mut}$; NG2-Cre mice (42%) compared to WT mice. Nuclei were counterstained with DAPI (blue). Representative images are shown. Scale bar, 50 μm. (**d**) *Nf1* deletion in *Nf1*$^{flox/mut}$; NG2-Cre mice was determined by GFAP/neurofibromin and NG2/neurofibromin double-labeling using *Nf1*$^{flox/flox}$ mice as controls. Similar to *Nf1*$^{flox/mut}$; GFAP-Cre mice with optic glioma, *Nf1*$^{flox/mut}$; NG2-Cre mouse optic nerves exhibit a 30% reduction in the percentage of GFAP/neurofibromin double-positive cells. No change in the percent of NG2/neurofibromin double-positive was observed in *Nf1*$^{flox/mut}$; NG2-Cre mice or *Nf1*$^{flox/mut}$; GFAP-Cre mouse optic nerves. Each error bar represents mean ± s.e.m. Asterisks denote statistically significant differences (*) $P = 0.044$.

other locations (white matter regions), highlights the innate heterogeneity of this population of cells.

NG2 has also been shown to label pericytes, which function as vital integrators of the neurovascular unit and coordinate a variety of homeostatic functions, including angiogenesis, blood–brain–barrier integrity, and clearance of toxic cellular byproducts.[28] To determine whether the NG2 + cells in the optic nerve were pericytes, we performed double-labeling experiments using two different pericyte markers (SMAα and PDGFRβ). Interestingly, NG2 + cells in the optic nerve and brain co-label with both of these markers. Consistent with this finding, our previous microarray studies similarly revealed increased expression of PDGFRβ in glial cell cultures from the optic nerve compared to other brain-region primary astrocyte populations.[11] In addition, we found that some of the NG2 + cells in the optic nerve were in close proximity to blood vessels, whereas others were uniformly distributed within the parenchyma. The fact that NG2 + cells express markers associated with pericytes as well as glial precursors highlights the difficulties associated with the use of currently available antibodies to distinguish between these NG2 + cell populations.

Support for the notion that NG2 + cells are potential glioma progenitor cells derives from immunohistochemical experiments demonstrating increased NG2 expression in invasive mouse glioma[29] and in the actively proliferating population of human high-grade glioma,[30] correlating with increasing glioma malignancy grade.[31] Similarly, we found robust SMAα immunolabeling in one representative NF1-associated optic glioma. Second, retroviral PDGF-induced rat gliomas arising in the spinal cord[30] and brainstem[13] contain a highly proliferative population of NG2 + progenitor cells. Third, overexpression of NG2 in mouse glioblastoma multiforme increases glioma growth, whereas NG2 knockdown decreases glioma growth *in vivo*.[33] Fourth, A2B5 + /CD133 − cells freshly isolated from human high-grade gliomas form tumors when injected into immunocompromised *nu/nu* mice.[34,35] Fifth, introducing p53/Nf1 mutations directly into OPCs cause malignant gliomas in adult mice.[36] These latter experiments were performed using the mosaic analysis with double markers approach, and revealed that high-grade gliomas arose in 8-month-old mice following the inactivation of both the *Nf1* and p53 genes in lv-SVZ OPCs. Similar to elegant studies by the Parada laboratory using mice with conditional *Nf1*, p53 and Pten inactivation,[37] SVZ progenitors serve as the putative cells of origin for malignant glioma in adult mice. In contrast to these adult malignant glioma studies, we have recently shown that low-grade *Nf1* mouse optic gliomas likely
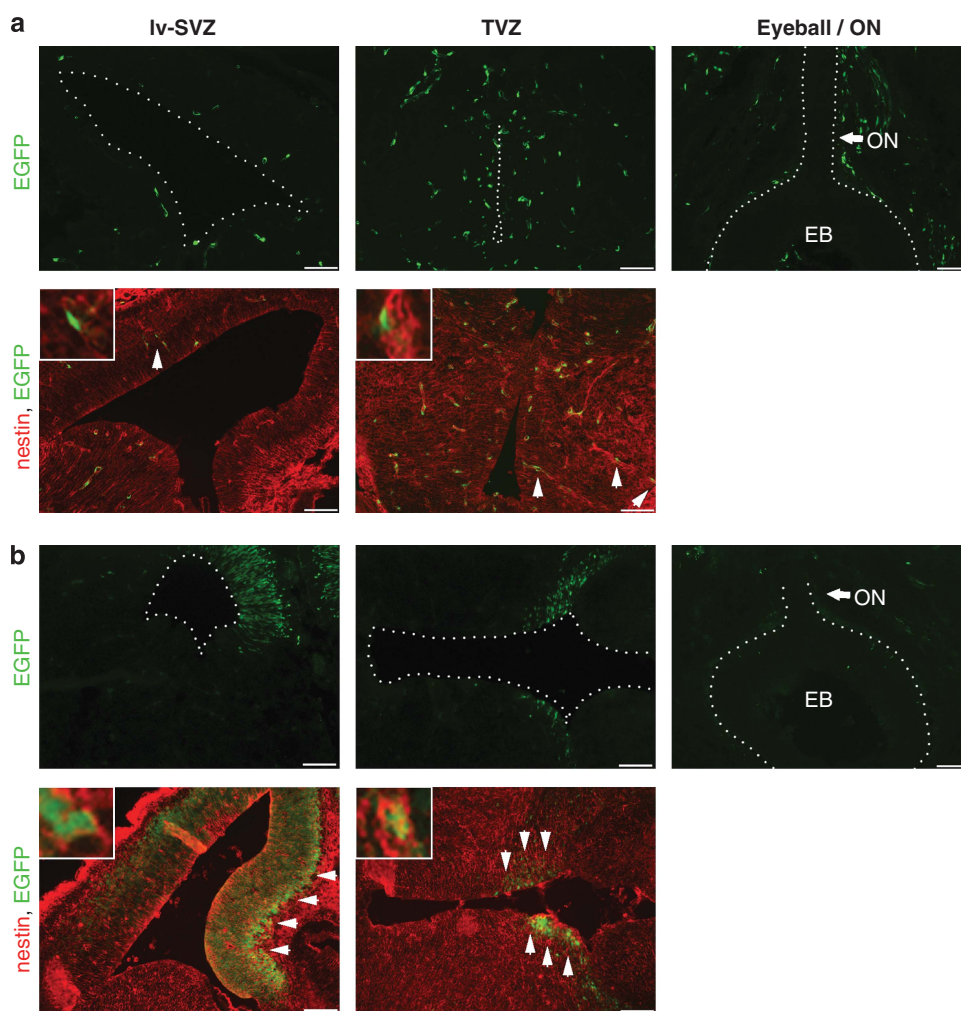
Figure 8. NG2-Cre is first expressed at E14.5 in cells in the third and lateral ventricles. (**a**) NG2-Cre- and (**b**) GFAP-Cre-mediated EGFP expression is first detected by embryonic day E14.5 in the lv-SVZ (dotted line) and TVZ (dotted line) of (**a**) Rosa-GREEN × NG2-Cre and (**b**) Rosa-GREEN × GFAP-Cre mice, respectively. Nestin/EGFP double-positive cells in Rosa-GREEN × NG2-Cre mice are randomly scattered in the areas around the lv-SVZ and TVZ, whereas nestin/EGFP double-positive cells in Rosa-GREEN × GFAP-Cre mice are preferentially localized to the TVZ, but not to the lv-SVZ, periventricular region. Representative images are shown with insets of immunopositive cells. Scale bar, 100 μm. EB = eye ball, ON = optic nerve.

arise from third ventricle progenitors, rather than lv-SVZ progenitors, during embryogenesis.[18] Consistent with this unique progenitor origin, *Nf1* loss in TVZ neural stem cells resulted in increased proliferation and glial differentiation, whereas *Nf1* loss in lv-SVZ neural stem cells did not.

To experimentally define the role of NG2+ cells in *Nf1* optic gliomagenesis, we employed a complementary series of *in vitro* and *in vivo* approaches. We initially demonstrated that NG2+ cells constitute a significant fraction of the non-oligodendrocyte glial cells in the normal optic nerve as well as in *Nf1* optic glioma. In the optic nerve, NG2+ cells represent a distinct population of glial cells *in vivo* and *in vitro*, lacking GFAP expression. Upon acute *Nf1* gene inactivation *in vitro*, NG2+ cells did not exhibit an increase in proliferation, whereas increased proliferation was observed in the GFAP+ glial cell population. Next, we showed that NG2+ cells from NG2-Cre mice in which Cre recombinase is expressed from the endogenous NG2 promoter can give rise to GFAP+ cells in the optic nerve *in vivo*. Similar to GFAP-Cre-driven *Nf1* inactivation, we found that NG2-Cre-driven *Nf1* inactivation resulted in reduced neurofibromin expression in GFAP+ cells in the optic nerve; however, in striking contrast to *Nf1*[flox/mut]; GFAP-Cre mice, *Nf1*+/− mice in which *Nf1* loss occurs in NG2+ cells

(*Nf1*[flox/mut]; NG2-Cre mice) do not form optic gliomas at 3 or 6 months of age *in vivo*. Further experiments are needed to clarify why the NG2+ cells in the *Nf1*[flox/mut]; NG2-Cre and *Nf1*[flox/mut]; GFAP-Cre mouse optic nerves retain *Nf1* expression, despite being derived from NG2-Cre- and GFAP-Cre-expressing progenitor cells.

The absence of optic gliomagenesis could reflect several differences between the GFAP-Cre and NG2-Cre mice used for these experiments. First, it is possible that NG2-Cre mice exhibit less efficient Cre-mediated *Nf1* inactivation. This possibility was excluded using Rosa-GREEN reporter mice, in which the percentage of cells with Cre-mediated excision is actually slightly higher than that observed in GFAP-Cre mice. Moreover, optic nerves from both *Nf1*[flox/mut]; GFAP-Cre and *Nf1*[flox/mut]; NG2-Cre mice exhibited similar levels of *Nf1* inactivation as assessed by recombination PCR as well as RNA FISH. Second, optic nerve astrocytes might not arise from NG2+ progenitors. However, we showed that both GFAP-Cre and NG2-Cre mice are capable of generating astrocytes in the optic nerve. Third, it is possible that Cre-mediated *Nf1* loss occurs at the wrong time point for glioma formation. Recent studies from our laboratory have shown that gliomas are only formed in *Nf1*+/− mice when *Nf1* gene loss occurs during embryogenesis.[18] As NG2-Cre-mediated recombination begins at E14.5,

similar to GFAP-Cre mice, it is unlikely that temporal differences account for the lack of optic gliomas in $Nf1^{flox/mut}$; NG2-Cre mice.

Fourth, it is possible that NG2+ cells are not located in a progenitor zone relevant to optic gliomagenesis. Recent studies from our laboratory have shown that $Nf1$ murine optic gliomas likely arise from progenitor cells lining the third ventricle, rather than the lateral ventricle.[18] This ventricular zone has previously been shown to contain the progenitor cells that give rise to optic nerve OPCs.[38,39] In addition, a recent gene expression study using human optic-glioma specimen supported the third ventricle as the likely germinal zone of origin for these tumors.[40] We have previously shown in both human and mice that the third ventricle contains nestin+ radial glia-like cells,[41] analogous to type B-cells, important for gliomagenesis in adult malignant glioma.[10,41–43] In this report, we demonstrate, using a Rosa-GREEN reporter mouse strain, that unlike the GFAP-Cre strain used to generate $Nf1$ murine optic gliomas, the EGFP+/nestin+ cells from NG2-Cre mice were not preferentially localized to the periventricular zone of the third ventricle.

This intriguing difference suggests that the failure of $Nf1^{flox/mut}$; NG2-Cre mice to develop optic glioma may reflect the requirement for a specific population of susceptible progenitor cells (potential tumor-initiating cells) within the third ventricle germinal zone to expand following $Nf1$ inactivation, and generate these common low-grade brain tumors in children.

MATERIALS AND METHODS

Mice
$Nf1^{flox/flox}$ (WT)[44] and $Nf1^{flox/mut}$; GFAP-Cre[6] mice were generated as previously described. For cell-fate mapping experiments, NG2-Cre mice (B6,FVB-Tg(Cspg4-cre)1Akik/J, Jackson Laboratory, Bar Harbor, ME, USA[17]) and GFAP-Cre mice[8] were intercrossed with Rosa-GREEN (B6.Cg-Gt(ROSA)26Sor$^{tm(CAGZsGreen1)Hze}$+/J; Jackson Laboratory) reporter mice,[45] respectively. To establish $Nf1$+/− background mice with NG2-specific $Nf1$ inactivation, $Nf1$+/− mice[46] were bred with $Nf1^{flox/WT}$ mice to produce $Nf1^{flox/mut}$ mice, which were subsequently intercrossed with $Nf1^{flox/flox}$; NG2-Cre mice to generate $Nf1^{flox/mut}$; NG2-Cre mice. $Nf1^{flox/WT}$; NG2-Cre mice were generated by crossing $Nf1^{flox/flox}$ mice with NG2-Cre mice. All mice were maintained on a C57Bl/6 background and used in accordance with approved animal studies protocols at the Washington University School of Medicine.

Primary astrocyte cultures
Primary astrocyte cultures were established from the optic nerves of PN day 1–2 $Nf1^{flox/flox}$ pups.[11] WT and $Nf1$-deficient ($Nf1$−/−) cultures were generated following infection with Adenovirus type 5 containing β-galactosidase (Ad5-LacZ) or Cre recombinase (Ad5-Cre) (University of Iowa Gene Transfer Vector Core, Iowa City, IA, USA), respectively. Neurofibromin loss was confirmed by western blot.

Cell proliferation
Approximately 50 000 astroglial cells were plated in 24-well dishes, allowed to adhere, and maintained in astrocyte growth medium for 24 h before exposure to the thymidine analog BrdU (5 mM) for 4 h.

NG2 immunopanning
Cell selection was based on the 'panning' technique described by Stallcup and Beasley.[23] Briefly, optic nerves were removed from decapitated PN1–2 pups and incubated for 30 min at 37 °C in 2 ml HEPES-buffered DMEM containing 1mg/ml collagenase (Sigma, St Louis, MO, USA). Following extensive washing, the dissociated optic nerve cells were subjected to immunopanning on six-well dishes precoated with 10 μg/ml NG2 monoclonal antibodies for 30–45 min (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Unbound cells were removed and cultured independently for comparison with bound cells. Bound cells were subsequently detached from the dishes by scraping. All cells were washed once and plated in cell culture plates. For the differentiation assay, cells were grown for 1, 3 or 7 days in astrocyte growth medium.

Immunocytochemistry
Astrocytes were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Following overnight incubation with primary antibodies (Supplementary Table 1), visualization was accomplished after incubation with either Alexa Fluor 488 or 568 IgG or IgM secondary antibodies (Invitrogen, Grand Island, NY, USA). Cells were counterstained with DAPI. To detect BrdU incorporation, cells were treated with 2 M HCl to denature DNA followed by overnight incubation with monoclonal anti-BrdU antibodies (1:200).

For each independent culture, at least 5 distinct microscopic fields were analyzed on a Nikon Eclipse TE300 fluorescence inverted microscope (Nikon, Tokyo, Japan) equipped with an optical camera (Optronics, Goleta, CA, USA) and MetaMorph image analysis software (Molecular Devices, Dowingtown, PA, USA).

Immunohistochemistry
For immunohistochemistry on paraffin sections, horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) were used in combination with Vectastain Elite ABC development and hematoxylin counterstaining. For immunofluorescence detection, appropriate Alexa-Flour tagged secondary antibodies (Invitrogen) were used, followed by DAPI counterstaining.

RNA in situ hybridization
FISH was performed using the QuantiGene ViewRNA kit (Affymetrix Inc., Frederick, MD, USA) according to the manufacturer's instructions with minor modifications. The proteinase K treatment was omitted in order to preserve the integrity of the glial cells. The oligonucleotide probe was designed commercially using the murine $Nf1$ sequence (accession number NM_010897.2). Following FISH, cells were incubated in blocking buffer (10% goat serum in 0.3% PBS-Triton X-100) for 1 h, and subsequently processed for immunofluorescence using standard methods. Images were obtained with a Nikon Eclipse TE300 fluorescence inverted microscope (Nikon) and analyzed using MetaMorph image analysis software (Molecular Devices, Dowingtown, PA, USA). Briefly, cell areas were measured by tracing the cell body, so that the cell body size would not affect the measurements. The $Nf1$ mRNA punctae in the cell body were counted and mRNA molecules per area were calculated. For FISH in tissue, the conditions were optimized to include a 10 min boiling and a 10 min protease treatment.

Western immunoblotting
Cells were lysed in 1% NP-40 lysis buffer, supplemented with protease and phosphatase inhibitors, and protein concentrations determined using the BCA protein assay (Pierce, Thermo Scientific, Rockford, IL, USA). Following SDS-PAGE separation and western blotting, neurofibromin expression was detected using rabbit anti-NF1GRP-D antibodies (1:200 dilution; Santa Cruz Biotechnology). Detection was accomplished by enhanced chemiluminescence using the ChemiDoc-It Imaging System (UVP, Upland, CA, USA). α-tubulin (Sigma) served as an internal control for protein loading and densitometric normalization.

Optic nerve measurements
Optic nerves with an intact chiasm were microdissected, photographed, and optic nerve diameters measured at the chiasm at ∼200 and ∼400 and ∼600 μ anterior to the chiasm to generate volumes as previously reported.[47]

Statistical analysis
All in vitro experiments were repeated at least three times with similar results. Statistical analysis was performed using GraphPad Prism 4.0 software (GraphPad, La Jolla, CA, USA). Data are presented as mean values with s.e.m. Statistical significance was assessed by using Student's two-tailed t-test. Grubbs outlier test was used to determine statistical outliers. Statistical significance was set at $P < 0.05$.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)