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Isolation of neural stem cells from the postnatal cerebellum

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Abstract

The cerebellum is critical for motor coordination and cognitive function and is the target of transformation in medulloblastoma, the most common malignant brain tumor in children. Although the development of granule cells, the most abundant neurons in the cerebellum, has been studied in detail, the origins of other cerebellar neurons and glia remain poorly understood. Here we show that the murine postnatal cerebellum contains multipotent neural stem cells (NSCs). These cells can be prospectively isolated based on their expression of the NSC marker prominin-1 (CD133) and their lack of markers of neuronal and glial lineages (lin⁻). Purified prominin⁺lin⁻ cells form self-renewing neurospheres and can differentiate into astrocytes, oligodendrocytes and neurons *in vitro*. Moreover, they can generate each of these lineages after transplantation into the cerebellum. Identification of cerebellar stem cells has important implications for the understanding of cerebellar development and the origins of medulloblastoma.

The cerebellum is required for motor coordination and is crucial for cognitive and affective processing¹. These functions depend on interactions among at least six types of neurons and two types of glia². Disruption of cerebellar structure and function is associated with disorders such as ataxia, autism and schizophrenia³⁻⁵. In addition, uncontrolled growth of cerebellar precursors results in medulloblastoma^{6,7}. Elucidating the mechanisms that control the generation of cerebellar neurons and glia during normal development is critical for understanding the basis of these diseases.

The cerebellum differs from most other brain regions in that it contains two distinct germinal layers: the ventricular zone (VZ), which is most active during embryonic development, and the external germinal layer (EGL), which contributes to neurogenesis after birth². There is strong evidence that Purkinje cells, the major output neurons of the cerebellum, originate from the VZ and that granule cells, the most abundant interneurons, arise from the EGL. However, the origin of the other cell types in the cerebellar cortex—including astrocytes, oligodendrocytes and stellate, basket, Lugaro and Golgi interneurons—is much less clear. Tissue grafting and transplantation studies suggest that many of these cells arise from VZ progenitors that migrate into the cerebellar cortex after birth^{8,9}. But whether each class of neuron and glial cell comes from a distinct progenitor or whether they all come from a common, multipotent progenitor is not known.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Here we purify a population of multipotent neural stem cells from the postnatal cerebellum. We show that this population can undergo self-renewal in culture and can generate neurons, astrocytes and oligodendrocytes both *in vitro* and after transplantation. Our findings suggest that cerebellar neurons and glia could be generated from a common progenitor. In addition, the approach we have used may be applicable for isolating NSCs from other parts of the nervous system.

RESULTS

Non-granule cell precursors proliferate in response to bFGF

Because the majority of cells in the postnatal cerebellum are granule cell precursors (GCPs), it has been difficult to study the precursors of other cell types. To circumvent this problem we used Math1-GFP mice, which express green fluorescent protein in their GCPs¹⁰. We isolated cells from the cerebellum of 7-d-old (P7) mice and analyzed them by flow cytometry. Among the cells we isolated, 90% were GFP⁺ GCPs (Fig. 1a). Approximately 10% of the cells were GFP⁻ and thus likely represented precursors of other lineages.

To study these cells in more detail, we sorted them by FACS and measured their responses to growth factors. Consistent with our previous findings¹¹, GFP⁺ GCPs proliferated robustly in the presence of Sonic hedgehog (Shh, Fig. 1b). Although some studies have suggested that basic fibroblast growth factor (bFGF) can be mitogenic for GCPs¹², we found that purified GFP⁺ cells did not proliferate in response to bFGF. In contrast, GFP⁻ cells showed little response to Shh but proliferated extensively in response to bFGF (Fig. 1b). These data indicate that at least two populations of precursors can be isolated from the postnatal cerebellum: Math1-GFP⁺, Shh-responsive GCPs, and Math1-GFP⁻, bFGF-responsive non-GCPs.

To identify the GFP⁻ cells, we stained them with antibodies specific for neuronal and glial markers (Table 1). The GFP⁻ population included cells with markers of neuronal (HNK-1, polysialated (PSA) NCAM, MAP-2; refs. 13-15), oligodendrocyte (O4, NG2; refs. 16,17) and astrocyte lineages (GFAP, TAPA-1, CD44; refs. 18-20). In addition, approximately one-third of GFP⁻ cells expressed markers associated with neural progenitors and stem cells, including nestin²¹, prominin-1 (ref. 22), Sox-2 (ref. 23) and Musashi²⁴ (Table 1 and data not shown). These studies suggested that the GFP⁻ population includes neurons, astrocytes, oligodendrocytes and stem cells.

Stem cells can be purified from the postnatal cerebellum

Our detection of cells expressing NSC markers raised the possibility that the postnatal cerebellum contains multipotent neural stem cells. To investigate this, we sorted the putative stem cells by FACS using antibodies specific for prominin-1 (CD133), a surface glycoprotein found on stem cells in the nervous and hematopoietic systems^{22,25,26}. The prominin⁺ progenitors we isolated were highly enriched for bFGF-responsive cells (Fig. 2). Because these cells co-isolated with GCPs, we sought to determine whether they were located in the EGL, where GCPs reside. *In situ* hybridization with probes specific for *Prom1*, the gene encoding prominin-1, identified *Prom1*-expressing cells throughout the cerebellar white matter (Fig. 3a-d). We also saw occasional *Prom1*-expressing cells in the internal granule layer (IGL) and Purkinje layer but not in the molecular layer or EGL. We observed a similar expression pattern when neonatal cerebellum was stained with antibodies to prominin (Fig. 3e,f). In addition, when cerebellum was microdissected into EGL and non-EGL regions and analyzed by FACS, prominin⁺ cells were found to be highly enriched in the non-EGL fraction (data not shown). Together these data suggest that the majority of prominin-expressing cells are located in the white matter and not in the EGL.

Among prominin⁺ cells, 50-60% expressed markers of neurons and 30-40% expressed markers of astrocytes and oligodendrocytes; only 10% lacked such markers and were considered 'lineage-negative.' To further purify these cells, we used antibodies to the surface markers PSA-NCAM, TAPA-1 and O4 to deplete cells associated with neuronal and glial lineages. The resulting prominin-positive, lineage-negative (prominin⁺lin⁻) population represented 1-3% of the Math1-GFP⁺ cells, or 0.1-0.3% of the cells that could be isolated from the neonatal cerebellum (Fig. 4a and Supplementary Fig. 1B online). Notably, only a subset of these cells (~20%) expressed nestin, suggesting that prominin and nestin are overlapping but not equivalent markers of NSCs.

Our identification of a population of cells with a phenotype associated with neural stem cells prompted us to determine whether these cells showed functional properties associated with neural stem cells as well.

Cerebellar stem cells generate neurospheres *in vitro*

Stem cells from many parts of the CNS proliferate and form macroscopic spheres when cultured on non-adhesive substrates in the presence of growth factors²⁷. To determine whether the cells we isolated could generate such neurospheres, we cultured them at clonal density in bFGF and EGF. Prominin⁺lin⁻ cells cultured in this manner reproducibly generated spheres within 6-10 d (Fig. 4b). These cells could also generate neurospheres when cultured at a density of one cell per well, confirming that neurospheres resulted from expansion of individual clones rather than from aggregation of cells. In contrast to prominin⁺lin⁻ cells, prominin⁻ cells cultured at clonal density survived poorly and never formed macroscopic spheres (Fig. 4c). Prominin⁺lin⁺ cells survived, but rarely formed free-floating spheres; instead, they usually gave rise to adherent colonies that showed signs of neuronal differentiation (extension of processes and expression of Map-2). The efficiency of neurosphere formation in various fractions of cerebellar cells is summarized in Supplementary Figure 1. Together these data demonstrate that prominin⁺lin⁻ cells are highly enriched in the ability to form neurospheres.

To determine whether the neurospheres generated from prominin⁺lin⁻ cells expressed stem cell markers, we stained them with antibodies specific for nestin, Sox-2, Musashi and GFAP (a marker often associated with astrocytes but recently shown to be expressed by radial glia and neural stem cells as well²⁸). These markers were expressed in all neurospheres examined (Fig. 4d-l). The majority of cells in each sphere expressed these markers at levels well above background (see controls, Fig. 4m-o), with a subset (3-6%) of cells in each sphere expressing particularly high levels. Thus, prominin⁺lin⁻ cells form neurospheres that maintain expression of stem and progenitor cell markers.

Cerebellar stem cells undergo extensive self-renewal

A key feature of stem cells is their ability to undergo self-renewal, that is, to proliferate and generate more stem cells. We examined the self-renewal of neurospheres generated from prominin⁺lin⁻ cells by dissociating them and testing their ability to re-form new neurospheres. Cells from dissociated neurospheres gave rise to secondary neurospheres that were morphologically and phenotypically identical to primary neurospheres (Supplementary Fig. 2 online). In addition, these neurospheres could be repeatedly dissociated and re-plated, allowing propagation for at least 10 weeks in culture.

The efficiency of neurosphere formation was maintained across multiple passages (Supplementary Fig. 2). When freshly isolated prominin⁺lin⁻ cells were cultured at clonal density, approximately 1 neurosphere was generated for every 30 cells plated (a frequency of 3%). When primary neurospheres were dissociated and re-plated at clonal density, the frequency of secondary and tertiary neurosphere generation was also ~3%. This is similar to

the percentage of cells that expressed high levels of stem cell markers, as mentioned previously. Like primary neurospheres, secondary and tertiary spheres retained expression of nestin, GFAP, Musashi and Sox-2. These studies indicate that prominin⁺lin⁻ cells isolated from the cerebellum are capable of undergoing extensive self-renewal.

Cerebellar stem cells generate neurons and glia *in vitro*

Another important characteristic of neural stem cells is multipotency: the ability to differentiate into neurons, astrocytes and oligodendrocytes. To test whether neurospheres derived from prominin⁺lin⁻ cells are multipotent, we withdrew bFGF and EGF and cultured the neurospheres in the presence of factors that support neuronal and glial differentiation²⁹⁻³¹. Neurospheres cultured in retinoic acid (RA), leukemia inhibitory factor (LIF) or platelet-derived growth factor (PDGF-AA) could each generate astrocytic (S100 β ⁺, GFAP⁺), oligodendroglial (NG2⁺) and neuronal (Map-2⁺) cells (Fig. 5a-k). Notably, mature oligodendrocytes expressing O4 were generated only in LIF (Fig. 5f) and PDGF-AA (Fig. 5i,k). These data demonstrate that a single prominin⁺lin⁻ cell can generate neurons, astrocytes and oligodendrocytes *in vitro*.

To determine the types of the neurons generated, we examined differentiated neurospheres for markers associated with different classes of cerebellar neurons. Neurons in the cerebellum can be distinguished on the basis of neurotransmitter secretion: granule cells secrete glutamate, whereas all other cerebellar neurons secrete γ -aminobutyric acid (GABA)^{32,33}. In our cultures, some neurons expressed GABA (Fig. 5l), whereas others expressed glutamate (Fig. 5m). This heterogeneity was also evident from staining with antibodies to lineage-specific transcription factors: some cells expressed Pax-2, a transcription factor found in stellate and basket cell progenitors³⁴ (Fig. 5n), whereas others expressed Zic-1 and Math-1, markers of the granule cell lineage^{10,35} (Fig. 5o and data not shown). Together these data indicate that cerebellar stem cells can generate various types of neurons and glia *in vitro*.

Stem cells generate neurons and glia after transplantation

To determine whether prominin⁺lin⁻ cells are also multipotent *in vivo*, we injected them into the cerebellum of neonatal mice and analyzed their ability to undergo differentiation. In one set of experiments (Fig. 6), freshly isolated prominin⁺lin⁻ cells were injected into hosts. This ensured that the ability of donor-derived stem cells to differentiate into neurons, astrocytes and oligodendrocytes was not influenced by exposure to growth factors or extensive cell culture. However, injecting large numbers of prominin⁺lin⁻ cells could not prove that all three lineages were derived from a single stem cell. To this end we also carried out experiments in which a single neurosphere (derived from a single stem cell) was transplanted into each host (Fig. 7). In each case, the fate of transplanted cells was assessed 2-3 weeks after injection.

Both freshly isolated prominin⁺lin⁻ cells and neurospheres gave rise to cells of all three lineages. S100 β ⁺ cells (astrocytes) were found in the Purkinje layer near the cell bodies of endogenous bergmann glia (Fig. 6a,d) or in the white matter (Fig. 7a,d). NG2⁺ cells (oligodendrocytes) were observed in the IGL (Fig. 6b,e) or near the surface of the cerebellum (Fig. 7b,e). TuJ1⁺ cells (neurons) were most commonly seen in the molecular and Purkinje layers (Figs. 6c,f and 7c,f), where basket, stellate and Purkinje neurons normally reside. These studies suggest that individual cerebellar stem cells can generate neurons, astrocytes and oligodendrocytes *in vivo* and that these cells can integrate into the cerebellum after transplantation.

The most abundant donor-derived cells were neurons. To further characterize these cells, we stained sections of host cerebellum with antibodies to GABA_A receptor $\alpha 6$ (a neurotransmitter receptor found specifically on granule cells), parvalbumin (a marker of mature basket, stellate

and Purkinje cells) and calbindin (a marker of Purkinje cells). Of the neurons we observed, none were found in the IGL and none expressed GABA_A receptor $\alpha 6$ (Fig. 7g,j). Rather, the majority of donor-derived neurons were small, parvalbumin⁺ cells located in the molecular layer (Fig. 7h,k), a phenotype consistent with stellate neurons. In addition, we also observed large calbindin⁺ cells in the Purkinje cell layer, alongside endogenous Purkinje cells (Fig. 7i,l). Together, these data indicate that cerebellar stem cells can give rise to astrocytes, oligodendrocytes and neurons both *in vitro* and *in vivo*.

DISCUSSION

Stem cells have been identified in many parts of the nervous system in embryonic life and in adulthood. However, there has been little evidence for the presence of stem cells in the cerebellum, and these cells have not been isolated or studied in detail^{36,37}. We have used flow cytometry to prospectively purify multipotent neural stem cells from the developing cerebellum. We show that these cells express stem cell markers, proliferate in response to bFGF and EGF, generate self-renewing neurospheres, and differentiate into neurons and glia *in vitro* and after transplantation into neonatal mice. These findings have important implications for our understanding of normal cerebellar development and tumorigenesis.

Our identification of cerebellar stem cells originated from studies of bFGF responsiveness in GCPs. Although previous reports have suggested that bFGF is a weak mitogen for GCPs¹², we have observed that it is a potent inhibitor of Shh-induced GCP proliferation¹¹. To explain this discrepancy, we purified GCPs to homogeneity by FACS sorting GFP⁺ cells from Math1-GFP mice. Analysis of the resulting cells showed that highly purified GCPs did not proliferate to bFGF. On the other hand, we observed a significant proliferative response to bFGF in a small population of cells that co-fractionates with GCPs during the purification procedure. Thus, Math1-GFP mice represent a powerful tool for purifying different classes of neurons and glia from the cerebellum.

Among the GFP⁺ cells in the postnatal cerebellum, a subset expressed markers associated with neural stem cells, including prominin-1. Prominin is associated with plasma membrane protrusions in embryonic and adult epithelial cells, and is also a marker of hematopoietic stem cells²². In the nervous system, prominin has been reported to colocalize with nestin in the ventricular zone²⁵. Moreover, antibodies to the human homolog of prominin, CD133, have been used to isolate multipotent stem cells from fetal brain and from brain tumor tissue^{26,38}. Thus, prominin is an important surface marker for neural stem cells. Expression of prominin has not been described previously in the postnatal cerebellum, but our studies suggest that it marks multipotent progenitors in this tissue as well.

We used antibodies to prominin to isolate progenitors from the postnatal cerebellum and tested their ability to form neurospheres. We found that prominin⁺ cells are highly enriched for neurosphere formation as compared to unfractionated cells from the postnatal cerebellum. Moreover, neurospheres derived from prominin⁺lin⁻ cells can undergo self-renewal and differentiate into both neurons and glia. The fact that these cells can be prospectively isolated has also allowed us to examine their potential without subjecting them to prolonged culture *in vitro*. This is important, because a number of recent studies have indicated that culturing cells under neurosphere-generating conditions can alter the types of cells generated³⁹. The fact that we can generate neurons, astrocytes and oligodendrocytes after transplantation of either single neurospheres or freshly isolated prominin⁺lin⁻ cells suggests that such de-differentiation has not taken place in our studies. Rather, the tri-lineage potential of our cells seems to reflect an intrinsic characteristic of the cells we have isolated.

Our demonstration that purified cerebellar stem cells can generate neurons, astrocytes and oligodendrocytes *in vitro* and after transplantation has important implications for cerebellar development. Although the postnatal cerebellum has long been known to contain precursors of basket and stellate neurons, astrocytes and oligodendrocytes², it has not been clear whether these cells arise from distinct precursors or from a single multipotent progenitor. Several studies have hinted at the latter possibility. For example, oncogene-immortalized cell lines derived from the postnatal cerebellum express neuronal and glial markers *in vitro* and can generate neurons and glia after implantation in the cerebellum and other parts of the brain^{40,41}. Although it has been suggested that these cells represent GCPs whose differentiation potential has been altered by oncogenes⁴⁰, our studies suggest they may be stem cells that have been selectively immortalized in culture. Similarly, an elegant series of retroviral lineage-tracing studies^{8,42} has shown that the white matter contains progenitors that give rise to interneurons and glia. In these studies, a small proportion of retrovirally marked cells did not stain with lineage markers, and it was suggested that these might represent uncommitted stem cells⁴². because we have detected expression of prominin primarily in white matter, the NSCs we have isolated may represent these uncommitted cells.

In addition to generating GABA and Pax2-expressing interneurons, prominin⁺lin⁻ cells also generated glutamate and Zic-1-expressing granule cells. Although both granule and non-granule cells are generated in the postnatal cerebellum, several studies have suggested that they arise from distinct progenitors^{8,9,43}. One interpretation of our results is that cerebellar NSCs have the potential to generate granule cells *in vitro*, but that *in vivo* they generate exclusively non-granule neurons and glia. Consistent with this view, we have not observed any granule neurons generated after transplantation of cerebellar stem cells into the neonatal cerebellum. On the other hand, we cannot rule out the possibility that some of the granule cells generated during postnatal development come from multipotent progenitors rather than from restricted GCPs in the EGL. These cells, if they are rare, would be difficult to detect using conventional lineage tracing and transplantation studies. More saturating methods of fate mapping⁴⁴ might shed light on this issue.

In addition to their importance for normal development, our studies also have significant implications for understanding medulloblastoma. The cell of origin for medulloblastoma has been a matter of longstanding debate. A subset of medulloblastomas express markers of GCPs and have mutations in the Shh pathway^{45,46}, and are therefore likely to arise from committed GCPs. However, the majority of medulloblastomas express distinct markers and show no evidence of Shh pathway activation^{46,47}. In light of recent studies demonstrating expression of CD133 and other NSC markers in human medulloblastoma^{38,48}, it is important to consider the possibility that some of these tumors arise from the stem cells we have described. If so, understanding the signaling pathways that control growth and differentiation of these cells may yield new approaches to the diagnosis or therapy of this devastating tumor.

METHODS

Animals

Math1-GFP mice¹⁰ were generated at UT Southwestern Medical Center. β -actin-cyan fluorescent protein (actin-CFP) transgenic mice were provided by B. Capel (Duke University). C57Bl/6 $\times$ CBAF1 mice were from the Jackson Laboratories. Animals were maintained in the animal facility at Duke University and used in accordance with protocols approved by the Duke Institutional Animal Care and Use Committee.

Isolation of non-GCPs and stem cells

Cells were isolated from the cerebellum as described previously¹¹. Briefly, cerebella from postnatal day 7 (P7) mice were digested with 10 U ml⁻¹ papain (Worthington), 200 µg ml⁻¹ L-cysteine and 250 U ml⁻¹ DNase (Sigma). Tissue was triturated to obtain a single cell suspension and then centrifuged through a Percoll gradient (Sigma). Cells were harvested, washed and resuspended in buffer consisting of Dulbecco's PBS with 5% FCS and 2 mM EDTA.

Non-GCPs were purified by FACS sorting of GFP-negative cells from Math1-GFP mice using a FACSVantage SE (BD Biosciences). Cerebellar stem cells were purified by sorting cells that expressed prominin-1 (detected with antibody 13A4; ref. ⁴⁹) and lacked the lineage markers O4, polysialated (PSA)-NCAM (Chemicon) and TAPA-1 (ebioscience). Prominin⁺lin⁻ cells were sorted into Neurobasal medium with B27 supplement (NBB27, Invitrogen) containing 10 mg ml⁻¹ BSA (Sigma), washed and resuspended in NBB27 with appropriate growth factors.

Proliferation assays

Cells isolated as described above were suspended in NBB27 and transferred to poly-D-lysine (PDL)-coated 96-well plates at a density of 2×10^5 cells per well. Growth factors were added immediately. After 48 h, cells were pulsed with methyl-[³H]thymidine (Perkin Elmer) and cultured for an additional 16-18 h. Cells were harvested onto filters using a Mach III Manual Harvester 96 (Tomtec) and incorporated radioactivity was quantified on a Wallac Microbeta scintillation counter (PerkinElmer) by liquid scintillation spectrophotometry.

Flow cytometry and immunofluorescence

To detect expression of surface markers, cells were stained for 1 h with primary antibodies and 30 min with secondary antibodies, and analyzed by FACS. To detect expression of intracellular markers, cells were plated on PDL-coated coverslips and allowed to adhere for 1-2 h before being fixed with 4% paraformaldehyde (PFA). Cells were incubated in normal goat serum (NGS) with 0.2% Triton X-100 to block nonspecific binding and permeabilize membranes, and stained overnight with primary antibodies and for 2 h with secondary antibodies. Coverslips were mounted with Fluoromount G (Southern biotechnology Associates). Immunofluorescence was detected using a Nikon TE200 inverted microscope and Openlab software (Improvision).

For immunostaining of tissue, cerebella were fixed in 4% PFA, cryoprotected in 25% sucrose and embedded in O.C.T. (Sakura Finetek). Cryosections (10-16 µM) were blocked and permeabilized overnight in NGS with Triton X-100 and stained with antibodies as described above. Sections were counterstained with DAPI (Molecular Probes) and mounted using Fluoromount G.

Antibodies used for flow cytometry and immunofluorescence included the following: antibodies to HNK-1 (CD57), nestin and GFAP (BD Pharmingen); to TuJ1 and Pax-2 (Covance); to CD44 (biosource); to Musashi, Sox-2, NG2, Zic-1 and GFP (Chemicon); and to S100β, Map-2, GABA, glutamate, parvalbumin and calbindin (Sigma). Normal mouse and rabbit IgG, NGS and secondary antibodies labeled with fluorescein isothiocyanate (FITC), aminomethylcoumarin (AMCA), tetramethyl rhodamine (TRITC), phycoerythrin (PE) and Cy5 were from Jackson ImmunoResearch.

In situ hybridization

P7 pups were perfused with 2.5% PFA and their brains were dissected, embedded in O.C.T., and cryosectioned at a thickness of 12 µM. Sections were post-fixed in 4% PFA, acetylated and incubated for 1 h at room temperature (20-25 °C) in pre-hybridization buffer (50% formamide, 5× SSC, 1× Denhardt's solution, 250 µg ml⁻¹ yeast tRNA, 500 µg ml⁻¹ herring

sperm DNA). Sections were hybridized overnight at 65 °C in hybridization buffer (50% deionized formamide, 1 Denhardt's solution, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 10 mM Na₂HPO₄, pH 7.4, 10% dextran sulfate, 0.5 mg ml⁻¹ yeast tRNA) containing digoxigenin (DIG)-UTP-labeled probes for *Prom1*. Probes were synthesized using a DIG labeling kit (Roche) according to the manufacturer's protocol. After hybridization, sections were incubated overnight at 4 °C with antibodies to DIG conjugated to alkaline phosphatase (Roche). Bound probe was visualized by incubating slides in NBT/BCIP overnight in the dark. Coverslips were mounted with Aqua Polymount (Polysciences).

Neurosphere growth and differentiation

To generate primary neurospheres, prominin⁺lin⁻ cells were cultured at clonal density⁵⁰ (1-2 cells per mm²) on uncoated plates in NBB27 containing 25 ng ml⁻¹ bFGF (Invitrogen) and 25 ng ml⁻¹ EGF (Peprotech). Cells were cultured for 10 d and neurospheres were harvested using AdvanTip-LT low-retention pipette tips (Hamilton). To generate secondary neurospheres, primary neurospheres were dissociated with papain and replated under identical neurosphere growth conditions for an additional 10 d. For differentiation, free-floating neurospheres were centrifuged, supernatant was aspirated and neurospheres were resuspended in NBB27 containing 10 ng ml⁻¹ PDGF-AA (Sigma), 1 U ml⁻¹ leukemia inhibitory factor (ESGRO/LIF, Chemicon) or 100 ng ml⁻¹ all-*trans* retinoic acid (RA, Sigma) and plated onto PDL-coated coverslips. After 7 d, coverslips were fixed and stained as described above.

Neurosphere and stem cell transplantation

To assess *in vivo* the differentiation of prominin⁺lin⁻ cells and neurospheres, cells were either labeled with chloromethylbenzamido-DiI (CM-DiI, Molecular Probes) or isolated from β -actin-CFP mice. Cells were injected into the cerebellum of P3 pups using a 1.0-mm capillary needle. Each pup received 1×10⁴ prominin⁺lin⁻ cells or one neurosphere. After 17-26 d, cerebella were fixed, cryosectioned and stained as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Non-granule cell precursors can be purified from the postnatal cerebellum. (a) Isolation of non-GCPs. Cells from neonatal Math1-GFP cerebellum were sorted into GFP-negative and GFP-positive populations by flow cytometry. (b) Proliferative responses of non-GCPs. The GFP⁻ and GFP⁺ populations were cultured with no stimulus (∅), Shh or bFGF and then harvested to measure thymidine incorporation. Data represent triplicates ± s.e.m.

Figure 2.

Proliferin⁺ cells proliferate in response to bFGF. GFP⁺ cells from Math1-GFP cerebella were sorted by FACS into proliferin⁺ and proliferin⁻ cells, and each population was cultured either with no stimulus (Ø) or with bFGF and then harvested for analysis of thymidine incorporation. Data represent triplicates $\pm$ s.e.m.

Figure 3.

Prominin⁺ cells are located in the cerebellar white matter. (a-d) Detection of prominin mRNA by in situ hybridization. Cryosections of P7 cerebellum were hybridized with digoxigenin (DIG)-labeled sense (control; a,b) or antisense (c,d) probes specific for mouse *Prom1* (prominin RNA) and then stained with alkaline phosphatase-conjugated antibodies to DIG and with NBT/BCIP substrate to detect bound probe. Sections were photographed at 10× (a,c) and 20× (b,d) magnification; boxes indicate locations of regions shown in b,d. Arrows indicate prominin⁺ cells (dark brown spots) in the white matter (WM). (e,f) Detection of prominin protein by immunostaining. cerebellar sections were stained with rat antibodies to prominin-1 and TRITC-conjugated secondary antibodies. Low-power (10×) image of cerebellum from a Math1-GFP mouse shows GFP fluorescence (green) in the outer EGL and prominin⁺ cells (red) in the white matter (e). The section is counterstained with DAPI (blue) to highlight cerebellar structure. High-power (20×) image of cerebellum from wild-type mouse shows prominin⁺ cells (red) in the white matter (f). No counterstain is shown. Red, green and blue images were photographed separately and merged with Openlab software. EGL, external germinal layer; WM, white matter.

Figure 4.

Prominin⁺lin⁻ cells generate neurospheres. **(a)** Isolation of prominin⁺lin⁻ cells. cerebellar cells from Math1-GFP mice were stained with antibodies to prominin-1 (x axis) and neuronal and glial lineage markers (PSA-NCAM, TAPA-1 and O4, y axis), and analyzed by flow cytometry. Prominin⁺lin⁻ cells represent 0.2% of the total population isolated from the postnatal cerebellum. **(b,c)** Neurosphere formation. FACS-sorted prominin⁺lin⁻ and prominin⁻ cells were cultured at clonal density in the presence of bFGF and EGF for 10 d. **(c-o)** Expression of NSC markers. Neurospheres from clonal cultures of prominin⁺lin⁻ cells were stained with rabbit antibodies specific for Sox-2 **(d)**, Musashi **(g)** or GFAP **(j)** and with mouse antibodies specific for nestin **(e,h,k)**; primary antibodies were detected with FITC-conjugated anti-rabbit (green) and TRITC-conjugated anti-mouse (red) antisera. Insets show high-magnification images of regions indicated by white boxes; high levels Sox-2, Musashi, GFAP and nestin are expressed by a subset of cells within each sphere. To control for nonspecific staining, some spheres from each experiment were stained with normal rabbit IgG **(m)** and normal mouse IgG **(n)** followed by the same secondary antibodies. Red and green pictures were taken at 40 $\times$ magnification and merged **(f,i,l,o)** using Openlab software.

Figure 5.

Prominin⁺lin⁻ cells exhibit multipotency *in vitro*. (a-k) Generation of neurons, astrocytes and oligodendrocytes. Prominin⁺lin⁻ cells were cultured at clonal density for 10 d to generate neurospheres. Neurospheres were transferred onto PDL-coated coverslips and cultured in the absence of bFGF and EGF and in the presence of all-trans retinoic acid (RA; a-c), leukemia inhibitory factor (LIF; d-f) or platelet-derived growth factor (PDGF-AA; g-k). After another 7 d, cultures were fixed and stained with antibodies specific for NG2 and S100β (red and blue, respectively; a,d,g), Map-2 (b,e,h), O4 (c,f,i) or GFAP (j). Also shown (k) is a single neurosphere cultured in PDGF-AA and stained with antibodies to O4 (red), Map-2 (green) and S100β (blue). (l-o) Generation of granule and non-granule neurons. Neurospheres differentiated in the presence of PDGF-AA were fixed and stained with antibodies specific for GABA (l), glutamate (m), and Pax-2 (n) and Zic-1 (o) followed by rhodamine-conjugated secondary antibodies. All pictures were taken at 40× magnification and processed using Openlab software.

Figure 6.

Prominin⁺lin⁻ cells differentiate into neurons and glia after transplantation into the cerebellum. Freshly isolated prominin⁺lin⁻ cells were labeled with CM-DiI (red) and injected into the cerebellum of 3-d-old mice (10,000 cells per host). After 2-3 weeks, host cerebella were fixed and stained with antibodies specific for S100β (**a**), NG2 (**b**) or TuJ1 (**c**). Primary antibodies were detected with FITC-labeled antisera (green). Double-labeled cells (orange-yellow in **a-c**) were identified and photographed at 40 magnification; 10× bright-field pictures (**d-f**) indicate the location of × transplanted cells in the corresponding fluorescent panels. PCL, Purkinje cell layer; IGL, internal granule layer; ML, molecular layer.

Figure 7.

Neurospheres derived from a single stem cell can generate neurons and glia *in vivo*.

Prominin⁺lin⁻ cells from actin-CFP transgenic mice were cultured at clonal density for 10 d to generate neurospheres. Neurospheres were injected into the cerebellum of 3-d-old mice (one neurosphere per host). After 2-3 weeks, host cerebella were fixed and stained with antibodies specific for S100 β (**a**), NG2 (**b**), TuJ1 (**c**), GABA_A receptor $\alpha 6$ (**g**), parvalbumin (**h**) or calbindin (**i**). These antibodies were detected with TRITCconjugated secondary antibodies (red).

Chicken antibodies to GFP and FITCconjugated anti-chicken antiserum were used to amplify the CFP signal (green). Double-labeled cells (orange-yellow in a-c and g-i) were identified and photographed at 40 $\times$ magnification; 10 bright-field pictures (**d-f** and **j-l**) indicate the location of transplanted cells in the corresponding fluorescent panels. WM, white matter; pia, pial membrane surrounding surface of cerebellum; ML, molecular layer; IGL, internal granule layer; PCL, Purkinje cell layer.

Table 1

Phenotype of non-granule cell precursors

Antibody	Specificity	Percentage positive (among GFPcells)
Prominin-1*	Stem cells	30
Nestin	Stem cells	29
GFAP	Astrocytes and stem cells	23
HNK-1*	Neuronal progenitors	73
PSA-NCAM*	Neuronal progenitors	47
Map-2	Neuronal progenitors	79
O4*	Oligodendrocytes	32
NG2*	Oligodendrocytes	16
TAPA-1*	Astrocytes	58
CD44*	Astrocytes	14

GFP- cells were stained with antibodies to surface antigens (asterisks) and analyzed by flow cytometry, or allowed to adhere to coverslips and then fixed and stained with antibodies specific for intracellular antigens (nestin, GFAP, Map-2). Data represent the percentage of GFPcells that express a given marker. For intracellular antigens, percentages represent averages of four fields.