A population of Nestin-expressing progenitors in the cerebellum exhibits increased tumorigenicity

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It is generally believed that cerebellar granule neurons originate exclusively from granule neuron precursors (GNPs) in the external germinal layer (EGL). Here we identified a rare population of neuronal progenitors in mouse developing cerebellum that expresses Nestin. Although Nestin is widely considered a marker for multipotent stem cells, these Nestin-expressing progenitors (NEPs) are committed to the granule neuron lineage. Unlike conventional GNPs, which reside in the outer EGL and proliferate extensively, NEPs reside in the deep part of the EGL and are quiescent. Expression profiling revealed that NEPs are distinct from GNPs and, in particular, express markedly reduced levels of genes associated with DNA repair. Consistent with this, upon aberrant activation of Sonic hedgehog (Shh) signaling, NEPs exhibited more severe genomic instability and gave rise to tumors more efficiently than GNPs. These studies revealed a previously unidentified progenitor for cerebellar granule neurons and a cell of origin for medulloblastoma.

Nestin, a type IV intermediate filament protein, was first identified in multipotent neural stem cells (NSCs)1. Since then, Nestin has been widely used as a marker for NSCs in various regions of the nervous system. It is generally believed that as NSCs differentiate into lineage-restricted progenitors, Nestin is replaced by neurofilament and glial fibrillary acidic protein (GFAP) in neurons and glial cells, respectively2. These events may reflect temporal and spatial control of intermediate filament expression, which facilitates changes in cellular shape and migratory potential. However, several studies have suggested that not all Nestin-expressing cells are NSCs and that some are lineage-committed neuronal and glial progenitors3,4. Furthermore, recent studies have suggested that Nestin expression is not limited to the nervous system: for example, Nestin+ cells have been described in the skin, pancreas and kidney5. These studies suggest that Nestin cannot be unambiguously interpreted as a marker for NSCs.

In the cerebellum, Nestin expression has been well-documented in both NSCs and radial (Bergmann) glia6–9. However, Nestin expression in GNPs has been controversial: whereas some reports have suggested that Nestin expression is extinguished before cells commit to the granule lineage10, others have suggested that GNPs can express Nestin11–13. In part, these discrepancies may be due to the fact that the EGL, where GNPs reside, is traversed by the processes of Bergmann glia, making it difficult to distinguish Nestin+ cells from Nestin− fibers. In many studies, animals that express Cre recombinase under the control of the nestin promoter have been used to target GNPs14,15. However, it is not known whether recombination occurs in GNPs themselves or in the NSCs that give rise to them.

In this study, we identified a population of progenitors in the developing cerebellum that express high levels of Nestin. Despite lacking the canonical GNP lineage marker Math1 (encoded by Atoh1, here referred to as Math1), these NEPs are committed to the granule neuron lineage. NEPs are distinct from conventional GNPs in terms of location, proliferative status and gene expression. In particular, genes associated with DNA repair are underexpressed in NEPs compared with GNPs. After activation of Shh signaling, NEPs exhibit more severe genomic instability and give rise to medulloblastoma more efficiently than GNPs. Our studies therefore revealed a unique population of neuronal progenitors in the developing cerebellum and suggest that the intrinsic properties of the cell of origin can serve as predisposing factors for tumorigenesis.

RESULTS
A rare cell population in cerebellar EGL expresses Nestin
In the cerebellum, Nestin expression has been well-described in both NSCs and Bergmann glia6–9. However, whether GNPs express Nestin still remains unresolved.

To evaluate Nestin expression in the developing cerebellum, we first performed immunohistochemical staining using antibodies to Nestin. We readily detected Nestin protein in the cerebellum of mice at postnatal day 4 (P4; Fig. 1a). However, because Nestin protein is distributed both in the cytoplasm and on cell fibers, it is difficult to clearly distinguish Nestin-expressing cells based on immunostaining. To circumvent this limitation, we used Nestin–CFP transgenic mice, which express a nuclear-localized form of CFP driven by the Atoh1 promoter and were used as a control for expression in the EGL. In the EGL, we observed nuclear and cytoplasmic staining with antibodies to Nestin, as expected. Moreover, we observed colocalization of nuclear-localized CFP with Nestin in the EGL (Fig. 1b).

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This protein does not label fibers and therefore makes it easy to identify the cell bodies of Nestin-expressing cells. In the cerebellum of Nestin-CFP mice at P4, at least three populations of cells were CFP+ (Fig. 1b). Consistent with previous reports, CFP+ cells included Bergmann glia in the molecular layer (S100β, Fig. 1c) and NSCs in the white matter (Musashi+, Fig. 1d). In addition, a small population of cells in the EGL expressed the Nestin-CFP transgene (Fig. 1e). All the CFP+ cells in the EGL (NEPs) did not express Math1 (Fig. 1f), a well-characterized marker for GNP. In addition, Math1+ GNP resided in the superficial part of the EGL, whereas NEPs were predominantly localized in the deep EGL of the developing cerebellum (Fig. 1f). These data suggest that NEPs may be a previously unidentified cell population that is distinct from conventional GNP. In Nestin-CFP transgenic mice, we found NEPs only in the cerebellum during early development (E16.5–P15). At embryonic day 14.5 (E14.5), we detected no Nestin-expressing cells in the EGL (Supplementary Fig. 1a) or in the rhombic lip (Supplementary Fig. 1b) where GNP originate. We first detected NEPs in the EGL at E16.5 (Supplementary Fig. 1c,d) and we did not find them in the postnatal cerebellum at P21 (Supplementary Fig. 1e). These data indicate that NEPs are a transient population that exists only during early cerebellar development.

To further characterize NEPs in the EGL, we purified these cells. For this purpose, we crossed Nestin-CFP mice with Math1-GFP mice, in which conventional GNP exclusively express GFP. We then prepared sagittal cerebellar slices from P4 Math1-GFP–Nestin-CFP mice, and microdissected cerebellar EGLs under a fluorescence microscope to exclude the Nestin+ cells in the molecular layer and the white matter (Fig. 1g). We dissociated the dissected EGLs (Fig. 1h) and analyzed them by flow cytometry to detect expression of GFP and CFP. The majority of cells in the EGL were GFP+ (Fig. 1i), indicating that cerebellar EGL is dominated by Math1+ GNP. Approximately 3–5% of cells in the EGL expressed Nestin-CFP, which is consistent with our immunohistochemical staining of cerebellar sections (Fig. 1b). Almost none of the cells were both GFP+ and CFP+ based on the flow cytometric analysis (Fig. 1i). Using confocal microscopy, we confirmed that GFP+ GNP and CFP+ NEPs were mutually exclusive in the EGL of Math1-GFP–Nestin-CFP mice at P4 (Fig. 1j). These data suggest that NEPs and GNP are two distinct cell populations in the postnatal EGL. As a control, we also dissected the white matter from the cerebellum of Math1-GFP–Nestin-CFP mice (Supplementary Fig. 2a,b). FACS analysis (Supplementary Fig. 2c–e) indicated that more than 18% of cells in the white matter were CFP+, and 35% of CFP+ cells expressed Prominin1, a marker for NSC, suggesting that at least some of the Nestin-expressing cells in the white matter are NSCs. To determine whether the CFP and GFP fluorescence in the cerebellum of Nestin-CFP–Math1-GFP mice faithfully reflects expression of endogenous Nestin and Math1 protein, we examined FACS-sorted CFP+ cells and GFP+ cells by immunocytochemistry. All GFP+ cells isolated from the EGL in Nestin–CFP–Math1-GFP animals at P4 were Math1+, but lacked Nestin protein (Supplementary Fig. 3a–d). Similarly, we detected Nestin protein in the cytoplasm of all sorted CFP+ cells, which lacked Math1 protein (Supplementary Fig. 3c–h). These data suggest that NEPs and GNP can be purified from cerebellum of Nestin-CFP–Math1-GFP mice by microdissection followed by FACS.

NEPs are committed to the granule neuron lineage
As Nestin is commonly used as a marker for NSC, we examined whether NEPs isolated from the EGL might represent NSC. NSC from many parts of the central nervous system proliferate and form macroscopic neurospheres when cultured in the presence of growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). To examine the capacity of NEPs to form neurospheres in culture, we purified NEPs and GNP from the EGL of the cerebellum P4 Nestin-CFP–Math1-GFP mice and cultured them at clonal density in the presence of bFGF and EGF. As controls, we also cultured NSC (Prominin1+Lin− cells) isolated from the same cerebellum as previously described. After 7 d, neurospheres were readily detectable in cultures of NSC, whereas almost no neurospheres were generated from NEPs and GNP (Fig. 2a,b). These data indicate that unlike NSC, NEPs have a limited capacity to form neurospheres in vitro.
Figure 2 Neuronal lineage commitment of NEPs in viva and in vitro. (a) Phase contrast image of neurospheres generated from NSC culture in the presence of EGF and FGF. (b) Number of neurospheres per 5,000 NSCs, GNPs and NEPs after 7 d in culture under stem cell culture conditions. (c) After 4 d under differentiation conditions, NEPs were stained for β-tubulin (red) and counterstained with DAPI (blue). (d) Percentage of neurons (β-tubulin*), Bergmann glia (S100β*) and oligodendrocytes (O4*) was quantified after culturing NSCs and NEPs under differentiation culture conditions. NEPs exclusively differentiate into neurons in vitro. (e-i) NEPs isolated from P4 Nestin-CFP–DsRed animals were transplanted into the cerebellum of P4 SCID mice. At P21, the recipient cerebellum was sectioned and immunostained with the indicated antibodies. NEPs gave rise to only neurons after the transplantation. (j-n) Cerebellar sections from Nestin-CreERT2–R26R-GFP animals at P21 were immunostained with the indicated antibodies. PL, Purkinje layer; ML, molecular layer. Error bars (b,d), s.e.m. (n = 3); *P < 0.01 and **P < 0.05 (two-tailed Student’s t test); NEPs versus NSCs in b, P = 0.0015, df = 2; neurons derived from NEPs versus NSCs in d, P = 0.0043; oligodendrocytes derived from NEPs versus NSCs in d, P = 0.0153. Scale bars, 2 mm (a); 200 μm (c,e,j); and 67 μm (f, same scale in g,i,k-n).

Another important characteristic of NSCs is their capability to differentiate into multiple cell lineages including neurons, astrocytes and oligodendrocytes22. To examine whether NEPs exhibit multipotency, we cultured purified NEPs and NSCs under differentiation culture conditions. After 3 d in vitro, NSCs differentiated into neurons (βIII-tubulin*), astrocytes (S100β*) and oligodendrocytes (O4*) (Supplementary Fig. 4). In contrast, NEPs exclusively gave rise to βIII-tubulin* neurons (Fig. 2c). Fewer than 2% of cells in NEP cultures were Bergmann glia or oligodendrocytes (Fig. 2d). These data suggest that NEPs represent lineage-restricted neuronal progenitors rather than multipotent stem cells.

To confirm the neuronal lineage commitment of NEPs, we examined their differentiation potential in vivo using intracranial transplantation assays. We purified NEPs from the EGL of P4 Nestin-CFP–Actin-DsRed animals (which express red fluorescent protein in all cells21), and then transplanted them into the cerebellum of strain CB17 severe combined immunodeficient (SCID) animals at P4. We detected no proliferation among NEPs after the transplantation based the immunohistochemical staining for Ki67 (data not shown). At P21, we sectioned recipient cerebella to detect the differentiation of transplanted cells (DsRed+, Fig. 2e). All DsRed+ cells were Zic1+ (Fig. 2f), a marker of cerebellar granule neurons22, and in the recipient cerebellum we found no DsRed+ Purkinje neurons (Calbindin*, Fig. 2g), Bergmann glial cells (S100β*, Fig. 2h) or interneurons (Parvalbumin*, Fig. 2i). As a comparison, NSCs (Prominin1*Lin*) purified from cerebellum of P4 Nestin-CFP–Actin-DsRed mice differentiated into neurons, Bergmann glia and Purkinje neurons after the transplantation (data not shown). These data confirm that NEPs are lineage-committed neuronal progenitors.

In the studies above we focused on the differentiation potential of NEPs after isolation. To determine the fate of NEPs in situ, we lineage-traced these cells by using a Nestin-CreERT2 mouse, in which expression of a tamoxifen-regulatable Cre recombinase is controlled by the nestin enhancer23. We crossed Nestin-CreERT2 mice to Gt(Rosa)26Sor reporter (R26R) mice expressing GFP preceded by aloxP-flanked stop sequence24. After tamoxifen treatment at P4, Nestin-CreERT2–R26RGFP mice were killed at P21 to locate the GFP+ cells in their cerebella by immunohistochemistry. 5–7% of the cells in the cerebellar internal granule layer (IGL) were GFP+ (Fig. 2j). All of the GFP+ cells in the IGL expressed Zic1 (Fig. 2k). We found no Calbindin* Purkinje neurons (Fig. 2l) or Parvalbumin* interneurons (Fig. 2m) among GFP+ cells. GFP+ cells in the molecular layer were glial cells that expressed S100β (Fig. 2n), presumably originating from Bergman glial cells expressing Nestin at P4. Some GFP+ fibers remained on the surface of the Nestin-CreERT2–R26R-GFP cerebellum at P21. These fibers did not have cell nuclei and were S100β+ (Supplementary Fig. 5), suggesting that they represent end-feet of the Bergmann glial cells mentioned above. These data suggest that in addition to GNPs, NEPs also contribute to the genesis of granule neurons during cerebellar development.

NEPs and GNPs represent distinct lineages

It is generally believed that cerebellar granule neurons originate predominately from Math1+ GNPs in the EGL25. Above we demonstrated that NEPs also generate granule neurons. We therefore examined the possible lineage relationships between NEPs and GNPs. For this purpose, we crossed Math1-Cre–R26R-GFP mice, which have previously been used to lineage-trace conventional GNPs25, with Nestin-CFP animals. We microdissected cerebellar EGLs from these animals at P4 and dissociated them for FACS analysis. 58% of cells in the EGL were GFP+, and ~4% of EGL cells at P4 were GFP+ (Fig. 3a). We detected no GFP+CFP+ cell
population among EGL cells at P4 (Fig. 3a) or at later stages examined (P8–P15; data not shown). These results suggest that Math1+ GNPs do not give rise to NEPs during cerebellar development.

To determine whether NEPs can give rise to GNPs, we crossed Nestin-CreER\(^{12}\) mice to R26R-GFP mice to lineage-trace NEPs in the postnatal cerebellum. We treated Nestin-CreER\(^{12}\)–R26R-GFP animals with tamoxifen at P4 and collected cerebella at P8 for immunostaining with antibodies to Math1 and GFP. Math1+ GNPs were preferentially located in the outer part of cerebellar EGL (Fig. 3b). No Math1+ GNPs expressed GFP at P8 or at later developmental stages (P10–P21, data not shown), suggesting that GNPs do not derive from NEPs in the developing cerebellum. The above data indicate that NEPs and GNPs are two independent cell lineages.

NEPs can proliferate in response to Sonic hedgehog
Shh protein, secreted by Purkinje neurons, is the major mitogen for GNPs in the EGL.\(^{26}\) As NEPs residing in the inner EGL are closer to the source of Shh than GNPs are, they might be expected to show increased Shh signaling. To test this, we used quantitative PCR to measure expression of Shh pathway target genes (Ccdn1 and Glil) in NEPs and GNPs purified from Math1-GFP–Nestin-CFP cerebellum at P4. As expected, we detected very high levels of Ccdn1 and Glil in GNPs (Fig. 4a), consistent with the fact that Shh signaling occurs in these cells. Expression of Ccdn1 and Glil was markedly lower in NEPs than in GNPs, suggesting that the Shh pathway was less active in NEPs.

To test whether NEPs can respond to Shh, we purified GNPs and NEPs from cerebellum of P4 Nestin-CFP–Math1-GFP mice, treated them with recombinant Shh protein in vitro and examined their proliferation by immunostaining for Ki67 and Ki67+ mice. Expression of both genes was significantly higher in GNPs than in NEPs in the EGL (Fig. 4b, c), indicating that they are highly proliferative. In contrast, the majority of NEPs were Ki67– at P4 (Fig. 4c) and at all other stages examined (P0–P15, data not shown), suggesting that in contrast to GNPs, NEPs in the EGL are quiescent. Thus, despite being located proximal to the source of Shh (Purkinje neurons), NEPs do not appear to exhibit an active Shh signal pathway in vivo.

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DNA repair–associated genes are downregulated in NEPs
The studies described above indicate that NEPs can give rise to granule neurons but are distinct from conventional GNPs. To determine the molecular basis for the difference between NEPs and GNPs,
we performed gene expression analysis. RNA from NEPs, GNPs and NSCs purified from cerebellum was subjected to microarray analysis using Affymetrix mouse 430 2.0 chips. We then performed principal component analysis (PCA), a statistical method that facilitates global comparison of gene expression among multiple samples. NEPs, GNPs and NSCs were well separated from one another (Fig. 5a), confirming that NEPs are a unique progenitor population in the developing cerebellum.

To gain insight into properties that distinguish NEPs and GNPs, we examined the genes differentially expressed between these two cell populations. Among the 45,101 probe sets on the arrays, 4,902 (10.87%) showed significant differences in expression (increased or decreased by ≥2-fold, false discovery rate < 0.01 by paired t test). Among those, expression for 2,755 was higher in NEPs, and that for 2,147 was lower. Gene enrichment analysis using NexusExp3 software identified four major categories of genes that were differentially expressed between NEPs and GNPs (Table 1). Consistent with our observation that NEPs are normally quiescent in vivo, expression of genes associated with cell proliferation and cell cycle was markedly decreased in NEPs compared with GNPs. Cell adhesion and migration genes were also upregulated in NEPs, in agreement with the known involvement of Nestin in cell migration5. Genes involved in neural cell fate commitment were upregulated in NEPs relative to GNPs, consistent with the neuronal lineage restriction of NEPs. Finally, of 179 genes associated with DNA damage and repair, 62 (34.64%) were differentially expressed between NEPs and GNPs (P < 0.001). All 62 genes were downregulated in NEPs compared with GNPs. We validated the reduced expression of several DNA repair–associated genes in NEPs (including Chek1, Lig3 and Parp1) by quantitative PCR (Fig. 5b).

The quiescent status of cells, particularly stem cells, has been suggested to be an essential protective mechanism that minimizes endogenous stress caused by cellular respiration and DNA replication28. To determine whether the decreased expression of DNA repair–associated genes in NEPs is due to their quiescent status, we induced the proliferation of NEPs and GNPs by exposing them to recombinant Shh. After 48 h, we harvested cells for quantitative PCR analysis. Expression of Chek1, Lig3 and Parp1 was significantly lower (two-tailed Student’s t test; Chek1, P = 0.00085; Lig3, P = 0.0171; Parp1, P = 0.0016) in proliferating NEPs than GNPs (Supplementary Fig. 6). These data suggest that decreased expression of DNA repair–associated genes in NEPs is independent of their quiescent state. The above results indicate that NEPs have a distinct genetic profile characterized by decreased expression of DNA repair–associated transcripts.

Table 1 Categories of genes differentially expressed in NEPs versus GNPs

| Gene name                      | Gene symbol | Log ratio | Gene function                  |
|--------------------------------|-------------|-----------|--------------------------------|
| **Cell cycle**                 |             |           |                                 |
| E2F transcription factor 1     | E2f1        | −2.2      | Cell cycle control              |
| Cyclin D1                      | Ccnd1       | −2.0      | Cell cycle G1-S transition      |
| Cyclin G2                      | Ccng2       | −1.7      | Cell cycle control              |
| Structural maintenance of       | Smc2        | −1.7      | chromatin cohesion and chromosome condensation |
| chromosomes 2                   |             |           |                                 |
| Cyclin-dependent kinase 6       | Cdc6        | −1.6      | Cell cycle G1-S transition      |
| DNA damage or repair            |             |           |                                 |
| Breast cancer 1                 | Brca1       | −1.9      | DNA damage response             |
| Rad51-associated protein-1      | Rad51ap1    | −1.6      | DNA recombination and repair     |
| Brcal/Brcal-containing complex, | Brcal       | −1.1      | DNA damage and repair           |
| subunit 3                       |             |           |                                 |
| Brca2 and Cdk5 interacting protein | Bccip   | −1.0      | DNA damage and repair           |
| Checkpoint kinase 1             | Chek1       | −0.9      | DNA damage and checkpoint and DNA repair |
| DNA ligase 3                    | Lig3        | −0.6      | DNA recombination and repair     |
| Poly (ADP-ribose) polymerase 1  | Parp1       | −0.5      | DNA repair                      |
| **Cell adhesion and migration** |             |           |                                 |
| Tenascin C                      | Tnc         | 8.2       | Fibronectin binding             |
| Procollagen, type 1, alpha 2    | Col1a2      | 6.7       | Extracellular matrix structural constituent |
| Vascular cell adhesion molecule 1 | Vcam1 | 6.4      | GPI anchor binding and protein binding |
| Neurophilin 1                   | Np1         | 5.0       | Semaphorin receptor regulation  |
| Developmentally downregulated 9 | Nedd9       | 4.3       | Cell adhesion and protein binding |
| Neural cell adhesion molecule 2 | Ncam2       | 3.3       | GPI anchor binding              |
| Proteoglycan 17                 | Pchd17      | 2.6       | Calcium ion binding             |
| **Neural fate commitment**      |             |           |                                 |
| Pancreas specific transcription factor 1a | Ptf1a | 7.8  | Neural fate commitment |
| Neurogenin 2                    | Neurog2     | 6.3       | Neural differentiation and lineage commitment |
| Neurogenin 1                    | Neurog1     | 5.0       | Neural differentiation and lineage commitment |
| notch gene homolog 1            | Notch1      | 4.5       | Notch1 signaling and fate commitment |
| SRY-box containing gene 6       | Sox6        | 4.3       | Neural lineage commitment       |
| Sprouty homolog 2               | Spy2        | 2.9       | Cell fate commitment            |

Proliferative stress causes DNA instability in NEPs

The cellular DNA repair machinery is critical for maintaining the genomic integrity that is constantly challenged by endogenous and exogenous stimuli29. The decreased expression of DNA repair genes in NEPs raises the possibility that these cells may be more susceptible to DNA damage in response to genotoxic agents and stress. Because hyperproliferation can cause DNA-replication stress and genomic damage30, we compared the genomic alterations in hyper-proliferating NEPs and GNPs. For this purpose, we crossed Math1-GFP–Nestin-CFP mice with Ptch1C/C mice, in which the loxP-flanked Patched1 (Ptch1) gene can be conditionally ablated in aCre recombinase–dependent manner31. Ptch1 is an antagonist of the Shh signaling pathway, so Ptch1 deletion causes aberrant activation of Shh signaling and hyperproliferation in both stem cells and progenitors in the nervous system7,32,33. We purified NEPs and GNPs from P4 Math1-GFP–Nestin-CFP–Ptch1C/C cerebella and deleted Ptch1 in these cells by infection with a lentivirus encoding Cre recombinase. 24 h after infection, we pulse-labeled the cells with BrdU for an additional 12 h and then collected them for analysis of BrdU incorporation and hyper-proliferating cells in both stem cells and progenitors in the nervous system7,32,33.
discovered that deletion of Ptch1 in GNPs causes medulloblastoma formation in mice. The fact that Ptch1 deletion promotes proliferation and genomic instability in NEPs led us to postulate that NEPs may be more susceptible to oncogenic transformation after loss of Ptch1. To test this, we crossed Ptch1C/C mice, with Nestin-CreERT2 mice. For comparison, we also deleted Ptch1 in GNPs using Math1-CreERT2 mice. We treated animals with tamoxifen at P4 and examined cerebellum at P21, a time point at which wild-type GNPs have exited the cell cycle, differentiated, and migrated inward from the surface of the cerebellum. As reported previously, we found large numbers of proliferating cells on the surface of the cerebellum in P21 Math1-CreERT2–Ptch1C/C animals. We detected far fewer ectopically proliferating cells in the Nestin-CreERT2–Ptch1C/C cerebellum at P21, consistent with the relatively small number of NEPs in the cerebellum at the time of tamoxifen treatment (NEPs account for only 3–5% of EGL cells at P4). Notably, both Nestin-CreERT2–Ptch1C/C animals and Math1-CreERT2/Ptch1C/C animals eventually developed tumors. These tumors resemble human medulloblastoma in terms of histology and expression profiles based on principal component analysis (Supplementary Fig. 7). Thus, NEPs can also give rise to medulloblastoma after loss of Ptch1. Despite being far less abundant than GNPs, NEPs give rise to tumors with the same penetrance (100%) and latency as Math1+ GNPs. These findings suggest that on a per-cell basis, NEPs may be more prone to give rise to tumors than GNPs.

To directly compare the tumorigenic potential of NEPs and GNPs, we performed limiting dilution transplantation assays. For this purpose, we generated Nestin-CreERT2–Nestin-CFP–Ptch1C/C and Math1-CreERT2–Math1-GFP–Ptch1C/C mice. After treatment of these animals with tamoxifen at P4, we microdissected EGLs at P8. We isolated NEPs and GNPs with deleted Ptch1 from the dissociated EGLs by FACS-sorting CFP+ and GFP+ cells, respectively. We confirmed comparable efficiency of Ptch1 deletion in the two purified cell populations by quantitative PCR as previously described (Supplementary Fig. 8a–c). Transplantation of more than 50,000 NEPs or GNPs resulted in tumor formation in 100% of recipients (Fig. 6h and Supplementary Fig. 8d). Transplantation of 20,000 GNPs resulted in tumors in 60% of recipients, whereas the same number of NEPs still generated tumors in 100% of recipients. Transplantation of fewer than 20,000 GNPs did not cause tumor formation, but as few as 5,000 NEPs generated medulloblastoma in 50% of recipients. These data confirm that NEPs have increased tumorigenic potential compared to GNPs.

**DISCUSSION**

Nestin is widely considered a marker for NSCs in various regions of the nervous system, but is commonly lost as NSCs differentiate into lineage-restricted neuronal and glial progenitors. Here we describe a previously unidentified population of Nestin-expressing cells in the EGL of the developing cerebellum. These cells did not exhibit stem cell properties such as neurosphere-forming capacity and multipotency, and exclusively generated granule neurons. These data suggest that some neuronal progenitors retain Nestin expression during differentiation.

Although they are committed to the granule neuron lineage, NEPs are apparently distinct from conventional GNPs in that NEPs account for only 3–5% of the cells that can be isolated from the EGL, whereas GNPs account for over 90% of EGL cells; NEPs do not express Math1, a transcription factor previously thought to be essential for specification of GNPs; NEPs reside in the deep part of the EGL; GNPs proliferate extensively in the EGL, whereas NEPs migrate and differentiate without initial proliferating; and the expression profile of NEPs is distinct from that of GNPs. Together, these data suggest that NEPs represent a unique population of granule neuron progenitors. Previous studies have suggested that cerebellar granule neurons predominately originate from Math1-expressing GNPs. However, our studies demonstrate that NEPs also contribute to the genesis of granule neurons. We have not observed any obvious phenotypic or morphological differences
between granule neurons originating from NEPs and those arising from conventional GNP5s, but additional studies will be necessary to determine whether there are functional distinctions between these two populations.

The ability of NEPs to give rise to granule neurons despite their lack of Math1 expression is somewhat surprising. Math1 is highly expressed in GNP5s located in the anterior rhombic lip as well as the outer part of the EGL17. The fact that Math1-deficient animals lack an EGL has often been interpreted to mean that Math1 is required for GNP5 regeneration. Our observation that NEPs can produce granule neurons suggests that Math1 is not absolutely required for specification or maintenance of granule neuron identity. Although our studies have revealed that NEPs and GNP5s are mutually exclusive during cerebellar development, it is possible that NEPs and GNP5s have a common cellular origin: NSCs in the ventricular zone15. It has been demonstrated that GNP5s originate from progenitors in the rhombic lip10. However, we detected no Nestin-expressing cells in the rhombic lip at embryonic stages (Supplementary Fig. 1), and NEPs are always found in the deep part of the EGL, implying that NEPs are not rhombic lip-derived progenitors. Other possible sources of NEPs include NSCs in the embryonic ventricular zone or postnatal white matter8, or astroglial cells in the EGL36.

A recent study identified a new population of GFAP+ cells in the EGL by using transgenic mice expressing DsRed driven by the human GFAP promoter38. DsRed+ cells expressed Nestin, reside in the deep part of EGL and to give rise to granule neurons. However, 60% of GFAP+ cells were Musashi1+, and almost half of GFAP+ cells were Ki67+. Based on these immunostaining data, the authors concluded that GFAP+ cells in the EGL represent NSCs. In contrast, the NEPs we identified in our study were quiescent and did not exhibit multipotency or the ability to form neurospheres. Given these differences, we believe that NEPs and GFAP+ cells in the EGL are distinct populations.

Shh is a potent mitogen for GNP5s in the developing cerebellum. Despite residing in a Shh-enriched environment, NEPs do not express Shh target genes and do not proliferate in vivo. However, NEPs markedly increase their proliferation in response to Shh in vitro. These data suggest that NEPs have the capacity to respond to Shh signaling. The failure of NEPs to respond to Shh in vivo may be due to factors in the inner EGL that override the mitogenic effects of Shh. For example, pituitary adenylate cyclase activating polypeptide37, bone morphogenetic proteins38, FGF-2 (ref. 39) and the extracellular matrix molecule vitronectin40 have all been shown to inhibit Shh-induced proliferation of GNP5s. Additional experiments will be necessary to delineate the contribution of these signals to the quiescent state of NEPs in vivo.

It has been reported that GNP5s can give rise to medulloblastoma after aberrant activation of Shh signaling7,41. In this study, we demonstrated that NEPs also have the capacity to initiate medulloblastoma formation after deletion of Ptch1. We did not find any obvious differences between tumors arising from GNP5s and those derived from NEPs based on thymidine incorporation, cell-cycle analysis and gene expression profiling. These studies suggest that both NEPs and GNP5s can represent cells of origin for Shh-associated medulloblastoma. Notably, expression of Shh in Nestin+ cells using the RCAS-TVA system also results in medulloblastoma12. Although previous studies have suggested that these tumors arise from GNP5s, our studies raise the possibility that they may actually originate from NEPs.

Cell proliferation involves many processes that need to be tightly coordinated to ensure the preservation of genome integrity and to promote faithful genome propagation. Efficient and error-free DNA replication is critical for faithful replication of chromosomes before their segregation. Coordination of DNA replication with DNA-damage repair ensures genome integrity during cell division, thus preventing tumorigenic mutations. It has been reported that disruption of a DNA repair pathway has the potential to expedite tumorigenesis by resulting in a cell that is hypermutable12,43. In this study, we demonstrated that NEPs exhibit more chromosomal aberrations after Ptch1 deletion than GNP5s. In our previous studies, we have demonstrated that loss of Ptch1 alone is not sufficient to initiate tumor formation and that additional epigenetic or genetic changes (‘second hits’) are required to fully transform GNP5s7,44. Comparable levels of proliferation of NEPs and GNP5s after deletion of Ptch1 (Fig. 5c) indicate that the differential tumorigenic potential of these two cell populations is not solely due to deletion of Ptch1. Deficiency in DNA repair may give NEPs advantages in terms of accumulating the oncogenic mutations necessary for tumor initiation, and this may result in the increased tumorigenicity of NEPs after deletion of Ptch1.

The fact that NEPs exhibit increased genomic instability and increased tumorigenic potential is consistent with previous studies showing that genomic instability facilitates formation of medulloblastoma in neuronal progenitors. Ionizing radiation dramatically increases tumor incidence and accelerates medulloblastoma formation in Ptch111− mice, suggesting that DNA damage results in predisposition to medulloblastoma tumorigenesis45,46. Moreover, inactivation of DNA repair–associated genes, including Lig4, Xrc4 and Brc2 in cerebellar neuronal progenitors (using a Nestin-Cre transgene), has been reported to cause formation of medulloblastoma in a p53-deficient background14,35,47. A very recent study has also shown that overexpression of YAP by a YAP-targeted promoter increases medulloblastoma tumorigenesis in cerebellar neuronal progenitors48. Our observation that NEPs are already deficient in DNA repair suggests that these cells may be particularly prone to transformation and may represent cells of origin in some of the above tumor models.

It is common to think of oncogenic transformation as resulting from a series of mutations that endow a cell with increased genomic instability, unlimited proliferative capacity, decreased ability to undergo apoptosis and altered ability to interact with the microenvironment. However, our studies suggest that these properties may not necessarily be the result of somatic mutations, but instead, may represent intrinsic characteristics of cells at certain stages of development. Indeed, transgenic expression of the same oncogene or conditional deletion of the same tumor suppressor gene in different tissues under the control of tissue-specific promoters frequently results in remarkable variations in tumor-initiating capability, phenotype, latency and penetrance49. These examples highlight the critical role of the cellular context in determining whether and when tumorigenesis will take place in response to particular oncogenic stimuli. Identification of ‘tumor-prone’ cell populations may provide critical insight into mechanisms of transformation and yield new approaches to targeting cancer.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: GSE50824.

Note. Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

Z.Y. and R.J.W.-R. conceived the project. P.L., ED., L.W.Y., T.L., R.E.M. and R.T. performed the experiments. Z.Y., P.L., J.W., A.B. and R.J.W.-R. analyzed the data. G.E. provided reagents. Z.Y. prepared the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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1. Lendahl, U., Zimmerman, L.B. & McKay, R.D. CNS stem cells express a new class of intermediate filament protein. Cell 60, 585–595 (1990).
2. Morshead, C.M. et al. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron* 13, 1071–1082 (1994).
3. Shi, H. et al. Nestin expression defines both glial and neuronal progenitors in postnatal sympathetic ganglia. *J. Comp. Neurol.* 508, 867–878 (2008).
4. Vukojevic, K., Petrovic, D. & Saraba-Bagic, M. Nestin expression in glial and neuronal progenitors of the developing human spinal ganglia. *Gene Expr. Patterns* 10, 144–151 (2010).
5. Hyder, C.L., Isionemi, K.O., Tovoldvass, E.S. & Eriksson, J.E. Insights into intermediate filament regulation from development to ageing. *J. Cell Sci.* 124, 1363–1372 (2011).
6. Lee, A. et al. Isolation of neural stem cells from the postnatal cerebellum. *Nat. Neurosci.* 8, 723–729 (2005).
7. Yang, Z.J. et al. Medulloblastoma can be initiated by deletion of Patched in lineage-restricted progenitors or stem cells. *Cancer Cell* 14, 135–145 (2008).
8. Sutter, R. et al. Cerebellar stem cells act as medulloblastoma-initiating cells in a mouse model and a neural stem cell signature characterizes a subset of human medulloblastoma. *Oncoogene* 29, 1845–1856 (2010).
9. Sotelo, C., Alvarado-Mallart, R.M., Frain, M. & Vernet, M. Molecular plasticity of adult Bergmann fibers is associated with radial migration of grafted Purkinje cells. *J. Neurosci.* 14, 124–133 (1994).
10. Alder, J., Cho, N.K. & Hatten, M.E. Embryonic progenitor cells from the rhombic lip are specified to a cerebellar granule neuron identity. *Neuron* 32, 17–24 (2001).
11. Ben-Arie, N. et al. Math1 is essential for genesis of cerebellar granule neurons. *Nature* 390, 169–172 (1997).
12. Umemori, E.A. et al. Math1-driven GFP expression in the developing nervous system of transgenic mice. *Gene Expr. Patterns* 3, 389–395 (2003).
13. Uchida, N. et al. Direct isolation of human central nervous system stem cells. *Proc. Natl. Acad. Sci. USA* 97, 14720–14725 (2000).
14. Reynolds, B.A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255, 1707–1710 (1992).
15. Vintersten, K. et al. Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. *Genesis* 40, 241–246 (2004).
16. Aruga, J. et al. A novel zinc finger protein, zic, is involved in neurogenesis, especially in the cell lineage of cerebellar granule cells. *J. Neurochem.* 63, 1880–1890 (1994).
17. Balordi, F. & Fishell, G. Mosaic removal of hedgehog signaling in the adult SVZ reveals that the residual wild-type stem cells have a limited capacity for self-renewal. *Neuron* 27, 14248–14251 (2005).
18. Nao, X., Fujwara, Y., Chapdelaine, A., Yang, H. & Orkin, S.H. Activation of EGFP expression by CRE-mediated excision in a new ROSA26 reporter mouse strain. *Blood* 97, 324–326 (2001).
19. Macphail, R. & Fishell, G. Math1 is expressed in temporally discrete pools of cerebellar rhombic-tip neural progenitors. *Neuron* 48, 17–24 (2005).
20. Wechsler-Reya, R.J. & Scott, M.P. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* 22, 103–114 (1999).
21. Kenney, A.M., Cole, M.D. & Rowitch, D.H. Nsectic upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. *Development* 130, 15–28 (2003).
22. Orford, K.W. & Scaffen, D.T. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat. Rev. Genet.* 9, 115–128 (2008).
23. Lombard, D.B. et al. DNA repair, genome stability, and aging. *Cell* 120, 497–512 (2005).
24. Burhans, W.C. & Weinberger, M. DNA replication stress, genome instability and aging. *Nucleic Acids Res.* 35, 7545–7556 (2007).
25. Ellis, T. et al. Patched 1 conditional null allele in mice. *Genes 36*, 158–161 (2003).
26. Ahn, S. & Joyner, A.L. In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. *Nature* 437, 894–897 (2005).
27. Balordi, F. & Fishell, G. Hedgehog signaling in the subventricular zone is required for both the maintenance of stem cells and the migration of newborn neurons. *J. Neurosci.* 27, 5936–5947 (2007).
28. Aguilera, A. & Gomez-Gonzalez, B. Genome instability: a mechanistic view of its causes and consequences. *Nat. Rev. Genet.* 9, 204–217 (2008).
29. Frappart, P.O. et al. Recurrent genomic alterations characterize medulloblastoma arising from DNA double-strand break repair deficiency. *Proc. Natl. Acad. Sci. USA* 106, 1880–1885 (2009).
30. Silberis, J. et al. Astroglial cells in the external granular layer are precursors of cerebellar granule neurons in neonates. *Mol. Cell. Neurosci.* 44, 362–373 (2010).
31. Nicot, A., Lelièvre, V., Tam, J., Waschek, J.A. & DiCicco-Bloom, E. Pituitary adenylate cyclase-activating polypeptide and sonic hedgehog interact to control cerebellar granule precursor cell proliferation. *J. Neurosci.* 22, 9244–9254 (2002).
32. Rios, I., Alvarez-Rodriguez, R., Marti, E. & Pons, S. Bmp2 antagonizes sonic hedgehog-mediated proliferation of cerebellar granule neuron precursors through Smad5 signalling. *Development* 131, 3159–3168 (2004).
33. Fagarty, M.P., Kessler, J.D. & Wechsler-Reya, R.J. Morphing into cancer: the role of developmental signaling pathways in brain tumor formation. *J. Neurobiol.* 64, 458–475 (2005).
34. Pons, S., Trejo, J.L., Martinez-Morales, J.R. & Marti, E. Vimentin regulates Sonic hedgehog activity during cerebellum development through CREB phosphorylation. *Development* 128, 1481–1492 (2001).
35. Schuler, U. et al. Acquisition of granule neuron precursor identity is a critical determinant of progenitor cell competence to form Shh-induced medulloblastoma. *Cancer Cell* 14, 123–134 (2008).
36. Kaye, J.A. et al. DNA breaks promote genomic instability by impeding proper chromosome segregation. *Curr. Biol.* 14, 2096–2106 (2004).
37. Jasin, M. Chromosome breaks and genomic instability. *Cancer Invest.* 18, 78–86 (2000).
38. Oliver, T.G. et al. Loss of patched and disruption of granule cell development in a pre-neoplastic stage of medulloblastoma. *Development* 132, 2425–2439 (2005).
39. Pazzaglia, S. et al. High incidence of medulloblastoma following X-ray-irradiation of newborn Ptc1 heterozygous mice. *Oncoogene* 21, 7580–7584 (2002).
40. Pazzaglia, S. et al. The genetic control of chemically and radiation-induced skin tumorigenesis: a study with carcinogenesis-susceptible and carcinogenesis-resistant mice. *Radiat. Res.* 158, 78–83 (2002).
41. Lee, Y. & McKinnon, P.J. DNA ligase IV suppresses medulloblastoma formation. *Cancer Res.* 62, 6395–6399 (2002).
42. Fernandez, L.A. et al. Oncogenic YAP promotes radioresistance and genomic instability in medulloblastoma through IGFl-mediated Akt activation. *Oncoogene* 31, 1923–1937 (2012).
43. Beer, S. et al. Developmental context determines latency of MYCl-induced tumorigenesis. *PLoS Biol.* 2, e332 (2004).
ONLINE METHODS

Animals. Ptc1+/C mice, Nestin-CFP mice and Nestin-CreERT2 mice have been described previously21,31. Math1-Cre mice, Math1-CreERT2 mice, Math1-GFP mice, Actin-DsRed mice and R26R-GFP reporter mice were purchased from the Jackson Laboratory. SCID mice (C.B-17 SCID) were bred in the Fox Chase Cancer Center Laboratory Animal Facility. All animals were maintained in the Laboratory Animal Facility at Fox Chase Cancer Center, and all experiments were performed in accordance with procedures approved by the Fox Chase Cancer Center Animal Care and Use Committee. In this study, we used mice of either sex at various ages, which are mentioned in the text and figure legends.

Cerebellum microdissection, cell isolation and cell culture. Cerebella from Nestin-CFP–Math1-GFP animals at P4. 300-µm slices were prepared using a VT1000S vibratome (Leica). Under a fluorescence microscope, EGL or white matter were carefully removed from the rest of the cerebellum using fine forceps. Dissected EGL or white matter were then digested in a solution containing 10 units/ml papain (Worthington) and 250 U/ml DNase then triturated to obtain a single-cell suspension. GNPs (GFP+) and NEPs (CFP+) were then purified using a FACSVantage SEM (BD Biosciences). For isolation of NSCs, the cell suspension dissociated from Nestin-CFP–Math1-GFP cerebellum was stained for 1 h with PE-conjugated Prominin1 antibody and with antibodies specific for lineage markers (PSA-NCAM, O4 and TAPA-1; sources, identifiers and dilutions used for all antibodies are listed in Supplementary Table 1). After staining with FITC-conjugated secondary antibodies, NSCs were FACs sorted for PE-positive and FITC-negative cells.

NEPs and GNPs were suspended in NB-B27 (Neurobasal with 1 mM sodium pyruvate, 2 mM t-glutamine, penicillin-streptomycin and B27 supplement; all from Invitrogen) and plated on poly(λ-lysine) (PDL)-coated coverslips (BD Biosciences). For the neurosphere-forming assay, cells were plated at 2 × 105 cells/ml in NSC proliferation medium (NeuroCult basal medium with proliferation supplement, Stem Cell Technologies) plus 10 ng/ml basic fibroblast growth factor and 20 ng/ml epidermal growth factor (PeproTech). Neurospheres were counted or harvested for immunostaining after 7 d in culture. To confirm the self-renewal capacity, neurospheres were mechanically dissociated and replated in fresh proliferation medium at 2 × 105 cells/ml. For differentiation assays, cells were plated on PDL-coated coverslips in NSC differentiation medium (NeuroCult basal medium with differentiation supplement; Invitrogen).

Immunohistochemical staining and histological analysis. For tissue staining, cerebella from PPA-perfused animals were fixed overnight in 4% PFA, cryoprotected in 30% sucrose, frozen in Tissue T ek-OCT (Sakura Finetek) and cut into 10–12 µm sections. For cell staining, GNPs cultured on PDL were fixed with 4% PFA for 15 min. Immunofluorescence staining of sections and cells was carried out according to standard methods. Briefly, sections or cells were blocked and permeabilized for 2 h with PBS containing 0.1% Triton X-100 and 1% normal goat serum, stained with primary antibodies overnight at 4 °C, and incubated with secondary antibodies for 2 h at room temperature. Sections or cells were counterstained with DAPI and mounted with Fluoromount-G (Southern Biotech) before being visualized using a Nikon TE200 microscope. Antibodies used in this study are listed in Supplementary Table 1. To compare the histological properties between human and mouse medulloblastoma, hematoyxlin and eosin stain was performed according to a standard procedure. Human medulloblastoma slides were purchased from US Biomax, Inc.

Tamoxifen treatment and intracranial transplantation. Tamoxifen (Sigma) was prepared as a 20 mg/ml stock solution in corn oil (Sigma) and was administered by oral gavage using a 24G gavaging needle (Fine Science Tools). Animals at P4 were given tamoxifen at a dosage of 0.6 mg/30 ml.

For the intracranial transplantsations, SCID mice were anesthetized using 100 mg/kg ketamine (Fort Dodge Animal Health) plus 9 mg/kg xylazine (Ben Venue Laboratories) and positioned in a stereotaxic frame with a mouse adaptor (David Kopf Instruments). An incision was made in the midline of the scalp over the cerebellum, and a small hole was made in the skull (1 mm lateral to midline) using a beveled 18G needle. A 24G Hamilton syringe loaded with cells was mounted on a micromanipulator and introduced through the hole at a 30° angle to the surface of the cerebellum at a depth of 1 mm. Freshly sorted (uncultured) cells were injected over the course of 2 min, and the needle was left in place for another 2 min to avoid reflux. After removing the mouse from the frame, 1–2 drops of 0.25% (2.5 mg/ml) bupivicaine (Hospira) were applied along the incision for postoperative analgesia and the skin was closed with 6.0 fast-absorbing plain gut sutures using a 3/8 PC-1 cutting needle (Ethicon).

Microarray analysis. RNAs isolated from NEPs, GNPs and NSCs were labeled and hybridized to Affymetrix Mouse Genome 430 2.0 arrays. Microarray data were preprocessed using robust multichip analysis (RMA). Principal component analysis (PCA) was performed on samples based on normalized expression of all genes using Partek Genomics Suite 6.3 software. Gene ontology analysis was carried out to examine the biological functions of the differentially expressed genes between NEPs and GNPs using NexusExp3 software.

Quantitative PCR. RNA was isolated using the RNAqueous kit (Ambion) and treated with DNA-free DNase (Ambion). cDNA was synthesized using oligo(dT) and Superscript II reverse transcriptase (Invitrogen). Quantitative PCR reactions were performed in triplicate using iQ SYBR Green (Bio-Rad) and the Bio-Rad iQ Multicolor Real-Time PCR Detection System. Gene expression was normalized to actin and expression of each gene was compared between NEPs and GNPs. Primer sequences are available upon request.

Metaphase spread. Metaphase preparations were carried out by the Laboratory Animal Facility at Fox Chase Cancer Center according to standard procedures.

Statistics. Student’s t tests and Chi-squared tests were performed to determine the statistical significance of the difference in means between samples in the experiments reported in Figures 2, 4, 5 and Supplementary Figures 6 and 8. P < 0.05 was considered statistically significant. Error bars represent s.e.m. No statistical methods were used to predetermine sample sizes, but these were similar to those employed in the field. We did not use randomization in this study, but for each experiment all genotypes were represented and appropriately blocked for data collection. We did not perform data collection and analysis blind to the conditions of the experiments because the properties of each cell type are so distinctive that the investigators would be able to tell the genotype of the animals by observation of the tissue sections. The data distribution was assumed to be normal, but this was not formally tested. Overall survival in Figure 6 was assessed using the Kaplan-Meier survival analysis and the Mantel-Cox log-rank test was used to assess the significance of difference between survival curves. Data handling and statistical processing was performed using Microsoft Excel and Graphpad Prism Software.