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Purification of Prominin-1+ Stem Cells from Postnatal Mouse Cerebellum

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TITLE:

Purification of Prominin-1$^+$ Stem Cells from Postnatal Mouse Cerebellum

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KEYWORDS:

cerebellum, prominin-1, postnatal, neural stem cells, interneurons, astrocytes

SUMMARY:

Demonstrated here is an efficient and cost-effective method to purify, culture, and differentiate white matter stem cells from postnatal mouse cerebellum.

ABSTRACT:

Most cerebellar neurons arise from two embryonic stem niches: a rhombic lip niche, which generates all the cerebellar excitatory glutamatergic neurons, and a ventricular zone niche, which generates the inhibitory GABAergic Purkinje cells, which are neurons that constitute the deep cerebellar nuclei and Bergman glia. Recently, a third stem cell niche has been described that arises as a secondary germinal zone from the ventricular zone niche. The cells of this niche are defined by the cell surface marker prominin-1 and are localized to the developing white matter of the postnatal cerebellum. This niche accounts for the late born molecular layer GABAergic interneurons along with postnatally generated cerebellar astrocytes. In addition to their developmental role, this niche is gaining translational importance in regards to its involvement in neurodegeneration and tumorigenesis. The biology of these cells has been difficult to decipher because of a lack of efficient techniques for their purification. Demonstrated here are efficient methods to purify, culture, and differentiate these postnatal cerebellar stem cells.

INTRODUCTION:

The cerebellum has been long recognized as a major neuronal circuit coordinating voluntary movement[1]. It receives input from the wide swathes of the neuroaxis, which includes proprioceptive information from the periphery, fine tune motor output, and coordinate motion. More recently, it has also been implicated in regulating cognition and emotions by potentially using similar information processing networks[2-4].

The adult cerebellum is composed of an outer cerebellar cortex and inner white matter. Interspersed within these structures are deep intracerebellar nuclei. Similar to the rest of the nervous system, the development of the cerebellum is driven by the proliferation of multipotent progenitor cells (stem cells) that migrate and differentiate to yield this well-organized structure. In early development (E10.5–E13.5), a ventricular stem niche around the developing fourth ventricle generates GABAergic neurons (i.e., Purkinje cells, Lugaro cells, Golgi cells) along with Bergmann glia[5-8].

Later in development (postnatal week one), a second stem cell niche in the rhombic lip generates MATH1- and Nestin-expressing progenitors that give rise to excitatory granule neurons[9-12]. Recently a third stem cell niche has been described[13]. These cells express prominin-1 (also known as CD133), a membrane-spanning glycoprotein that defines a subset of stem cells in the intestine and hematopoietic systems[14-16]. In vivo fate mapping shows that these stem cells generate key molecular layer interneurons (i.e., basket cells and stellate cells), along with astrocytes, during the first three postnatal weeks. In the past, it has been difficult to study these cells in vitro because prior methods have required costly and time-consuming techniques (i.e., fluorescence-activated cell sorting [FACS]) that are dependent on prominin-1 staining[12,13,17]. This protocol describes an immunomagnetic-based method for the isolation of these stem cells that can then be readily culture and differentiated.

PROTOCOL:

All animal experiments were performed in compliance with the NIH's Guide for the Care and Use of Laboratory Animals (2011) and were viewed and approved by the Northwestern University IACUC (Protocol IS00011368).

1. Preparation of solutions

1.1. Prepare tissue dissociation solution by adding 100 U/mL papain, 0.2 mg/mL cysteine, and 250 U/mL DNase in 25 mL of sterile phenol red-containing Dulbecco's phosphate-buffered saline (DPBS).

1.2. To prepare DNase solution, 100 mg of the lyophilized powder of DNase I (one bottle) in 50 mL of H_2O. Mix well and filter the stock solution. Prepare 10 mL stock aliquots. Divide one stock tube into 0.5 mL single use aliquots. Store these single use aliquots at -80 °C.

1.3. Prepare magnetic separation reagents by preparing magnetic column buffer X: 0.5% bovine serum albumin (BSA) and 2 mM EDTA solution.

1.4. To prepare neurosphere media, use neurobasal medium containing penicillin/streptomycin with L-glutamine and supplement with 2% B27, 20 ng/mL human recombinant epidermal growth factor (EGF), and 20 ng/mL human recombinant basic fibroblast growth factor (bFGF).

1.5. To prepare differentiation media, use neurobasal medium and supplement with 10 ng/mL differentiated factor platelet-derived growth factor (PDGF-AA) or 10 ng/mL leukemia inhibitory factor (LIF) and 2% B27.

1.6. Use ultra-low attachment 12 well (3.5 cm^2) and 6 well (9.6 cm^2) culture plates.

2. Dissection of cerebellum

2.1. Anesthetize mouse pups (P3-P7) with isoflurane and decapitate using surgical scissors.

2.2. Spray the separated head of the pups with 70% ethanol.

2.3. Transfer each head into an empty sterile 10 cm culture dish. Separate the skin using the microdissection scissors, then remove the skull by running the scissors sagittal along the midline.

2.4. Using #7 forceps, peel off the skull bones starting caudally from the brainstem. Carefully lift the brain using a spatula, keeping the cerebellum intact, and transfer the brain to a fresh sterile 10.0 cm dish containing 15 mL of ice-cold HBSS solution.

2.5. Place the dish containing the brain under a dissection microscope. Using fine #5 forceps, remove the meninges and large blood vessels from the cerebellum and separate the cerebellum from the brainstem using the spatula.

2.6. Transfer the cerebellum into a 15 mL centrifuge tube containing 5.0 mL of ice-cold HBSS solution. Wash the cerebellum by rinsing and decanting 3x with 5.0 mL of HBSS.

NOTE: Each cerebellum should be placed in its own tube for further processing.

3. Cell suspension preparation

3.1. After the last rinse, add 5 mL of papain-based tissue dissociation solution (based on previous work[13,18]) that has been pre-warmed to 37 °C. Incubate the tissue for 15 min at 37 °C in a water bath. Slowly mix the content by inverting the tube up and down 3x–5x every 3 min either using a nutating mixer or by hand.

3.2. Prepare a wide diameter (regular glass pipette) and narrow diameter Pasteur pipette (fire-polished by heating over a Bunsen burner) as described previously[19].

3.3. Wash the tissue 3x with 5 mL of HBSS solution, avoiding loss of the tissue while decanting by hand.

3.4. Remove the last HBSS wash and add 5 mL of DPBS solution containing 250 µL of DNase solution to the tissue. Dissociate the tissue by triturating 10x–15x using the wide diameter Pasteur pipette. Perform this step gently to avoid the formation of bubbles.

3.5. Then incubate the slurry for an additional 10 min at 37 °C in the water bath, mixing by inverting and straightening the tube.

3.6. Use the reduced diameter Pasteur pipette to further triturate the tissue slurry 10x. If large pieces of tissue remain, press the tissue pieces against the bottom of the tube with the tip of the pipette gently and continue pipetting until the cells reach a fine suspension.

3.7. Incubate the tissue at 37 °C for an additional 10 min, repeating the previous mixing steps.

4. Immunolabeling of stem cells

4.1. Place the centrifuge tubes on ice and use ice-cold solutions for the next steps. Strain the dissociated cells through a 40 µm cell strainer into a 50 mL centrifuge tube. Top the filter with 10.0 mL of HBSS solution to ensure that cells pass through the mesh in this additional solution.

4.2. Transfer the filtered cells into a fresh 15 mL centrifuge tube and centrifuge the cell suspension at 300 *x g* for 10 min at 4 °C. Aspirate and discard the supernatant completely and carefully using a vacuum aspirator.

4.3. Resuspend the pellet in 160 µL of magnetic column buffer. To obtain the single-cell suspension before magnetic labeling, pass cells through a 30 µm nylon mesh to remove cell clumps, which can otherwise clog the column.

4.4. Add 40 µL of anti-prominin-1 microbeads to each 15 mL tube, mix, and incubate in a refrigerator for 15 min in order for the antibody to bind to prominin-1-expressing cells.

4.5. Wash the cells by adding 1.0–2.0 mL of column buffer X and centrifuge at 300 *x g* for 10 min. Aspirate the supernatant completely.

4.6. Resuspend the pellet in 1.0 mL of column in buffer X.

NOTE: If small clumps are seen after the resuspension of pellet with 1.0 mL of column buffer X, it should be removed carefully using a tip of the Pasteur pipette; otherwise, these clumps can block the magnetic column during cell sorting.

5. Magnetic column preparation, cell sorting, and plating

NOTE: The magnetic separation from different genotype conditions (disease vs. control) must be performed at same time, since any delay may affect the neurosphere morphology.

5.1. Prepare the magnetic columns by placing them on the magnetic stand exposed to the magnetic field. Rinse the column once with 500 µL of buffer X by applying buffer that drips into a centrifuge tube to be discarded.

NOTE: Prepare fresh magnetic column buffer X for each experiment; if not, then stem cell yield will be low.

5.2. Apply the labeled cell suspension onto the column. Collect the flowthrough containing unlabeled cells that mainly consist of cerebellar neuronal/glial mixed cells into fresh 15 mL tubes (**Figure 1A**).

5.3. Wash the column 3x with 500 µL of buffer X (each wash takes around 2–4 min).

NOTE: Cerebellar neuronal/glial mixed culture enriched with cerebellar granular neurons can serve as a useful byproduct of this purification step.

5.4. Remove the column from the magnetic field and place in a 1.5 mL tube. Add 1.0 mL of culture medium (neurosphere medium) to the column and push the plunger into the column to flush out the cells tagged with prominin-1 beads into a fresh 1.5 mL falcon tube.

5.5. To enhance the purity of prominin-1 labeled cells, pass the eluted cells over a second column following steps 5.1–5.4.

5.6. Count the cells with a hemocytometer. The typical yield is 10^7 cells per cerebellum. Plate the cells onto ultra-low attachment plates (6 or 12 well, based on the density required for downstream experiments).

6. Passaging of neurospheres and differentiation

6.1. Plate the prominin-1-labeled stem cells on ultra-low attachment 12 well plates in neurosphere medium (5,000 cells/well).

6.2. After 7–10 days, the cells divide to yield ball shaped floating neurospheres (primary neurospheres).

NOTE: In this experiment, secondary neurospheres that expand in numbers are generated for further use. Primary neurosphere populations are not used for these experiments, since they may contain contaminating cells that do not have stem cell properties and can clump together with neurospheres.

6.3. For passaging, transfer the primary neurospheres along with the culture media using 1.0 mL pipette tips to a 15 mL sterile centrifuge tube. Pellet the neurospheres by centrifugation at 300 *x g* for 5 min and discard the supernatant.

6.4. Resuspend the pellet in 5 mL of tissue dissociation media that contains papain or 0.05% trypsin solution. Incubate at 37 °C for 10 min.

6.5. Centrifuge the cell suspension at 300 *x g* for 5 min. Resuspend the cells in 5 mL of neurosphere medium and dissociate the cells mechanically using plastic Pasteur pipette by pipetting up and down 10x, slowly.

6.6. Plate the cells (again) in neurosphere media as described earlier. After 7–10 days in culture, the plate should be enriched with secondary neurospheres.

NOTE: These cultures can be passaged up to 8x with good efficiency, after which neurosphere size tends to be smaller and suggestive of a decrease in proliferation.

6.7. Count the cells on the hemocytometer and plate the optimal number for future experiments on poly-D-lysine coated plates (6 or 12 wells).

6.8. To differentiate the stem cells, collect neurospheres from the second to eighth passage by centrifugation as described earlier, except add differentiation medium to the pellet.

NOTE: Upon 7 days in vitro, the stem cells differentiate into neurons, astrocytes, and oligodendrocytes, as demonstrated by staining with the neuronal marker β-III tubulin, astrocytic marker GFAP, and oligodendrocyte maker O4.

REPRESENTATIVE RESULTS:

Prominin-1-positive postnatal cerebellar stem cells formed neurospheres in neurosphere medium rich in growth factors (EGF and bFGF). These neurospheres were positive for prominin-1-staining, the marker used for isolation, and also as a stain for other stem cell markers such as Nestin and GFAP[13] (**Figure 1**). The stem cell marker expression was maintained throughout culture and for up to at least eight passages[20]. Upon withdrawal of growth factors and in the presence of LIF and PDGF-AA (which are factors that support neuronal and glial differentiation[21,22]), the neurospheres differentiated into neuronal and glial lineages (**Figure 2**).

FIGURE LEGENDS:

Figure 1: Isolation of prominin-1 stem cells from postnatal cerebellum. (**A**) Cerebellar stem cells were isolated using immunomagnetic prominin-1 beads. Top panel: Purified stem cells (column-bound) formed neurospheres with extensive proliferation and self-renewal properties. The cells unbound to the column (flowthrough) were unable to form neurospheres; instead, they became cerebellar neuronal/glial mixed cells (β-III tubulin/GFAP). Bottom panel: the neurospheres formed from prominin-1^{+} cells expressing stem cell-specific markers: Nestin, prominin-1, and GFAP. (**B**) Cells in the flowthrough stained negative for stem cell markers prominin-1 and nestin and positive for neuronal marker β-III tubulin.

Figure 2: Differentiation of prominin-1 positive neurospheres. In the presence of differentiation factors (PDGF-AA or LIF), prominin-1-positive neurospheres differentiated into neurons (β-III tubulin), astrocytes (GFAP), and oligodendrocytes (O4).

DISCUSSION:

Prominin-1-expressing cerebellar stem cells reside in the prospective white matter during the first 3 weeks of postnatal life. Their proliferation is tightly controlled by the sonic hedgehog pathway supported by Purkinje cells[17]. These stem cells/progenitors contribute to later-born GABAergic interneurons called basket cells and stellate cells. These interneurons reside in the molecular layer, where they synapse onto Purkinje cells and sculpt PC topography and function via GABAergic inhibition[13,17,23]. Besides forming interneurons, this stem cell population also generates all postnatally derived cerebellar astrocytes[17,24].

This protocol describes an easy and cost-effective method to purify prominin-1/CD133 stem cells from the postnatal mouse cerebellum. Stem cells must be cultured in ultra-low attachment plates. Culturing these stem cells in normal plates may cause the neurospheres to attach the surface and lead to low stem cell proliferation and differentiation. Here, the yield of 200–300 neurospheres per 5,000 cells corresponds to a stem cell yield of around 1 x 10^7 cells from a single cerebellum. This is comparable to what has been described for an FACS-based strategy that is 10x more expensive. Moreover, FACS equipment requires an expensive set-up and highly trained personnel and is not readily available.

These stem cells are also gaining increasing translational significance in research on cancer as well as neurodevelopmental and neurodegenerative disorders[25-27]. Uncontrolled proliferation of these stem cells in early life leads to medulloblastoma[28], while research from our own lab suggests that their abnormal proliferation and differentiation can contribute to later cerebellar degeneration in the genetic disease spinocerebellar ataxia type 1[20]. These new protocols will be valuable for studying these cells and provide novel insight into their roles in health and disease. These methods may also lead to advances in regenerative therapies after stroke or trauma and other insults to the brain that warrant neuroregeneration. It is conceivable that these techniques can be generalized to extract prominin-1-expressing stem cells from other tissues, such as intestine and bone marrow, where they are also expressed[15,29].

ACKNOWLEDGEMENTS:

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DISCLOSURES:

No conflicts of interest are declared.

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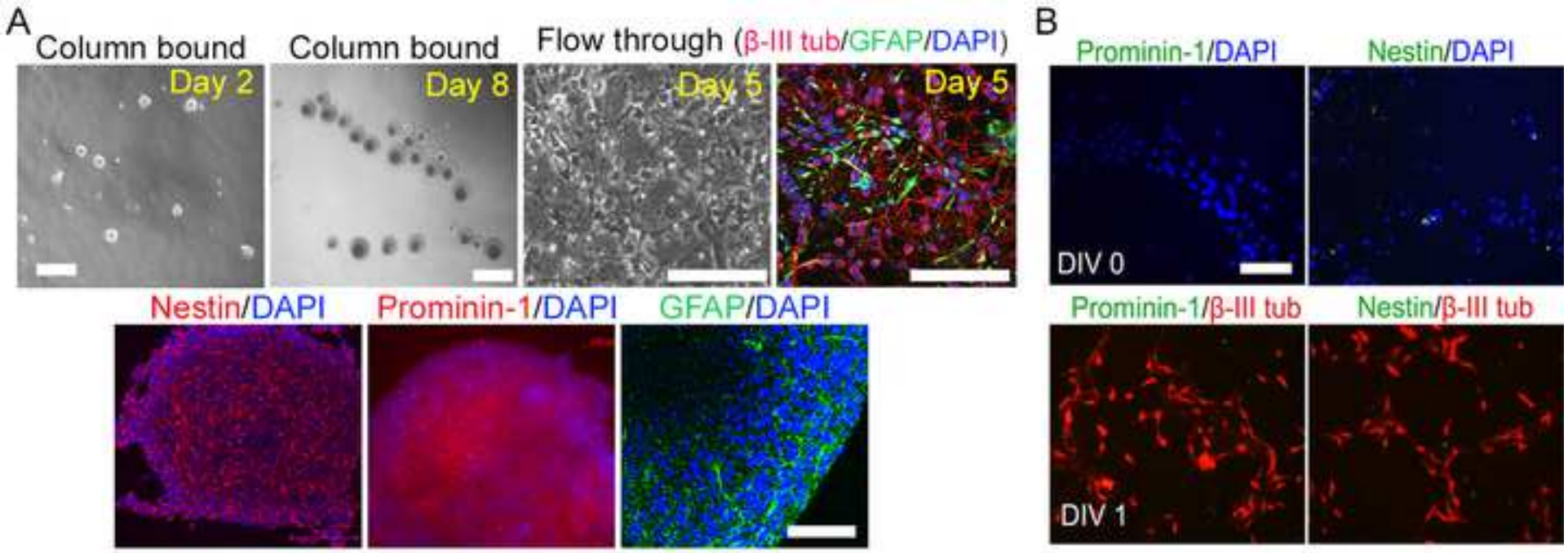

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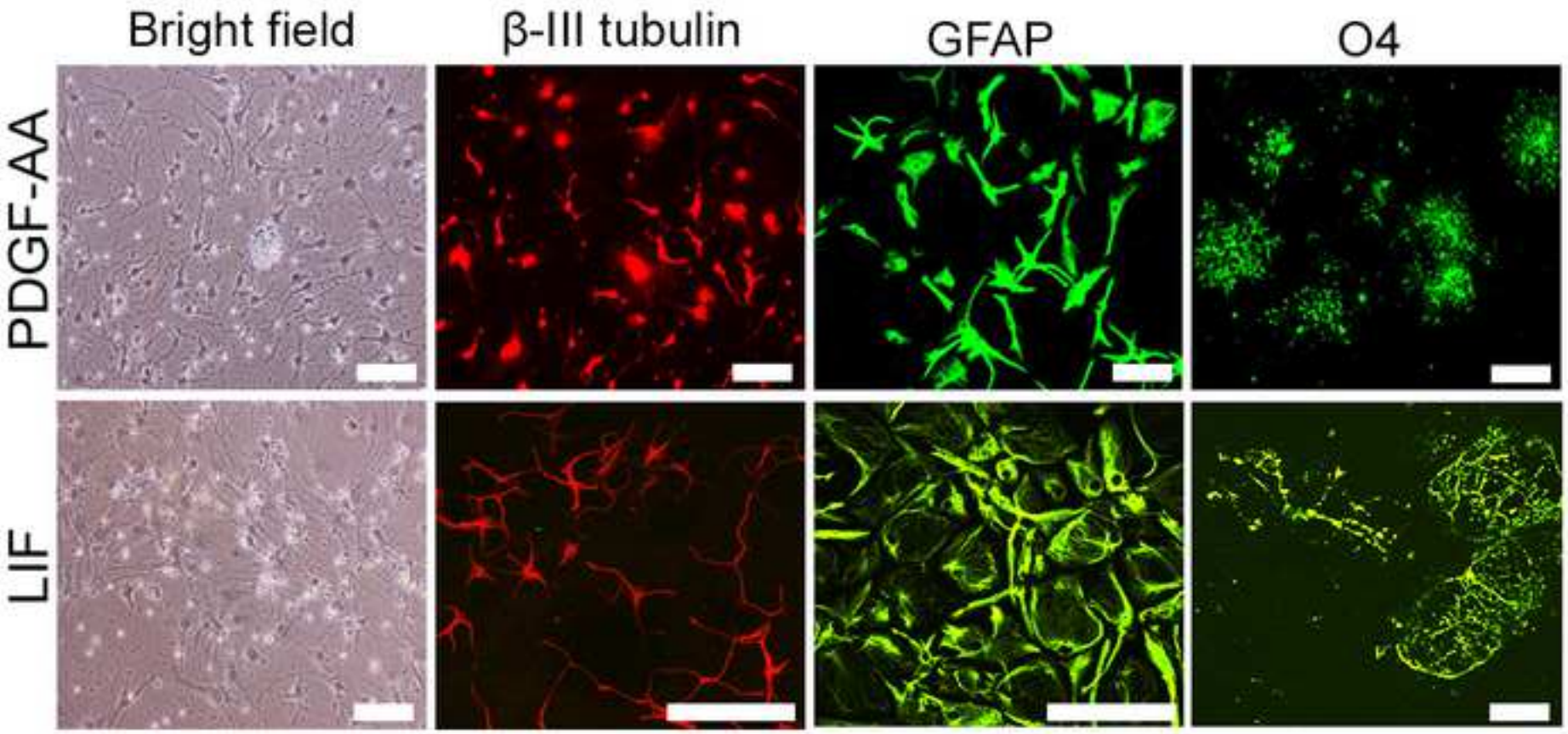

Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
0.05%Trypsin	Thermo Fisher Sc	25300054	0.05%
2% B27	Gibco; Thermo Fish	17504001	
2mM EDTA solution	Corning	46-034-CI	
Anti- Prominin-1 microbeads	Miltenyi Biote	130-092-333	
bovine serum albumin	Sigma	A9418	
Column MultiStand	Miltenyi Biotec	130-042-303	
culture plates ultra - low attachm	Corning	3473	
cysteine	Sigma	C7880	
DNase	Sigma	D4513-1VL	250 U/ml
Dulbecco's Phosphate Buffer Saline	Thermo Fisher Scier	14040141	
Hank's balanced salt solution-HI	Gibco	14025-092	
Human recombinant Basic Fibro	Promega	G507A	20 ng/ml
Human recombinant Epidermal (	Promega	G502A	20 ng/ml
Leukemia Inhibitory Factor	Sigma	L5158	
l-glutamine	Gibco	25030081	
Microscopy	Lieca TCS SP5 confocal microscopes		
MiniMACS separator	Miltenyi Biotec	130-042-102	
mouse anti-Prominin-1	Affymetrix eBiosci	14-1331	1 in 100
Nestin	Abcam	ab27952	1 in 200
Neurobasal medium	Thermo Fisher	25030081	
O4	Millopore	MAB345	
Papain	Worthington	LS003126	(100 U/ml)
Platelet- Derived Growth Factor	Sigma	H8291	10 ng/ml
Poly-D-Lysine	Sigma	P6407	
rabbit anti-tubulin, β-III	Sigma	T2200	1 in 500
Rabit anti-GFAP	Dako	Z0334	1 in 500
Separation columns-MS column	Miltenyi Biotec	130-042-201	
Sterile cell strainer	Fisher Scientific	22363547	40um

Rebuttal Letter
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Dear Dr. Bajaj,

We are pleased to submit our revised manuscript "**Purification of Prominin-1$^+$ stem cells from mouse postnatal cerebellum**" (Manuscript number: JoVE60554).

We appreciate the comments of the reviewers, who have found our results novel and of interest. We have performed additional experiments and rewritten the manuscript to accommodate the referees' concerns. We respond to each point below with each referee's comments quoted in *italics* for ease of reference. We have also marked all the changes in the manuscript with yellow highlights making them easier to spot.

Reviewer #1:

We are pleased that reviewer #1 "found our manuscript to be "carefully executed and clearly presented" with no major concerns. The reviewer raised a few points that we address below:

1. *Authors need to provide information on the manufacturer and catalog numbers for the reagents: 1) Cysteine (0.2mg/ml); 2) DPBS (Dulbecco's Phosphate Buffer Saline; 3) Magnetic column buffer* X.

   We have provided this information in table of materials (see Line: 78, Line: 79 and Line 83-84)

Reviewer #2:

This reviewer agrees that "this protocol would potentially be of use to researchers interested in neuronal development and the development of hindbrain tumors such as medulloblastoma". The reviewer has a few comments that are addressed below:

1. The first are several related comments.

More prominin-1 staining of cells isolated via this protocol (vs those that are present in the flow through) needs to be included. If we do not see an absence of prominin-1 in the flow through cells, how does one know that the purification is efficiently selecting all positive cells? Typically, validation of such strategies would include staining for multiple markers on both the selected and non-selected populations by analytical flow cytometry or high throughput immunostaining, to confirm purity of the resulting desired population and selectivity for all possible positive cells.

   As suggested, we have performed immunostaining for Prominin-1 and Nestin on the cells that have not bound the magnetic column (the flow through). In a revised **Figure 1B**, we have confirmed that there are indeed no stem cells defined by these markers in the flow through. This is a substantive improvement to the manuscript and thank the reviewer for this suggestion.

Furthermore, is Prominin-1 expression maintained during culture?

Yes, Prominin-1 expression is indeed maintained during culture and up to at least eight passages (this has been added to Line: 203-204) with a reference to our previous publication where this finding was first described (J Clin Invest. 2018 Jun 1;128(6):2252-2265)

Are these cells able to become cerebellar cells after differentiation? The current markers shown do not distinguish cerebellar cells specifically; only glial / neuronal identity.

The goal of this study was not to differentiate these precursors into regional specific neuronal and glial subtypes; rather our goal was to demonstrate that these prominin-1 positive cells have indeed stem cell properties in vitro. Differentiation of these stem cells into cerebellar GABAergic interneuron and glial cells has been shown by others in vivo (by fate mapping or stem cell transplantation); (Nat Neurosci. 2005 Jun;8(6):723-9; Nat Neurosci. 2013 Dec;16(12):1737-44)) (Line: 64-66).

2. More broadly, the authors assert multiple times that this protocol is more cost effective and less time consuming than FACS, but provide no calculations or numbers to back this up. Given that this approach requires consumable kits from the manufacturer, it's not immediately apparent to this reviewer that it would be less expensive or faster than FACS executed in a lab with low-cost flow cytometer access.

We have now addressed these concerns. In Line 211-212, we mention that our protocol generates approximately 10^7 cells per cerebellum, typically yield 250-300 neurospheres per 5000 sorted cells. This is comparable to FACS based strategies (J Clin Invest. 2018 Jun 1;128(6):2252-2265; Nat Neurosci. 2005 Jun;8(6):723-9). This is a significant appeal given that FACS sorting is approximately 10 times more expensive costing an average of 150$ - 200$ per run compared to 15$ per run on a magnetic column ($300 for 25 magnetic columns). Moreover, the FACS equipment is expensive, requires trained personnel, and is not readily available (described in Lines: 214- 217).

3. As described, this is essentially an implementation of a commercial kit in tissue from a brain subregion where it has not been used previously. If the protocol is not systematically compared to prior approaches, or to the use of this kit in other regions where it has been published (e.g. postnatal stem cell niches of the forebrain, or prenatal stem cells), it's not clear that this is a resource that will be broadly useful to the field.

The value of our protocol lies precisely in our ability to exclusively purify the cerebellar stem cell niche away from other progenitor populations in the nervous system. Thus, it is not meant to be generalized to other CNS stem cell populations. It is however possible that our techniques could be generalized to extract prominin-1 expressing stem cells from other tissues (for instance, the intestine, etc) (stated in line: 224-226).

Specific items:

1. Line 14 - rather than "neuronal" stem cells it would be more appropriate to say "neural" stem cells - progeny are not exclusively neurons

We have made this change (Line: 14).

2. Line 68 - Papain is used for tissue dissociation. Have the authors tested to confirm that this does not cleave any of the surface epitopes they are using for prospective isolation? Has papain been compared versus other enzymes?

We have benefited from prior work (Nat Neurosci. 2005 Jun;8(6):723-9; Nat Neurosci. 2013 Dec;16(12):1737-44), where we already know that papain is in fact the best enzyme to preserve prominin-1 surface expression (trypsin on the other hand cleaves cell surface antigens) (Stem Cells. 2007 Jun;25(6):1560-70; Nat Neurosci. 2005 Jun;8(6):723-9). We have mentioned this in line: 114-115.

3. Line 67 - 0.05% trypsin is listed as a possible method to dissociate neurospheres derived from sorted cells, as is the papain-based dissociation solution. Can the authors comment on whether one of these is preferable, and why they are used versus Accutase, which is often used in neurospheres cultured from other brain regions?

Both trypsin and papain work equally well and that is why we mentioned both (Line: 181-182). We did not test accutase.

4. Line 101 - "peel off" not "peel of"

We have made this change (Line: 101).

5. Line 116 - Is the incubation at 37 degrees in an incubator? A water bath? Can this be done on a nutating mixer rather than inverting by hand?

Corrected to “37 degrees in a water bath”. (line: 116)
Yes, it can be done either on nutating mixer or by hand (see revised line 117).

6. Line 118 - Please give more information on what constitutes "wide" and "narrow" diameter Pasteur pipettes - diameters in microns? Are these beveled or fire polished? These pipettes were also not listed in the Materials section.

For trituration for a “wide” pipette orifice, we used regular Pasteur pipette. To “narrow” the orifice diameter, we polished the glass over a Bunsen burner flame (fire polished). We have added this information to the manuscript (Line: 118-119). This is an established

procedure for generating narrow bore tips and the diameter is not typically measured (Nat Protoc. 2012 Sep;7(9):1741-54.).

7. Line 120 - says to add DNase to the sample. Is this in addition to the DNase that is already in the dissociation solution?

Yes, DNAse is added at this step-in addition to the DNAse added in the dissociation step.

8. Line 134 - What is the rationale for using "ice-cold" solutions versus 37 degrees Celsius? Was this tested?

Miltenyi Biotec—the company that produces the Prominin-1 magnetic beads (#130-092-333) recommends that the temperature of the solutions be ice-cold. We therefore used their instructions (we did not test binding at 37 degrees).

9. Line 156 - What is the expected fraction of cells in the sample that will flow through vs. bind?

We did not specifically test for this. But a previous publication (Nat Neurosci. 2005 Jun;8(6):723-9) reported 0.1- 0.3% of cells extracted from the cerebellum will be Prominin-1 positive.

10. Line 157 - How long does each wash take?

It takes around 2-4 min for each wash (Mentioned in Line: 158)

11. 3. In lines 165-167, the authors seem to indicate that they plate and grow the isolated cells along with the beads used to isolate them, does this impact how the cells grow?

We did not test whether the beads impact cell growth. However, since cells are typically grown even in the presence of beads we did not try to isolate them away.

12. Line 166 - Open parentheses.

Closed the parenthesis (Line: 168).

13. Line 177 - Demonstrating self-renewal capacity is an assertion here; either explain how this is quantitatively assessed or remove.

We have removed this assertion (Line:177).

14. Line 181 - See comments above (item 3) regarding dissociation questions.

Addressed above

15. Line 215 - "proliferation" typo

Changed (Line: 220).

16. Line 253 - "From" not "form"

Changed (Line: 264)

17. Line 266 and Figure 2 generally - What is the duration of exposure to PDGF-AA or LIF? References for why these factors are used?

We differentiate the neurospheres in presence of either PDGF-AA or LIF for 7 days as has been described before (Nat Neurosci. 2005 Jun;8(6):723-9; Nat Neurosci. 2013 Dec;16(12):194-195) (Line: 207-208).

Also, please confirm that the "B27" used here in the media is the same as the "B2" mentioned in the procedures section of the authors' preceding JCI paper using this approach.

Yes, Both B27 or B2 are the same. This was an error on our part.

Reviewer #3:

We are pleased that reviewer 3 finds that "The article is well presented, with appropriate details, and should be useful to many. Also the basic tips should be applicable to other protocols".

The reviewer only has a few minor comments that we address below:

1) *Could it be possible to write a troubleshooting section and to identify crucial steps? For example, is mycoplasma contamination a common problem and can it be easily monitored or prevented?*

We have added a troubleshooting section – Line: 228-239

2) Is it possible to freeze and store the cells at some point?

In general, stem cells are typically not passed through cycles of freeze-thawing. Therefore, we avoided storing cells.

Minor Concerns:
- Abstract typo: "marker" not "maker"

Changed (Line: 23)

- Line 80: it might be better to give the diameters of the wells, if some want to use different plates.

Added the diameters of the plate wells (Line: 88)

- Line 100:" lambda to bregma" is an expression which will be cryptic to many readers. This would require either a picture, a reference or an explanation.

We have removed the expression “lambda to bregma” from the sentence (Line 101); we instead now write that one should “remove the skull by running the scissors sagitally along the midline”.

- Line 116 and later. "°C" is the usual abbreviation for Celsius degrees.

We have made this change throughout the manuscript

- Line 158: the flowthrough fraction probably contains an heterogenous cell population, not only granule cells. Do the authors know whether it is better than a whole cerebellum suspension to prepare a primary culture of granule cells?

We agree that it was a mistake to mention only granule neurons. The flow through contains a mixed culture, mostly enriched with granular neurons and glial population. We have corrected the text accordingly (Line 159-160).

- Line 183: is it required/useful to monitor cell dissociation under the microscope?

It is not required to monitor the cell dissociation under a microscope since even a few residual clumps of cells do not interfere with the overall protocol.

- Line 185: one can conveniently buy disposable plastic transfer pipettes. Do the authors know whether these can replace the modified glass pipettes?

Yes, for this step we can also use plastic transfer pipettes. This has been added Line: 184

- Line 192: does it make a visible difference to use different passages?

No, we don’t see any visible difference with passage number.

Reviewer #4:

We are pleased that this reviewer finds that “The work is very clearly written and concise. The introduction very clearly explains why PROM1+ cells are of interest, and provides a good

rationale for using this method to purify them. The methods are clearly described. The figures are excellent and helpful. I strongly feel that the work should be published".

The reviewer has only one concern addressed below:

1. I note that there is no step to inactivate the papain after dissociation, or even to spin down to remove the papain by dilution. Many protocols that use papain to dissociate brain cells call for inactivation of papain with ovomucoid inhibitor, serum, or dilution. Is this an omission, or is no inactivation removal of papain needed? With this question addressed, the work is ready for publication and will be of interest to the readers of JOVE.

> Thank you very much for recognizing this oversight. We have now mentioned the washing step where we describe that the tissue is washed three times with 5ml of HBSS solution. Remove last HBSS wash, and add 5ml of DPBS solution containing 250µl of DNase to the tissue (Line: 120-1123).

Minor Concerns:
on line 91, O4 antibody is missing a source and catalog #

> Thanks for correction. Added in table of materials.

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Author(s): CHANDRAKANTH REDDY EDAMAKANTI & PUNEET OPAL

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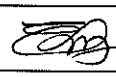
Signature:	
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