HHS Public Access

Author manuscript

J Vis Exp. Author manuscript; available in PMC 2023 September 16.

Modeling Human Cerebellar Development *In Vitro* in 2D Structure

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Abstract

The precise and timely development of the cerebellum is crucial not only for accurate motor coordination and balance but also for cognition. In addition, disruption in cerebellar development has been implicated in many neurodevelopmental disorders, including autism, attention deficit-hyperactivity disorder (ADHD), and schizophrenia. Investigations of cerebellar development in humans have previously only been possible through post-mortem studies or neuroimaging, yet these methods are not sufficient for understanding the molecular and cellular changes occurring *in vivo* during early development, which is when many neurodevelopmental disorders originate. The emergence of techniques to generate human-induced pluripotent stem cells (iPSCs) from somatic cells and the ability to further re-differentiate iPSCs into neurons have paved the way for *in vitro* modeling of early brain development. The present study provides simplified steps toward generating cerebellar cells for applications that require a 2-dimensional (2D) monolayer structure. Cerebellar cells representing early developmental stages are derived from human iPSCs *via* the following steps: first, embryoid bodies are made in 3-dimensional (3D) culture, then they are treated with FGF2 and insulin to promote cerebellar fate specification, and finally, they are terminally differentiated as a monolayer on poly-l-ornithine (PLO)/laminin-coated substrates. At 35 days of differentiation, iPSC-derived cerebellar cell cultures express cerebellar markers including ATOH1, PTF1α, PAX6, and KIRREL2, suggesting that this protocol generates glutamatergic and GABAergic cerebellar neuronal precursors, as well as Purkinje cell progenitors. Moreover, the differentiated cells show distinct neuronal morphology and are positive for immunofluorescence markers of neuronal identity such as TUBB3. These cells express axonal guidance molecules, including semaphorin-4C, plexin-B2, and neuropilin-1, and could serve as a model for investigating the molecular mechanisms of neurite outgrowth and synaptic connectivity.

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A complete version of this article that includes the video component is available at http://dx.doi.org/10.3791/64462.

Disclosures

The authors have no conflicts of interest to declare.

This method generates human cerebellar neurons useful for downstream applications, including gene expression, physiological, and morphological studies requiring 2D monolayer formats.

Introduction

Understanding human cerebellar development and the critical time windows of this process is important not only for decoding the possible causes of neurodevelopmental disorders but also for identifying new targets for therapeutic intervention. Modeling human cerebellar development *in vitro* has been challenging, yet over time, many protocols differentiating human embryonic stem cells (hESCs) or iPSCs with cerebellar lineage fates have emerged[1, 2, 3, 4, 5, 6, 7, 8]. Furthermore, it is important to develop protocols that generate reproducible results, are relatively simple (to reduce error), and are not heavy on monetary costs.

The first protocols for cerebellar differentiation were generated from 2D cultures from plated embryoid bodies (EBs), inducing cerebellar fate with various growth factors similar to *in vivo* development, including WNT, BMPs, and FGFs[1, 9]. More recent published protocols induced differentiation primarily in 3D organoid culture with FGF2 and insulin, followed by FGF19 and SDF1 for rhombic liplike structures[3, 4], or used a combination of FGF2, FGF4, and FGF8[5]. Both cerebellar organoid induction methods resulted in similar 3D cerebellar organoids as both protocols reported similar cerebellar marker expression at identical time points. Holmes and Heine extended their 3D protocol[5] to show that 2D cerebellar cells can be generated from hESCs and iPSCs, which start as 3D aggregates. In addition, Silva et al.[7] demonstrated that cells representing mature cerebellar neurons in 2D can be generated with a similar approach to Holmes and Heine, using a different time point for switching from 3D to 2D and extending the time of growth and maturation.

The current protocol induces cerebellar fate in feeder-free iPSCs by generating free-floating embryoid bodies (EBs) using insulin and FGF2 and then plating the EBs on PLO/laminin-coated dishes on day 14 for 2D growth and differentiation. By day 35, cells with cerebellar identity are obtained. The ability to recapitulate the early stages of cerebellar development, especially in a 2D environment, allows researchers to answer specific questions requiring experiments with a monolayer structure. This protocol is also amenable to further modifications such as micropatterned surfaces, axonal outgrowth assays, and cell sorting to enrich the desired cell populations.

Protocol

The human subjects research was approved under the University of Iowa Institutional Review Board approval number 201805995 and the University of Iowa Human Pluripotent Stem Cell Committee approval number 2017–02. Skin biopsies were obtained from the subjects after obtaining written informed consent. The fibroblasts were cultured in DMEM with 15% fetal bovine serum (FBS) and 1% MEM-non-essential amino acids solution at 37 °C and 5% CO_2. Fibroblasts were reprogrammed using an episomal reprogramming kit following the manufacturer's protocol (see Table of Materials) using a nucleofector for electroporation. All procedures were performed in a Class II Type A2 biological safety

cabinet (“hood” for short). All cell culture media were antibiotic-free; therefore, every component that entered the hood was cleaned with 70% ethanol. All cell culture media and components were sterile filtered or opened in the hood to maintain their sterility.

1. Experimental preparation

1. Prepare basement membrane matrix (BMM)-coated plates.

   NOTE: BMM solidifies more quickly at warmer temperatures. Plates must be prepared rapidly and immediately placed at 4 °C for storage.

   1. Thaw the BMM (see Table of Materials) on ice, at 4 °C for at least 2 h, or overnight.
   2. Mix DMEM/F12 and BMM to a final concentration of 80 μg/mL. Distribute the BMM solution in tissue culture dishes (1 mL for 35 mm and 2 mL for 60 mm dishes) and incubate at 37 °C for at least 1 h or overnight before plating the cells.

      NOTE: The unused dishes can be stored at 4 °C for 2 weeks.

2. Prepare poly-L-ornithine/laminin (PLO/laminin)-coated plates.

   1. Prepare 20 μg/mL PLO (see Table of Materials) in sterile DPBS+/+ and add 1 mL to each well of a 6-well plate. Incubate overnight at 37 °C in the incubator.
   2. The following day, aspirate the PLO with a vacuum aspirator and wash two times with DPBS+/+. Air dry in the hood.

      NOTE: Air-dried plates can be stored at 4 °C for up to 2 weeks, wrapped in aluminum foil, for future use.

   3. Prepare 10 μg/mL laminin (see Table of Materials) in DPBS+/+ and add 1 mL to each well of a 6-well plate. Incubate at least for 3 h or overnight at 37 °C in the incubator.
   4. Aspirate the laminin with a vacuum aspirator and add either 1 mL of medium or sterile DPBS+/+.

      NOTE: The laminin coating must not dry out. PBS or an appropriate culture medium should be added immediately to prevent this. Coated plates can be stored at 4 °C for up to 2 weeks.

3. Prepare PSC passaging solution.

   NOTE: An osmometer (see Table of Materials) is required to make PSC passaging solution[10].

   1. Dissolve 11.49 g of potassium chloride (KCl) and 0.147 g of sodium citrate dihydrate (HOC(COONa) ($CH_2COONa)_2$*$2H_2O$) in 400 mL of sterile cell culture grade water (see Table of Materials).

2. Measure the volume of the solution and record it as the initial volume (Vi).
3. Measure the osmolarity and adjust it to 570 mOsm by adding sterile cell culture grade water using the formula Vi × Oi = Vf × 570 mOsm.
4. Filter-sterilize the solution with a 0.20 µm filter and make 10 mL aliquots. Store the aliquots at room temperature (RT) for up to 6 months.

4. Prepare pluripotent stem cell (PSC) medium.

   NOTE: iPSCs are maintained in a medium containing heat-stable FGF2 (see Table of Materials), providing a weekend-free feeding schedule. Other commercially available PSC media have not been tried for this protocol.

   1. Thaw the PSC medium supplement overnight at 4 °C.
   2. Add 10 mL of PSC medium supplement to 500 mL of basal PSC medium. Make 25 mL aliquots and store at −20 °C.

      NOTE: Frozen aliquots can be stored at −20 °C for 6 months. Thawed aliquots must be stored at 4 °C and used within 2 weeks. Cells can be frozen for long-term storage in a PSC medium with 10% (v/v) dimethyl sulfoxide (DMSO).

5. Prepare PSC thawing medium.

   1. Supplement PSC medium with 50 nM chroman, 1.5 µM emricasan, 1x polyamine supplement, and 0.7 µM trans-ISRIB (CEPT cocktail[11]) (see Table of Materials).
   2. Filter-sterilize with a 0.20 µm filter and store at 4 °C for up to 4 weeks.

6. Prepare pulled glass pipettes.

   1. Pull 22.9 cm (9 in) glass Pasteur pipettes into two pieces above a Bunsen burner, ~2 cm below the neck, creating two pipettes, with the thinner side being ~4 cm shorter than the other.
   2. With the help of the flame, bend the tips of the pulled side to create a smooth “r”.
   3. Place the pulled pipettes in autoclave sleeves and autoclave for sterilization.

7. Prepare cerebellar differentiation medium (CDM).

   1. Mix IMDM and Ham’s F12 nutrient mix in a 1:1 ratio. Supplement the mix with 1x L-alanine-L-glutamine supplement, 1% (v/v) chemically defined lipid concentrate, 0.45 mM 1-thio-glycerol, 15 µL/mL apo-transferrin, 5 mg/mL bovine serum albumin (BSA), and 7 µg/mL insulin (see Table of Materials).

2. Filter-sterilize with a 0.20 μm filter, store at 4 °C, and use within 1 month.

8. Prepare cerebellar maturation medium (CMM).

1. Supplement neurobasal medium with 1x L-alanine-L-glutamine supplement and 1x N-2 supplement (see Table of Materials).

2. Filter-sterilize with a 0.20 μm filter, store at 4 °C, and use within 2 weeks.

2. Feeder-free iPSC culture

1. Thaw the cells following the steps below.

1. Place a BMM plate (step 1.1) into the 37 °C incubator to gel for at least 1 h or overnight before thawing the cells.

2. Pre-warm 10 mL of PSC thawing medium (step 1.5) at 37 °C.

3. Transfer the cryovial with cells from liquid nitrogen to a water bath at 37 °C.

NOTE: To avoid generating a source of contamination in the cell culture space, using a standard water bath is not recommended. Instead, fresh water can be heated in a small beaker to 37 °C to generate a one-time-use water bath.

4. When there is a small piece of ice left in the tube, remove it from the water bath, dry the tube, and spray with 70% ethanol. Transfer the tube to the hood and use a 2 mL or 5 mL serological pipette (see Table of Materials) to gently transfer the cells to a 15 mL conical tube.

5. Add 8 mL of PSC thawing medium to the cells dropwise while swirling the conical tube. Centrifuge at 200 × *g* for 5 min at RT.

6. Carefully aspirate the supernatant with a vacuum aspirator without disturbing the cell pellet. Resuspend the cells in 2 mL of PSC thawing medium.

7. Aspirate the BMM from the plate and add the resuspended cells dropwise all over the plate for even distribution.

8. Place the plate in the incubator at 37 °C, 5% CO_2. Refresh the medium the next day with PSC medium. After that, change the medium daily.

2. Maintain the cells following the steps below.

1. Pre-warm the necessary volume of PSC medium at 37 °C (e.g., 1 mL of medium per 35 mm plate).

2. Investigate the plates for differentiated cells or colonies and remove the differentiated cells with a pulled glass pipette (step 1.6).

NOTE: iPSC colonies have smooth edges with morphologically identical cells.

3. Change the spent medium daily and passage every 3–4 days or when 70% confluency is reached.

3. Passage the cells.

1. Place the necessary amount of BMM plates into the incubator for at least 1 h or overnight before passaging. Pre-warm the necessary amount of PSC medium (step 1.3) at 37 °C.
2. Using a pulled glass pipette, clean off any differentiated cells or colonies.
3. Aspirate the spent medium with a vacuum aspirator and add PSC dissociation medium, 1 mL per 35 mm dish. Incubate at 37 °C for 1–3 min.

   NOTE: If colonies are lifting off in the PSC passaging solution before adding PSC medium, the incubation time can be reduced.
4. Aspirate the PSC passaging solution and add pre-warmed PSC medium.
5. Using a sterile 200 µL pipette tip, scratch lines parallel to each other in one direction, turn the plate 90°, and make a second set of scratch lines perpendicular to the previous ones (making a cross-hatched pattern across the plate).
6. Aspirate the BMM solution from the set plates. Collect the colonies using a serological pipette and distribute them to new BMM plates.
7. Incubate at 37 °C, 5% CO_2. Change the medium daily.

4. Freeze the cells.

1. Prepare cryovials by labeling the content and sterilize under ultraviolet (UV) light in the hood (see Table of Materials) for 30 min.
2. Prepare PSC freezing medium by supplementing PSC medium with 10% (v/v) DMSO. Follow steps 2.3.2–2.3.5.
3. Transfer the cells to a 15 mL conical tube with a 5 mL serological pipette and centrifuge at 200 × *g* for 5 min at RT.
4. Carefully aspirate the medium and resuspend the cell pellet in PSC freezing medium.
5. Distribute into cryovials. Place the vials into a freezing container and place the container in a −80 °C freezer.
6. The following day, transfer the cryovials to liquid nitrogen for long-term storage.

3. Cerebellar differentiation

NOTE: Before starting the differentiation, iPSCs are passaged to six 35 mm dishes and are ready for the differentiation when they are at 70% confluency. Each 35 mm plate will be transferred to one well of the 6-well plate.

1. On day 0, lift the healthy iPSC colonies for EB formation.
    1. Add 1 mL of CDM (step 1.7) supplemented with 10 μM Y-27632 and 10 μM SB431542 (see Table of Materials) to each well of a 6-well ultra-low attachment (ULA) plate and place in the incubator until the lifted colonies are ready to be added to the wells.
    2. Clean the differentiated cells using a pulled glass pipette. Aspirate the medium and add 1 mL of PSC passaging solution for each 35 mm dish. Incubate for 3 min at 37 °C, aspirate, and add 2 mL of CDM supplemented with 10 μM Y-27632 and 10 μM SB431542.

        NOTE: If colonies are lifting off in the PSC passaging solution before adding CDM, the incubation time can be reduced.
    3. Under a transmitted-light inverted microscope, gently lift the colonies using the bent edge of the pulled glass pipette using 4x magnification. Once every colony is lifted, gently transfer all to one well of the 6-well ULA plate using a 10 mL serological pipette. Repeat this process for each iPSC plate. Incubate the cells at 37 °C with 5% CO_2.

        NOTE: If the colonies are too large or are merged, they can be sliced with the tip of the pulled glass pipette to create smaller EBs.
2. On day 2, add FGF2 to each well to a final concentration of 50 ng/mL.

    NOTE: The FGF2 used for cerebellar differentiation is not heat-stable. This protocol has not been tested with heat-stable FGF2.
3. On day 7, do a 1/3 medium change. For 3 mL of total medium in a well, with a 1,000 μL pipettor, gently aspirate 1 mL of spent medium and replace it with 1 mL of fresh CDM. Incubate the EBs for 7 days.
4. On day 14, gently aspirate nearly all the spent medium using a 1,000 μL pipettor. To ensure minimal damage to the EBs, swirl the plate to gather all the EBs in the center of the plate, and then tilt the plate and aspirate slowly from the edge.
    1. As the medium amount decreases, slowly lay the plate flat and continue to aspirate. Leave enough medium to avoid drying the EBs out. Afterward, add 3 mL of fresh CDM supplemented with 10 μM Y-27632. Transfer the EBs using a 10 mL serological pipette to a PLO/laminin-coated dish (step 1.2).

        NOTE: Depending on the downstream application, the EBs can be transferred to a 6-well PLO/laminin plate or single EBs can be transferred to a single well of a PLO/laminin-coated 24-well plate or coverslip.

5. On day 15, aspirate the medium and replace it with fresh CDM.

   NOTE: It is important to add enough medium to the wells to ensure enough medium between feedings as, over time, there will be evaporation (e.g., for a well in a 6-well plate, add 3 mL of medium). If the medium has started to acidify (turn clear and yellow), refresh the medium even if it is not on the feeding schedule until the end of the protocol.

6. On day 21, aspirate the spent medium and replace it with CMM. On day 28, change the medium with fresh CMM. On day 35, harvest the cells for further applications.

4. Sample preparation for RNA isolation

1. Aspirate the medium and add 500 µL of cell dissociation reagent (see Table of Materials) to each well of the 6-well plate. Leave it on for a couple of seconds and aspirate.

2. Incubate the plate, with the lid on, at room temperature for 2 min. Tap the sides of the plate to detach the cells.

3. Add 1 mL of CMM (step 1.8) and collect the cells into a 1.5 mL tube.

4. Pellet the cells by centrifugation using a benchtop mini centrifuge (see Table of Materials) for 30 s at RT (max speed 2000 × *g*), discard the medium, and add 1 mL of 1x PBS pH 7.4 to wash the cell pellet.

5. Pellet the cells again using a benchtop mini centrifuge for 30 s at RT and wash with PBS once more.

6. Pellet the cells and discard the PBS. Lyse the cells in phenol and guanidine isothiocyanate containing reagent (see Table of Materials).

   NOTE: Cells lysed in phenol and guanidine isothiocyanate containing reagent can be stored at −80 °C for up to 1 year. Cells can be lysed directly in the dish depending on the RNA isolation method and reagents being used. Due to the low number of cells in a dish, isolating RNA using silica spin columns (see Table of Materials) will result in higher yields of RNA.

5. Preparing cells for immunofluorescent staining

NOTE: For a 24-well plate, one EB per well is sufficient.

1. On day 35, aspirate the spent medium and wash the cells by adding 1 mL of DPBS+/+ per well (for one well of a 24-well plate).

2. Aspirate the DPBS+/+ and add 500 µL of cold 4% paraformaldehyde (PFA, see Table of Materials) prepared in DPBS+/+ per well. Incubate for 20 min at RT.

3. Remove 4% PFA and wash the cells twice with DPBS+/+, as in step 5.1. Add 1 mL of DPBS+/+ and store the cells at 4 °C until staining is done.

   NOTE: It is advised to do the immunofluorescent staining within 1 week after fixation to avoid cell detachment and the loss of antigens. However, depending

on the antibody and the cellular location of the target, staining can be done at later times up to 4 weeks after fixation.

Representative Results

Overview of the 3D to 2D cerebellar differentiation

Cerebellar cells are generated starting from iPSCs. Figure 1A shows the overall workflow and the addition of major components for differentiation. On day 0, EBs are made by gently lifting the iPSC colonies (Figure 1B) using a pulled glass pipette in CDM containing SB431542 and Y-27632 and placed into ultra-low-attachment plates. FGF2 is added on day 2. On day 7, one-third of the medium is changed, and EB formation is observed (Figure 1C). On day 14, enlarged EBs (Figure 1D) are plated on PLO/laminin-coated dishes in CDM supplemented with Y-27632. On Day 15, the medium is replaced with CDM without Y-27632. The cells start migrating outward from the EBs (Figure 1E) along the coated surface. On day 21, a complete medium change is performed, and the medium is switched to CMM. After that, the medium is changed once weekly (or more frequently if the medium acidifies quickly). By day 35, there is a monolayer of cells with neuronal-like morphology and complexity (Figure 1F,G).

2D cells express cerebellar cell markers

Cells are harvested on day 35 for RNA isolation and immunofluorescent labeling. The expression of genes known to be present during cerebellar development is measured by RT-qPCR (Figure 2). The cells express early cerebellar progenitor markers such as ATOH1[12, 13] (rhombic lip, glutamatergic progenitors) and PTF1α[14] (ventricular zone, GABAergic progenitors), as well as the Purkinje progenitor cell markers KIRREL2[15] and SKOR2[15]. In addition to the early developmental cerebellar cell markers, the expression of later-stage development genes, OTX2 and SIX3, is also observed. The immunofluorescent labeling of cells shows positive staining for the cerebellar markers EN2 and PTF1α (Figure 3A,D), the neuronal marker TUBB3 (Figure 3G), as well as for the proliferation marker Ki67 (Figure 3J). Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) shows cell nuclei (Figure 3B,E,H,K). To further validate this protocol, iPSCs derived from patients with a neuropsychiatric disorder were used to generate cerebellar cells and analyze cerebellar cell marker expression at day 35. The RT-qPCR data indicate a similar expression profile as the control iPSCs (Supplementary Figure 1). Additionally, the expression of axonal guidance molecules relevant for cerebellar development is present both in control and patient-derived cerebellar cells (Supplementary Figure 2).

Discussion

The ability to model human cerebellar development *in vitro* is important for disease modeling as well as furthering the understanding of normal brain development. Less complicated and cost-effective protocols create more opportunities for replicable data generation and broad implementation across multiple scientific labs. A cerebellar differentiation protocol is described here using a modified method of generating EBs that does not require enzymes or dissociation agents, using growth factors reported by

Muguruma et al.[4] and a modified 2D monolayer cell growth protocol similar to the method from Holmes et al.[5].

The overall protocol starts by generating EBs from iPSCs, followed by the induction of cerebellar differentiation, and, finally, plating for 2D monolayer culture. During this process, a significant amount of cell death was observed between days 7–14. Due to this cell loss, it is advised to start with a large number of EBs; for a 6-well plate of 2D cells, it is recommended to start with a minimum of six plates (either 35 mm or 60 mm plates) of iPSCs. Moreover, the addition of Y-27632 at the time of plating EBs onto PLO/laminin significantly increases the attachment of the EBs to the substrate. It is important to check the medium in the cultures intermittently visually. If the medium is turning yellow (acidifying), it is advised to change the medium even if it is not in the feeding scheme. The total number of cells that attach will vary from experiment to experiment, necessitating more frequent or less frequent refreshing of nutrients in the medium.

The RT-qPCR results revealed that the iPSCs differentiated into cells representing the early stages of the developing cerebellum. The present data suggest that, at day 35 of differentiation, there are cells expressing the neuronal cell fate marker (TUBB3[16, 17]), glutamatergic and GABAergic progenitor markers (ATOH1[12, 13] and PTF1α[14], respectively), midbrain-hindbrain boundary markers (EN1[18], EN2[18], GBX2[19]), isthmic organizer markers (WNT1[18], FGF8[19]), rhombic lip derivative cell marker (PAX6[18]), and Purkinje cell progenitor markers (KIRREL2[15], SKOR2[20]). Expression of the rhombic lip marker OTX2[21] was also observed. Previous cerebellar organoid protocols using hESCs have observed that FGF2 induction results in GBX2 expressing cells but very few OTX2 positive cells, while a similar protocol using iPSCs showed identical mRNA expression of GBX2 and OTX2 in cerebellar organoids[8]. However, mouse embryonic stem cell (mESC)-derived cerebellar neurons contain separate OTX2 and GBX2 positive cell clusters[4], and it has been shown that OTX2 is expressed throughout mouse[22] and human[21, 23] cerebellar development. The differential expression profile of OTX2 between protocols using the same fate specification factors might be due to other differences between the protocols or individual differences between hESCs and iPSCs; this merits further investigation. SIX3 expression was also observed in the culture on day 35. SIX3 is expressed in the anterior neural tube during development, and its expression in the human cerebellum remains low throughout development and adulthood[23, 24]; however, it is expressed in the neonatal and adult mouse cerebellum[25]. This suggests that there might be a subpopulation of cells that differentiate toward an anterior fate, or they may represent a subpopulation of cerebellar cells expressing SIX3 during development. These cells could be further explored.

Differentiation protocols are often developed using hESCs or iPSCs from healthy subjects, but it is important to confirm that these protocols can be applied to patient-derived iPSCs for dissecting the molecular and cellular changes in disease. To further test our protocol, alongside our control iPSC lines, we differentiated iPSC lines that were reprogrammed from fibroblasts obtained from patients diagnosed with schizophrenia. The previous literature has shown that patients diagnosed with schizophrenia have functional and anatomical cerebellar abnormalities[26, 27, 28]. These changes are observed in adult patients but may begin during development and require further investigation. Overall, it was observed that

cerebellar cells from schizophrenia patients expressed the cerebellar markers tested on day 35 and morphologically were not different from the cerebellar cells derived from control iPSCs (Supplementary Figure 1). This suggests that this protocol can investigate human cerebellar development in the disease context. Moreover, the expression of axonal guidance markers at day 35 was also examined, both in control and schizophrenia cell lines, since one of the major components of development is axonal pathfinding and neuronal connectivity[29, 30, 31, 32]. Indeed, on day 35, axonal guidance molecules were verified that are indicated in cerebellar development, including semaphorin-4C, plexin-B2, and neuropilin-1 (Supplementary Figure 2). During development, plexin-B3 expression is low in the human cerebellum[23], and a lower expression of plexin-B3 was also observed compared to the other axonal guidance molecules for the differentiations. Together with the expression of the cerebellar markers, this is a strong indication that this differentiation protocol generates cerebellar cells that express the correct cues for neuronal connectivity in that structure.

It is important to note that the cell types generated using this FGF2 and insulin-induced cerebellar differentiation protocol were not identified *via* single-cell analysis. Nayler et al.[8] recently published a dataset of single-cell profiling of cerebellar organoids generated using a similar induction protocol, and it is anticipated that future research will increasingly employ single-cell methods to address these questions. The cells beyond day 35 also were not tested for expression or morphology. The expression of cerebellar markers at later time points and how they change over time will give more insights into the maturity of the cells. Co-culturing stem cell-derived cerebellar cells with mouse cerebellar granule cell precursors[4] or human fetal cerebellar slices[33] has been shown to generate mature cerebellar cells, especially Purkinje neurons, which are often absent in cerebellar organoids. Notably, a recent study showed that cerebellar organoids dissociated on differentiation day 35 and plated for 2D growth also give rise to mature cerebellar neurons without needing co-culturing[7]. These applications aim to investigate the later stages of cerebellar development and neural maturation in comparison to this protocol, which can be utilized to investigate the earlier stages of development. Another interesting comparison could arise from comparing cultured embryonic mouse cerebellar neurons[34] to iPSC-derived human cerebellar neurons, potentially highlighting differences in development and fate specification between the two species.

In summary, the present protocol can be used for applications requiring *in vitro* 2D cerebellar cells generated from iPSCs. This protocol does not include complex steps or materials, is cost-efficient, and can be used as a model for early cerebellar development to investigate gene expression, cell morphology, and physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jenny Gringer Richards for her thorough work in validating our control subjects, from which we generated the control iPSCs. This work was supported by NIH T32 MH019113 (to D.A.M. and K.A.K.), the Nellie Ball Trust (to T.H.W. and A.J.W.), NIH R01 MH111578 (to V.A.M. and J.A.W.), NIH KL2 TR002536 (to

A.J.W.), and the Roy J. Carver Charitable Trust (to V.A.M., J.A.W., and A.J.W.). The figures were created with BioRender.com.

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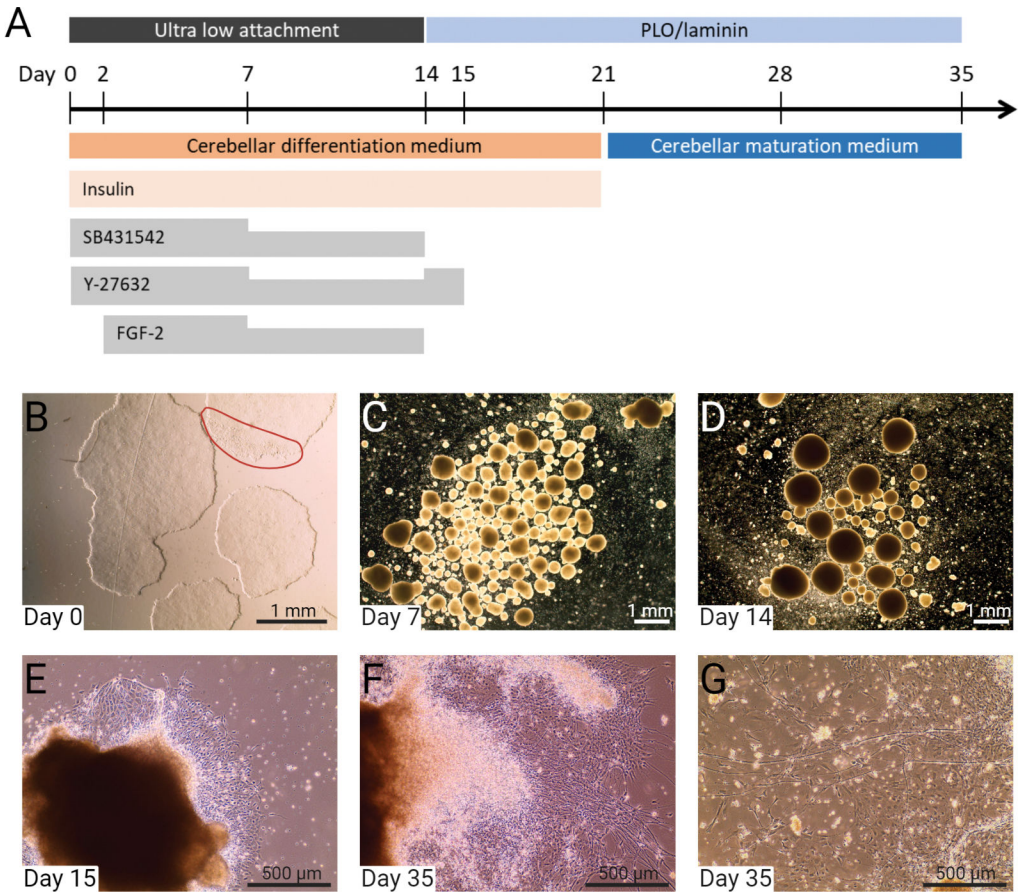

Figure 1: Overview of the protocol timeline and representative images.

(**A**) A schematic outline of the cerebellar differentiation protocol depicting the type of culture medium, the supplements added to the culture medium, the days when a medium change is required (indicated with vertical lines), and the surface coating of the culture dish. (**B**) Representative bright field images of iPSCs on day 0 and differentiated cells that will be cleaned off before making EBs (shown in red circle), (**C**) EBs on day 7 and (**D**) day 14, (**E**) the day after plating the EBs, and (**F**,**G**) maturing cells on day 35. Scale bar (**B**-**D**): 1 mm; (**E**-**G**): 500 µm.

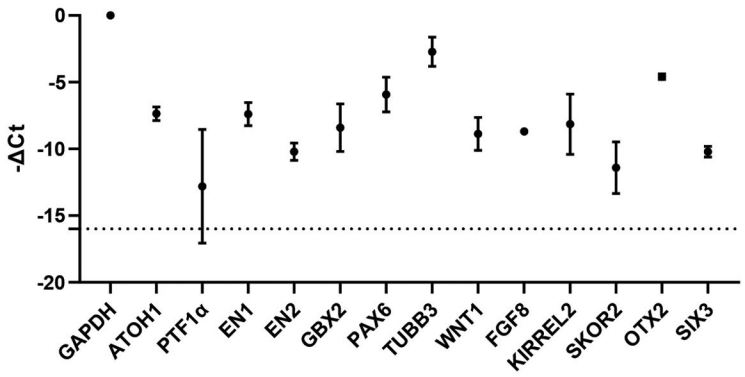

Figure 2: Expression of cerebellar cell markers in 2D cerebellar cells at day 35.

RT-qPCR gene expression results at day 35 for selected genes representing different cerebellar development markers and cell types, normalized to GAPDH. The −ΔCt values represent GAPDH-Ct values subtracted from target-Ct values; values closer to zero indicate higher expression. Any value below the ticked line represents expression below the detectable limit. N = 2 iPSC lines, data are presented as mean ± SD.

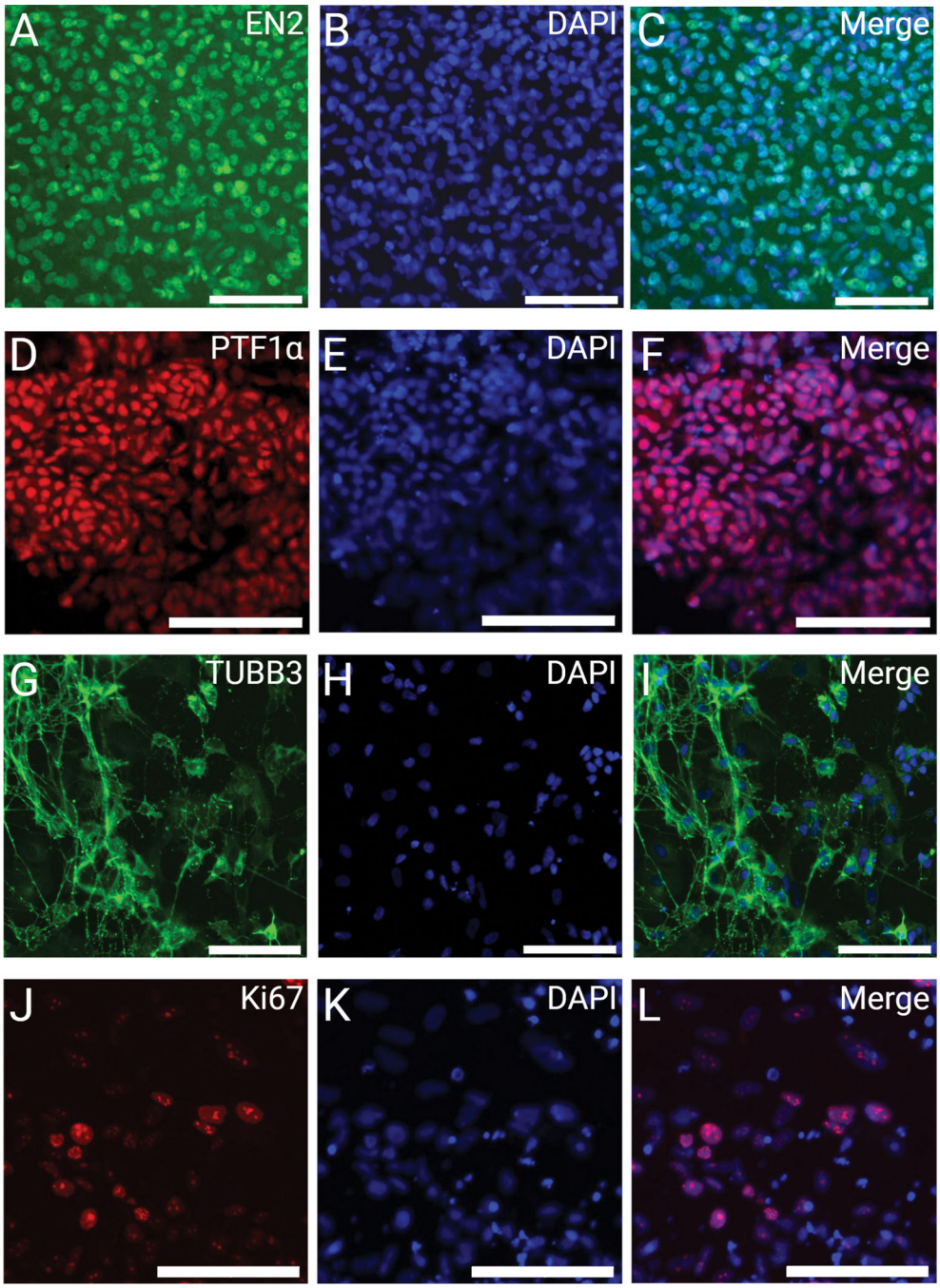

Figure 3: Immunofluorescent labeling of 2D cerebellar cells at day 35.

Cells are fixed with 4% PFA at day 35 and are nuclear stained with DAPI and immunolabeled for (**A**) EN2 (green), (**B**) DAPI (blue), (**C**) EN2-DAPI merged; (**D**) PTF1α (red), (**E**) DAPI (blue), (**F**) PTF1α-DAPI merged; (**G**) TUBB3 (green), (**H**) DAPI (blue), (**I**) TUBB3-DAPI merged; and (**J**) Ki67 (red), (**K**) DAPI (blue), (**L**) Ki67-DAPI merged. Scale bars: 100 μm.

Materials

Name	Company	Catalog Number	Comments
10 mL Serological pipette	Fisher Scientific	13-678-26D	
1-thio-glycerol	Sigma	M6145	
2 mL Serological pipette	Fisher Scientific	13-678-26B	
250 mL Filter Unit, 0.2 μm aPES, 50 mm Dia	Fisher Scientific	FB12566502	
35 mm Easy Grip Tissue Cluture Dish	Falcon	353001	
4D Nucleofector core unit	Lonza	276885	Nucleofector
5 mL Serological pipette	Fisher Scientific	13-678-25D	
60 mm Easy Grip Tissue Culture Dish	Falcon	353004	
6-well ultra-low attachment plates	Corning	3471	
9” Disposable Pasteur Pipets	Fisher Scientific	13-678-20D	
Apo-transferrin	Sigma	T1147	
Bovine serum albumin (BSA)	Sigma	A9418	
Cell culture grade water	Cytiva	SH30529.02	
Chemically defined lipid concentrate	Gibco	11905031	
Chroman 1	Cayman	34681	
Class II, Type A2, Biological safety Cabinet	NuAire, Inc.	NU-540-600	Hood, UV light
Costar 24-well plate, TC treated	Corning	3526	
Costar 6-well plate, TC treated	Corning	3516	
DAPI solution	Thermo Scientific	62248	
DMEM	Gibco	11965092	
DMEM/F12	Gibco	11320033	
DMSO (Dimethly sulfoxide)	Sigma	D2438	
DPBS+/+	Gibco	14040133	
Emricasan	Cayman	22204	
Epi5 episomal iPSC reprogramming kit	Life Technologies	A15960	
Essential 8-Flex	Gibco	A2858501	PSC medium with heat-stable FGF2
EVOS XL Core Imaging system	Life Technologies	AMEX1000	
Fetal bovine serum - Premium Select	Atlanta Biologicals	S11150	
FGF2	Peprotech	100-18B	
GlutaMAX supplement	Gibco	35050061	L-alanine-L-glutamine supplement
Ham’s F12 Nutrient Mix	Gibco	11765054	
HERAcell VIOS 160i CO2 incubator	Thermo Scientific	50144906	
Human Anti-EN2, mouse	Santa Cruz Biotechnology	sc-293311	
Human anti-Ki67/MKI67, rabbit	R&D Systems	MAB7617	
Human anti-PTF1a, rabbit	Novus Biologicals	NBP2-98726	
Human anti-TUBB3, mouse	Biolegend	801213	
IMDM	Gibco	12440053	

Name	Company	Catalog Number	Comments
Insulin	Gibco	12585	
Laminin Mouse Protein	Gibco	23017015	
Matrigel Matrix	Corning	354234	Basement membrane matrix
MEM-NEAA	Gibco	11140050	
Mini Centrifuge	Labnet International	C1310	Benchtop mini centrifuge
Monarch RNA Cleanup Kit (50 µg)	New England BioLabs	T2040	Silica spin columns
Monarch Total RNA Miniprep Kit	New England BioLabs	T2010	Silica spin columns
N-2 supplement	Gibco	17502-048	
Neurobasal medium	Gibco	21103049	
PBS, pH 7.4	Gibco	10010023	
PFA 16%	Electron Microscopy Sciences	15710	
Polyamine supplement	Sigma	P8483	
Poly-L-Ornithine (PLO)	Sigma	3655	
Potassium chloride	Sigma	746436	
SB431542	Sigma	54317	
See through self-sealable pouches	Steriking	SS-T2 (90×250)	Autoclave pouches
Sodium citrate dihydrate	Fisher Scientific	S279-500	
Syringe filters, sterile, PES 0.22 µm, 30 mm Dia	Research Products International	256131	
Trans-ISRIB	Cayman	16258	
TRIzol Reagent	Invitrogen	15596018	Phenol and guanidine isothiocyanate
TrypLE Express Enzyme (1×)	Gibco	12604039	Cell dissociation reagent
Vapor pressure osmometer	Wescor, Inc.	Model 5520	Osmometer
Y-27632	Biogems	1293823	